Antibacterial Peptides – A Bright Future or a False Hope

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Abstract: The increasing number of antibiotic resistant bacterial strains presents an emerging world health problem that demands continued effort to develop new antibacterial compounds. Endogenous antibacterial peptides (ABPs) that are constitutively and/or inducibly produced in tissues exposed to external surroundings represent new candidates for development of such compounds. Most ABPs target bacterial membranes initially by electrostatic interactions between positively charged amino acids and negatively charged molecules present on bacterial walls, followed by compromise of the permeability barrier of bacterial membranes through the formation of pores, leading to rapid cell death and efficient bacterial elimination. Other mechanisms, such as the inhibition of bacterial protein and DNA synthesis or receptor-mediated stimulation of host defense mechanisms are also ascribed to these molecules. The activities of ABPs correlate positively with a gradient of hydrophobicity along the peptide backbone, net positive charge at neutral pH, and secondary structure. More than 850 sequences with antibacterial activity have been described, and this number continues to grow with the addition of newly discovered, as well as synthetic, peptides. Many strategies, including increasing net positive charge, increasing net hydrophobicity, conjugation of peptides with lipophilic acids, incorporation of carbonate bonds, and synthesis of their hybrids and truncated sequences that omit hemolytic regions, have been proposed to increase efficiency of synthetic ABPs. Additionally peptide-mimicking molecules such as cationic steroid antibiotics (CSAs) may be useful alternatives to natural ABPs. Current challenges for practical application of ABPs are the high cost of synthesis/isolation, inactivation by blood plasma, confinement by anionic polyelectrolytes, and possible unknown toxicity.

Keywords: Cationic antibacterial peptides, endotoxin, lipoteichoic acid, NF-κB.

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ANTIBACTERIAL PEPTIDES AS A PART OF INNATE IMMUNE SYSTEM

Antibacterial peptides (ABPs), which were originally described in insects (*Hyalophora cecropia*) [1, 2] and plants [3], and subsequently identified in vertebrates [4-6], constitute a key component of the innate immune system. Most of the known ABPs (currently more than 850 characterized) are active against multiple microorganisms, such as viruses, bacteria, fungi and protozoa, and can be ascribed to one of three main groups: 1) α-helical peptides without cysteine, 2) peptides with three disulphide bonds or 3) peptides rich in proline or tryptophan [7] (Table **1**). However, in most cases, peptide sequences are unique to each species, and even to tissues within a species. Consequently, in nature there exists tremendous sequence diversity among bacteria-killing peptides.

ABPs are widely expressed in the skin, mouth, airways, digestive tract and genitourinary system as components of complex secretions, and function in the first line of defense against infectious agents [8]. Combinations of numerous peptides, often acting synergistically, prevent infection and serve to control resident microbial populations. Such a synergy has been demonstrated between β-defensin 2 (HBD2) and lactoferrin, as well as between β-defensins 2,4 (HBD2/HBD4) and lysozyme [9, 10]. Production of some ABPs occurs constitutively (e.g. α-defensins), or constitutively with significant increase during infection (e.g. LL37 peptide), however production of others (e.g. β-defensins) is modulated by inflammatory cytokines which can cause a local increase in ABP concentration [11]. For example, oral epithelial cells produce HBD2 and β-defensin 3 (HBD3) when exposed to a range of lipopolysaccharides (LPS) and inflammatory mediators [12, 13]. Similarly, heat-killed bacteria and TNF-α induced tracheal antimicrobial peptide (TAP), epithelial beta-defensin and lingual antimicrobial peptide (LAP) expression in bovine epithelial cells [14, 15]. Also, the concentration of cathelicidin-derived LL37 peptides and HBD2 was found to increase in airways of cystic fibrosis patients with bronchial inflammation [16], and upregulation of the human cathelicidin gene was observed in inflammatory skin disorders [17].

Expression of ABPs

Epithelial cells [18, 19], Paneth cells of the digestive tract [20], platelets [21, 22], and white blood cells recruited to infection sites by chemokines [4, 23-25], represent the major sources of endogenously produced ABPs (Table **2**). The inducible expression is mediated by a variety of different signaling pathways. The gene encoding HBD3 has NF-IL-6 consensus sequences, but does not have an NF-κB consensus, and unlike HBD4, the gene coding HBD3 is upregulated by IF-γ [10]. The existence of multiple regulatory motifs suggests that the expression of ABP genes is tightly controlled by distinct pathways [26]. Most antimicrobial peptides are derived from post-translational, and often ex-

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Table 1. Structure of Some Representative Antimicrobial Peptides. For Amino Acids the One Letter Code is Used. Subscript numbers Represent Amino Acids that are Joined by Disulfide Bridges

Peptide	Group	Structure
LL37	α-helix	**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**
magainin II	α-helix	**GIGKFLHSAKKFGKAFVGEIMNS**
temporin L	α-helix	**FVQWFSKFLGRIL**
β-defensin-1	with disulfide bridges	**DHYNC$_1$VSSGGQC$_2$LYSAC$_3$PIFTKIQGTC$_2$YRGKAKC$_1$C$_3$F**
lactoferricin	with disulfide bridges	**FKC$_1$RRWQWRMKKLGAPSITC$_1$VRRAF**
bactenecin-1	with disulfide bridges	**RLC$_1$RIVVIRVC$_1$R**
bactenecin-5	rich in proline	**ERPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLRFP**
PR39	rich in proline	**RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP**
indolicidin	rich in proline	**ILPWKWPWWPWRR**

tracellular, cleavage of precursor proteins that can have functions independent of the antibacterial activity of their cleavage products [27]. The generation of antimicrobial peptides from a promicrobicidal protein can occur through different mechanisms in related species. Of the 3 known serine proteases from azurophil granules, proteinase 3 was solely responsible for cleavage of hCAP-18 between alanyl and leucyl residues to liberate the antibacterial LL37 peptide. This site differs from cleavage sites in the bovine and porcine cathelicidins, which are cleaved by elastase [28].

Table 2. Dominant Cells Involved in Production of Human Antibacterial Peptides

Cells	Antibacterial peptides
Epithelial cells	**β-defensin (HBD1, HBD2), LL37, antimicrobial ribonuclease – RNase 7**
Granulocytes	**α-defensin (HNP1, HNP2, HNP3, HNP4), LL37,**
Paneth cells	**α-defensin (HNP5, HNP6)**
NK cells	**Granulysin**
Lymphocytes	**LL37, granulysin**
Platelets	**connective tissue activating peptide 3 (CTAP-3), fibrinopeptide B (FP-B), fibrinopeptide A (FP-A), platelet factor 4 (PF-4), RANTES**

Hormonal Activity of ABPs

As predicted from the index of binding potential, some antibacterial peptides may have hormonal activity and affect cell function by stimulating membrane receptors [7]. In endothelial cells, LL37 was found to induce angiogenesis, mediated by the formyl peptide receptor-like 1 (FPRL1) [29]. Similarly, regeneration of human corneal epithelium was found to occur through multiple pathways involving LL37-mediated activation of a G protein-coupled receptor and the epidermal growth factor (EGF) receptor [30]. Proliferation of A549 lung epithelial cells were also shown to increase in the presence of neutrophil-derived defensin [31]. Interestingly, both cathelicidins and defensins were found to activate chemotaxis (partially through FPRL1 activation), a process that may contribute to innate and adaptive immunity by recruiting neutrophils, monocytes, eosinophils and T cells to sites of microbial invasion [32-36]. The mechanism of ABP-induced chemotaxis is potentially important *in vivo*, because the chemotactic activity of LL37 *in vitro*, unlike its antibacterial action [37], was not significantly inhibited by the presence of human serum [33]. However, it is not clear if ABPs represent classical receptor agonists, as quantitative data showing direct binding of these peptides to receptors are lacking, and their mechanism of action may occur by less well-defined, non-specific, membrane-active effects that affect protein function by insertion into membrane, followed by membrane-structural reorganization and subsequent regulation of receptor signaling [38]. The varied and numerous effects of HBD2 present in significant amounts in human milk represent a strong case for receptor-mediated action, as well as the less specific membrane-reorganizing effect of ABPs. HBD2 profoundly alters the innate immune response of breast and intestinal epithelial cells and may contribute to breast feeding-associated decreases in maternal breast cancer risk, as well as lower rates of infections and inflammatory-allergic diseases detected in breast-fed infants [39]. A similar mode of action may explain the significant effect of the LL37 peptide during wound closure, where its reduction impairs re-epithelialization contributing to a failure to heal [40]. Human airway smooth muscle (HASM) stimulated with LL37 releases IL-8, an effect dependent on the activation of ERK1/2. The purinergic receptor (P2X7) antagonist suramin blocks LL37–induced ERK1/2 phosphorylation and IL-8 release, suggesting that LL37 activates the P2X7 receptor in HASM cells and that LL37 may regulate the inflammatory process in various lung diseases by enhancing IL-8 production [41], although this result does not exclude the possibility of LL37 activating P2X7 by affecting the membrane proximal to receptor. Induction of IL-8 production was also observed upon HNP stimulation of human lung epithelial cells. Transfection of lung epithelial cells with antisense

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oligonucleotides targeting specific purinergic P2Y receptors revealed that the P2Y6 (UDP receptor) signaling pathway plays a predominant role in mediating HNP-induced IL-8 production [36].

Interaction of ABPs with Bacterial Wall Components

In addition to the direct binding of ABPs to lipid membranes, these peptides can neutralize the inflammatory activity of bacterial wall components, which results in inhibition of cytokine and histamine release upon stimulation of eukaryotic cells by lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [42, 43]. This finding indicates that TLR activation by bacterial molecules, as well as binding of LPS to CD14/MD2 proteins, may be prevented by ABPs, but presently no direct interaction of ABPs with membrane receptors involved in LPS or LTA recognition has been reported [44]. In the context of LPS signaling, ABPs may also function as a negative feedback element in the regulation of ABP production. In this case, LPS or LTA-mediated induction of ABP production is limited by the release of ABPs which inactivate the bacterial wall molecules that initiated their expression. The ability of ABPs to prevent systemic inflammation, in addition to their bactericidal activity, may represent a significant advantage, compared to antibiotics that may temporarily increase inflammation by molecules released from dying bacteria [45]. However, the inhibition of LPS by ABPs may represent only one aspect of the ABPs-LPS interaction. Theoretically, ABP-LPS complexes may also activate cell receptors [44], as has been shown for the soluble form of the LPS receptor, the sCD14 protein, that can either inhibit or enhance cellular response to LPS [46]. An LPS binding protein (LBP) can also either enhance or inhibit the cellular response to LPS, depending in part on the presence of other LPS-binding molecules to which LBP may facilitate transfer [47]. Although there is no data showing that ABPs compete directly with LBP for binding LPS in plasma or at the site of inflammation, the delivery of LPS to cellular receptors, lipoproteins, and other targets mediated by LBP is likely to be modulated by ABPs or their source proteins. The dual role of LBP and CD14 in innate immunity has been recently established [48], and the potential that ABPs may modulate the interactions of these proteins with LPS deserves future investigation.

ABPs in Tumor Growth

In addition to their functions in inflammation, antimicrobial proteins including cathelicidins have been proposed to play a role in the non-specific defense against tumors [49-52]. Contrary to this assertion, it was found that hCAP18/LL37 is strongly expressed in tumor cells, and transgenic expression of hCAP18 in human epithelium, as well as addition of LL37 to cultured cells, resulted in increased proliferation. These findings do not support the hypothesis that LL37 has an anti-tumor effect at physiological concentration, but rather suggest that hCAP18/LL37 may promote tumor cell growth [49]. In this study the ability of LL37 to damage epithelial cells was tested in the presence of blood serum [49], a condition that inactivates LL37 bactericidal activity [50]. In the absence of serum, cell death associated with mitochondrial injury was observed following addition of HNP1-3 and LL37 peptides to cultured lung epithelial A549 cells [51]. Based on these observations, cationic antibacterial peptides may have some potential as natural-source drugs for the treatment of human cancers [52]. In some host cells, ABPs may compromise survival by induction of apoptosis. As an example, recombinant granulysin normally released by NK cells via granule-mediated exocytosis was found to induce apoptosis of Jurkat cells. This apoptosis was associated with an increase in the ceramide/sphingomyelin ratio, implicating the activation of sphingomyelinases [53].

ABPs - MECHANISMS OF BACTERIA KILLING

Antibacterial peptides exert their activity by both intracellular and extracellular killing of microbes, attacking multiple molecular targets to cooperatively penetrate and disrupt microbial surfaces and membrane barriers, resulting in membrane depolarization, a loss of pH gradient, impaired osmotic regulation, and inhibition of respiration [26]. When bacterial cells are exposed to ABPs, within minutes more and more peptide molecules dock to the surface of the bacterial membrane. Charge based interactions between negatively charged bacterial wall moieties such as LPS and LTA, and positively charged sites on the peptides initiate adhesion of ABPs to bacterial surfaces [54] and hydrophobic interaction may further enhance binding. Although electrostatics is assumed to be the critical force driving attachment of ABPs, some antibacterial peptides permeate both zwitterionic and negatively charged phospholipid vesicles [55]. The hydrophobicity of these molecules does not ensure their binding to phospholipids [56], but it does favors their penetration into phospholipid monolayers if docking is enabled, thereby destabilizing membranes of both eukaryotic and prokaryotic cells [57]. It appears that the membrane permeation function of antibacterial peptides is associated with an amphipathic character that promotes partitioning to hydrophobic-hydrophilic interfaces. Measurement of the intrinsic surface activity of cathelicidin LL37, melittin, magainin II, and PBP10 peptides revealed a positive correlation between their surface pressure activity and their antibacterial function [58].

Membrane-Disruption Models for ABP Bactericidal Activity

The interaction between peptides absorbing into a lipid monolayer and the molecules composing this surface is defined by a number of factors: the nature of the lipid polar head groups, their arrangements and net charge; the length, extent of saturation, and lateral packing density of the hydrocarbon chains; the intrinsic surface activity of the peptide; the strength of the interaction between the peptide and lipids; the valence of ions in the aqueous medium; and the accessibility of interacting groups in the lipids and peptides to one another [59, 60]. Surprisingly, no significant head group dependency was observed for the interaction of either LL37 or PBP10 peptides with monolayers of PC, PC/CH, PC/PS, PC/DPPG, PC/CL and a mixture of bacterial lipids (BL), suggesting little or no specificity of these peptides for the polar head groups of the lipids [58]. This observation suggests that negative charge, even in the context of a zwitterionic lipid, and its density on the bacterial surface could be

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more important than the specific type of phospholipid present in the bacterial membrane.

Three general mechanisms have been proposed to describe the process of phospholipid membrane permeation by membrane-active peptides. These are the barrel-stave model, the toroidal model, and the carpet model Fig. (**1**) [61, 62]. According to the barrel-stave model [63] as few as three molecules are required to form a membrane pore. To allow pore formation, the inserted molecules should have distinct structures, e.g. amphipathic α-helices. Some peptides appear to induce transmembrane pores that conform to the toroidal model, in which the lipid monolayer bends continuously through the pore so that the water core is lined by both the peptides and the lipid headgroups [61]. On the other hand, according to the carpet-model, peptides bind to the phospholipid membrane surface until a threshold concentration is reached, and only then permeate it in a detergent-like manner [62, 64]. Consequently, perturbation of membrane phospholipid packing after peptide insertion results in membrane permeabilization. A change of membrane phospholipid packing may also directly inhibit mechanosensitive channels, as recently observed for gating modifier peptide GsMTx-4 from spider *Grammostola spatulata* that effectively kills bacteria [65]. EM and AFM images of bacteria treated with ABPs justify the assertion that bacterial membranes represent structural targets for these molecules, and that membrane structural changes precede bacterial death Fig. (**2**).

In the case of some anti-bacterial peptides (e.g. LL37, HLP-2), changes in the structure of both individual peptides and self-assembled peptide aggregates were observed at the outer membrane surface at the time of ABP membrane insertion [66]. Despite their various folding characteristics in solution (ABPs may adopt α-helical, β-sheet or both α-helical and β-sheet structure), most ABPs adopt an amphipathic arrangement with opposing hydrophobic and positively charged faces when they are in contact with bacterial walls [62]. Changes in ABP folding are influenced by environmental factors such as salt concentration, pH, and the presence of charged lipids, all of which may represent important mechanisms in regulation of ABP activity. At micromolar concentration in water, LL37 exhibits a circular dichroism spectrum consistent with a disordered structure. A cooperative transition from disordered to helical structure was observed as the LL37 peptide concentration was increased, consistent with the formation of an oligomeric structure. The extent of LL37 α-helicity was found to correlate with both antibacterial and lytic activity against host cells [37].

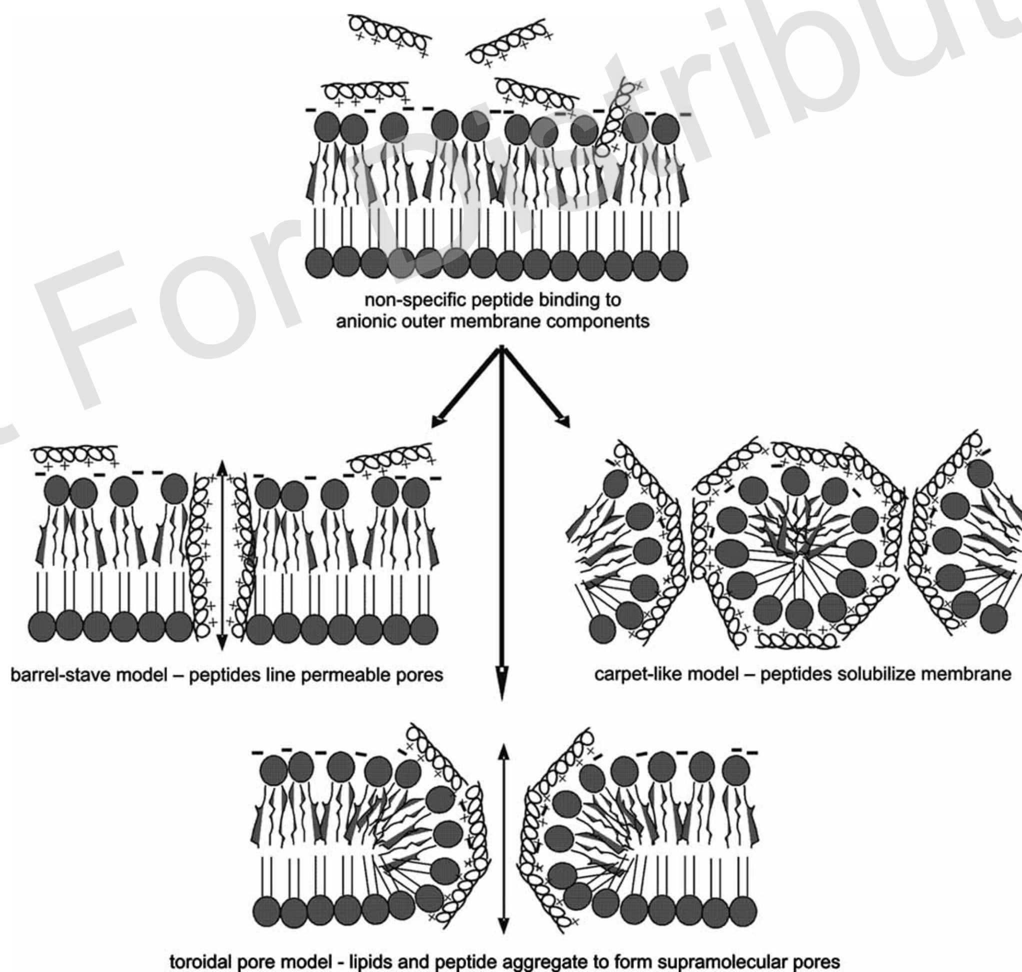

Fig. (1). Model showing interaction of antibacterial peptides with Gram-negative bacteria. Some general mechanisms: "carpet-like" and "barrel-stave" have been proposed to describe the process of phospholipid membrane permeation by membrane-active peptides that result in membrane permeabilization and depolarization.

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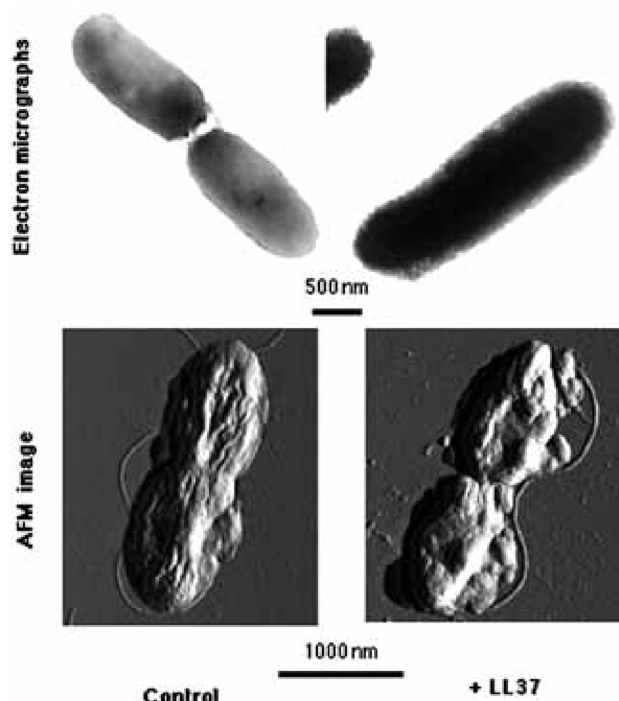

Fig. (2). Bacterial morphology after treatment with 5μM LL37. Electron micrographs show that LL37 causes an increase in *Escherichia coli* membrane permeability as concluded from the enhanced staining by PTA. LL37 mainly targets *Pseudomonas aeruginosa* (PAO1) membrane. This action results in irregular disruption and blebbing of the bacteria membrane as visualized by AFM.

Non-Membrane Disrupting ABP Mechanisms

Recently, it was described that in addition to bacterial membrane disruption, ABPs may kill bacteria and avoid bacterial resistance mechanisms by entering bacterial cells and manipulating bacterial genetic machinery. By examining the transcription profiles of *Escherichia coli* treated with sublethal and lethal concentrations of the cationic antimicrobial peptide cecropin A, significant changes were found in the transcription level of 26 genes. Some of these transcripts correspond to protein products with unknown function, and the pattern of response was distinct from the bacterial response to nutritional, thermal, osmotic, or oxidative stress. The pathway of cecropin A control of bacterial gene transcription is not known, but may potentially represent a surprising and novel mechanism by which ABPs kill bacteria [67].

ABPs may also expand the outer bacterial membrane by competing with divalent cations for binding sites on LPS and displace the cations which normally stabilize bacterial outer membrane structure [68]. Permeabilization of bacterial membrane allows passage of ABPs, mediated by self-promoted uptake pathways [69]. This uptake is an important step for the action of peptides for which the lethal targets are cytoplasmic. This model is proposed for the PR39 peptide which was originally isolated from the upper part of porcine intestine and was found to inhibit both DNA and protein synthesis [70]. Even though this mechanism is an example of direct interaction between an antibacterial peptide and cytoplasmic enzymes, this result is only applicable to a small class of ABPs known to effect intracellular targets. New observations show comparable antibacterial activities between natural and synthetic (all-D) cecropin [71] and PBP10 peptides [58], which lack the ability to interact with natural L-amino acid protein targets [57].

In addition to their bactericidal activity, antibacterial peptides, as a part of the host immunes system, have shown the ability to inhibit viral infection caused by DNA and enveloped RNA viruses [72]. ABPs interfere with viral entry based on interaction with heparan sulfate [73], viral entry by interaction with specific cellular receptors [74], viral spread from infected cells to neighboring cells [75] and viral envelope, causing changes in envelope assembly that prevent cellular infection [76].

Prokaryotic Specificity of ABPs

It is generally accepted that the permeability barriers of the outer and inner bacterial membranes are breached by antimicrobial peptides, but these same peptides are not lytic to the eukaryotic host cells. Several explanations have been proposed for the lytic effect on prokaryotic membranes without damage to eukaryotic membranes, including the absence of cholesterol from prokaryotes and bacterial targets distinct from membrane constituents, but the best documented hypothesis is based on the fact that the outer leaflets of bacteria are highly anionic whereas eukaryotic cell membranes, with few exceptions, are composed exclusively of zwitterionic lipids, with anionic phospholipids such as phosphatidylserine (PS) and phosphoinositides (PPI) avidly restricted to the inner leaflet. Even though RBCs contain a large amount of negatively charged sialic acid-containing carbohydrate moieties in the form of glycoproteins and glycosphingolipids on their surface (glycocalix layer), they are not affected by most natural ABPs, likely because the glycocalix traps the cationic ABPs and prevents them from partitioning into the zwitterionic cell membrane [62]. However, when the asymmetric distribution of membrane phospholipids in RBCs is lost after calcium-ionophore (A23187) treatment, an increase in hemoglobin release following addition of LL37 or melittin [54] was observed, suggesting ABP-induced RBC lysis. These findings indicate that the asymmetric distribution of membrane surface phospholipids in eukaryotic cells (i.e. confinement of negatively charged phospholipids to the inner leaflet) may prevent lytic attack of ABPs on host cells. This hypothesis may also relate to reports of anti-tumor cell activity of ABP since the asymmetric distribution of lipids is partly compromised in some transformed cells .

MECHANISMS OF BACTERIAL RESISTANCE TO ANTIBACTERIAL PEPTIDES

Based on unchanged MIC values after many passages of *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa* strains cultured in the presence of sublethal concentration of ABPs, it was concluded that most bacteria are unable to develop resistance against ABP molecules [26, 77]. This inability was proposed to derive from the hypothesis that the necessary modification of bacteria to build such a resistance may be lethal to the bacteria by compromising their ability to survive in changing environments (e.g.

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changes to bacterial wall molecules). New experimental studies have demonstrated the ability of several important human pathogens to resist killing by host antibacterial peptides [78]. Not only do different bacterial strains differ in their intrinsic susceptibility to ABPs, but also a number of bacterial pathogens have global regulatory networks that coordinate regulation of virulence factors (some of which determine bacterial resistance to ABPs), dependent on the growth phase and environmental conditions in different tissue niches of the host organism. Although the general, non-specific mechanism of ABP killing activity appears to minimize the possibility of bacterial resistance to membrane active peptides, the ability to escape killing has been described for several bacterial strains.

Modification of Bacterial Wall Components

In the case of Gram-negative bacteria, LPS molecules represent the first contact and principle target molecules for ABPs. Modifications in LPS molecules may result in bacterial resistance to ABPs Fig. (**3**). Activation of the *Salmonellae* pagP gene, after infection of vertebrate tissues with limited access to Mg^{2+} and Ca^{2+} resulted in increased acylation of lipid A, which in turn correlated with *Salmonellae* resistance to membrane active ABPs. The pagP mutants that demonstrate increased outer membrane permeability in response to ABPs support the hypothesis that increased lipid A acylation is a bacterial ABP resistance mechanism [79]. Mutation of genes in *Burkholderia cenocepacia* that encode enzymes involved in the modification of heptose sugars prior to their incorporation into the LPS core oligosaccharide results in mutant strains that produce complete lipid A-core oligosaccharide, but lack the polymeric "O" antigen. Due to this lack of "O" antigen, this strain was found to be insensitive to polymyxin B or melittin [80]. In *Haemophilus influenzae* acylation of encoded lipooligosaccharide by htrB gene [81] and expression of phosphorylcholine [82] was found to cause resistance to human β-defensin and cathelicidin LL37, respectively. Similar to surface modification of Gram-negative bacteria, modification of moieties in Gram-positive bacterial walls may result in those bacteria gaining resistance to ABPs [83]. Specific modification of teichoic acid, normally highly charged due to the presence of deprotonated phosphate groups, can be achieved by esterification with D-alanine, which reduces net negative charge [84]. In the *Staphylococcus aureus* wall, incorporation of D-alanine reduces cell wall negative charge and helps trap or repel ABPs before they reach inner membrane targets [83]. Modification of teichoic acid to reduce ABP sensitivity with D-alanine was also detected in *Bacillus subtilis* [85, 86].

Protein-Mediated Degradation and Entrapment

Alteration of bacterial wall molecules is not the only pathway by which bacteria develop resistance to ABPs. Enzymatic degradation of ABPs by proteases released from bacteria also occurs. Proteolytic degradation of the LL37 peptide was demonstrated for a number of pathogens including *Pseudomonas aeruginosa, Streptococcus pyogenes* [87] *Burkholderia cepacia* [88] and *Bacillus anthracis* [89].

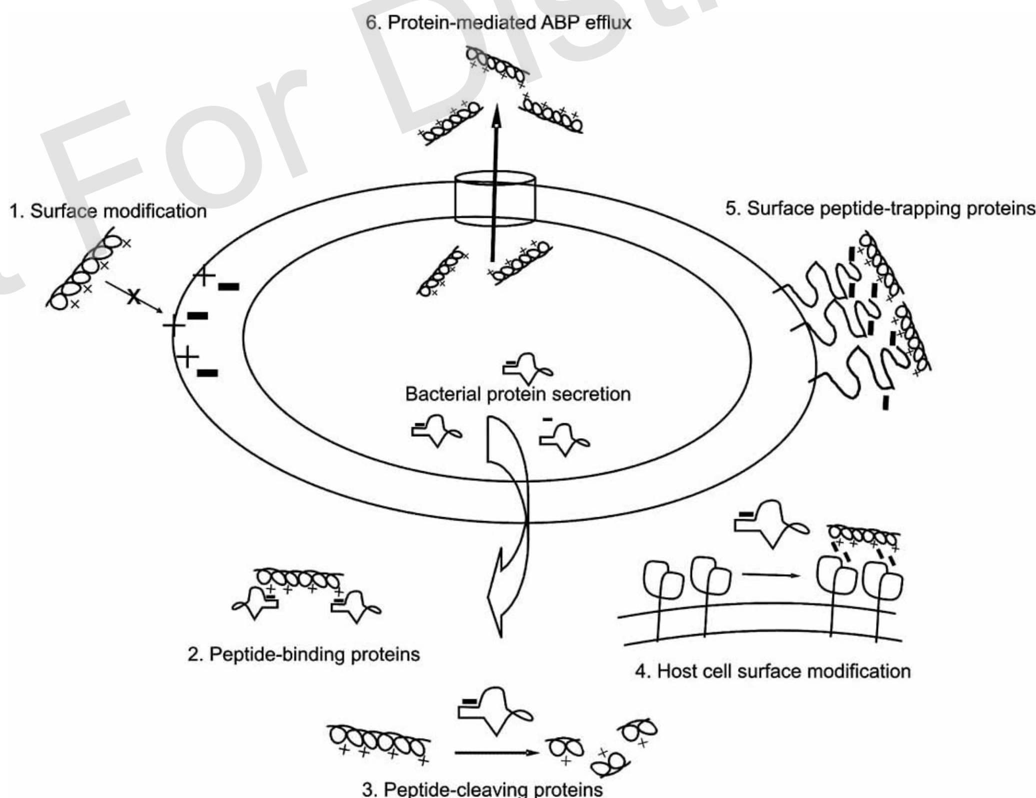

Fig. (3). Basic mechanisms that result in bacterial resistance to antibacterial peptides. 1-modification of bacterial wall molecules that limit ABP absorption to the surface, 2-proteins secreted by bacteria that bind ABPs before they reach bacterial surface, 3-secreted proteases that cause ABP degradation, 4- proteins that affect the function of host cells causing changes in host cell surfaces that trap ABPs, 5- production of proteins that trap ABP on the bacterial surface, 6- active transporters that mediate efflux of ABPs that reach the intracellular environment.

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Analysis by mass spectrometry of LL37 fragments generated by *Pseudomonas aeruginosa* elastase *in vitro* revealed that initial cleavages occurred at Asn-Leu and Asp-Phe, followed by two breaks at Arg-Ile, thus inactivating the peptide [87]. This degradation was blocked by the metalloproteinase inhibitors GM6001 and 1, 10-phenanthroline, both of which inhibited *Pseudomonas aeruginosa* elastase, *Proteus mirabilis* proteinase, and *Escherichia faecalis* gelatinase. This study indicates that the efficacy of addition of exogenous peptides to sites of infection may be increased by the addition of protease inhibitors. In addition to bacterial proteases, the host cysteine proteases cathepsins B, L, and S can also degrade ABPs. All three cathepsins were found to be present and active in CF bronchoalveolar lavage, and incubation of HBD2 and HBD3 with CF bronchoalveolar lavage caused their degradation, which was completely (HBD-2) or partially (HBD-3) inhibited by a cathepsin inhibitor. This finding indicates that in chronic lung diseases associated with infection, overexpression of cathepsins may lead to increased degradation of β-defensins, thereby favoring bacterial infection and colonization [90].

Other mechanisms by which bacteria avoid the killing effects of ABPs are based on bacteria-secreted or surface-associated proteins such as the SIC protein [91], staphylokinase [92], and host cell dermatan sulfate [93] that mediate external trapping of ABPs. Antibacterial peptides that reach the bacterial intracellular environment can also be specifically eliminated by efflux transporters, (e.g. ATP-binding cassette transporter) a mechanism observed in the case of β-defensin [94], LL37 peptide, and protegrin-1 [95, 96].

ACTIVITY OF ANTIBACTERIAL PEPTIDES IN THE PRESENCE OF POLY-ANIONIC BIOPOLYMERS

In addition to active bacterial mechanisms to escape ABP-induced destruction, the function of host antibacterial peptides can be mitigated by environmental factors present at infection sites. Anionic polyelectrolytes such as DNA and F-actin released from dead neutrophils and other cells lysed as the result of inflammation interact electrostatically with positively charged ABPs. The counterion condensation around polyelectrolytes strongly favors the binding of larger valence ABPs over smaller valence ions, and when a critical concentration of ABPs is present, the polyelectrolytes collapse into bundles that trap both polyelectrolyte and ABPs in dense, stable structures. Large bundles containing both F-actin and DNA are a very common feature of CF sputum, and one of the ligands holding these polymers together was identified as the cathelicidin/LL37 peptide [97]. ABPs with multiple positive charges are able to overcome the strong electrostatic repulsion between DNA/F-actin filaments that cannot be overcome by physiological concentrations of divalent ions [98]. *In vitro*, anionic oligomers with more then 5 negative charges bundle actin at concentrations in the μM range, below the concentration of cationic peptides present in airway fluid [98]. The concentration of polyvalent cations needed to form such bundles is likely to be even lower in sputum, because molecular crowding effects characteristic of airway fluid enhance electrostatic effects that would tend to form polyelectrolyte bundles [99]. The ability of polyanionic biopolymers to inactivate ABP activity may present an important obstacle for the practical use of exogenous ABPs. The addition or overexpression of these peptides at the infection site may be insufficient, requiring the depolymerization of DNA and F-actin for restoration of antibacterial activity. Depolymerization of DNA and F-actin in CF sputum using recombinant DNase I and recombinant human gelsolin, respectively, was shown to solubilize CF bundles and decrease CF sputum bacterial load [100], indicating recovery of the activity of ABPs following their release from bundle structure. Addition of poly-anions, such as poly-aspartate and poly-glutamate [101] presents a possible alternative to the specific treatment with DNase and gelsolin. The presence of polyanionic polymers at the site of infection may also cause changes in the pathogenic organisms that grow in these environments. By changing bacterial patterns of growth from planktonic to biofilm, certain strains may become less sensitive to ABP killing action [102].

SYNTHESIS OF MORE EFFECTIVE ANTIBACTERIAL PEPTIDES AND PEPTIDE-MIMICKING MOLECULES

The approach to enhance the desired bactericidal activity and reduce the hemolytic effect (a measure of peptide toxicity for host cells) of ABPs is based on rational modifications of existing peptide sequences. Since minor differences in amino acid sequence can produce significant differences in antimicrobial activity [105], the possibility of strategically varying key amino acids was explored by many investigators [72, 103, 107-108]. Sequence modifications offer an enormous number of combinatorial possibilities including deleting, adding, or replacing one or more residue, as well as truncating the peptide or assembling chimeric peptides based on sequences from the same or different species. Conjugation of peptides with lipophilic acid [109], rhodamine B [58], incorporation of carbonate bond(s) [110], peptoid residues (Nala) [111], or α-amino acid epimerization [112] was found to change peptide physico-chemical properties that often translated to better biological activity.

Since most organisms are equipped with at least a few antibacterial peptides, an obvious alternative to peptide sequence modification is identification and characterization of new natural peptides. Recent examples of this approach include four new antibacterial peptides that were isolated from bovine hemoglobin subjected to peptic digestion (three of these peptides correspond to fragments of the alpha-chain of bovine hemoglobin: alpha107-141, alpha137-141, and alpha133-141, and one peptide to the beta-chain: beta126-145) [113], and the discovery of antimicrobial activity of heparin-binding regions from matrix glycoproteins [114].

Yet another approach to production of new ABPs is a minimalist approach to designing synthetic antibacterial peptides by incorporating the features that define the function of known antibacterial molecules. This approach can result in the synthesis of powerful antibacterial agents that share common characteristics with ABPs (they are cationic with at least three positive charges, small and with at least 50% hydrophobic residues), but this approach may potentially result in high cytotoxicity [108]. Because the possible combinations of even relatively short sequences of synthetic ABPs present a dauntingly large number of molecules, the need for development of screening systems to choose candidates for

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synthesis raise an important issue parallel to optimal sequence design. Synthetic or biological combinatorial libraries, active sequences from a large collection of naturally occurring host defense peptides, and mutagenesis of DNA sequences encoding such peptides are all available methods for developing new molecules that can be screened to obtain an optimal synthetic anti-bacterial peptide [108]. After several years of intense study and identification of many sequences with antibacterial activity only a few peptides have entered clinical trials, with none yet progressing to a commercially available form. Currently, more than 10 biotechnology companies are designing ABPs for the purpose of specific local application (acne, Candidosis) or general use against infection by drug resistant bacteria [11].

As an alternative to creating a peptide, attempts have been made to synthesize peptide-mimicking molecules with the requisite antibacterial activity; for example, membrane-active cationic steroid antibiotics (CSA). This type of antimicrobial agent was first isolated from tissues of the dogfish shark [115], and has bactericidal activity against both Gram-positive and Gram-negative bacteria [116]. Similar to the antibacterial peptides [26, 58], CSA molecules display cationic facial amphiphilicity with positive charges arranged on one face and hydrophobic residues on the other. Because CSA molecules target membranes based on charge interaction, they effectively kill multiple different types of bacteria [116]. CSA may also have other applications, as their inhibitory effect on angiogenesis and HIV replication was recently reported (www.ceragenix.com). Another interesting design of ABP-mimicking molecules was based on the unique structural patterns of these peptides. Eight-residue oligoureas designed to mimic globally amphiphilic alpha-helical host-defense peptides are effective against both Gram-negative and Gram-positive bacteria (including methicillin-resistant *Staphylococcus aureus*) and exhibit selectivity for bacterial versus mammalian cells [117].

POTENTIAL APPLICATIONS FOR ANTIBACTERIAL PEPTIDES

As diseases caused by antibiotic resistant bacteria become a growing threat to human health, effective antibacterial components represent an emerging need. Many traditional therapies are just beginning to take into account the polymicrobial causes of diseases and the possible repercussions of treatment and prevention [118]. The large spectrum of antibacterial peptide activity against Gram-positive, Gram-negative bacteria, viruses and fungi based on non-specific membrane destruction represents a potential solution for these human health concerns. However, at present, the high cost of synthesis and the limited stability and activity of the known ABPs in some body compartments have to be recognized as unsolved obstacles. In addition to the use of ABPs in relation to human health, there exist other important applications for these molecules. ABPs may be used in prevention and treatment of infectious diseases in animals and plants, of bacterial growth on biological materials, as food preservatives, or as supplements to food and drink, whether as additives or by exogenous expression in modified plants or animals. As an example of these potential applications, overexpression of ABPs in transgenic fish was proposed to limit losses from infectious diseases in aquaculture based on investigations that showed the ability of cationic antimicrobial peptides to protect against infection by the fish pathogen *Vibrio anguillarum* [119]. The small size, simple structure, bactericidal effect, and stability to protease degradation of head-to-tail cyclic peptides of 4-10 residues consisting of alternating hydrophilic (Lys) and hydrophobic (Leu and Phe) amino acids make them potential candidates for the development of effective antibacterial agents for use in plant protection [120]. The past years of intense research have encouraged the hope that ABPs will fulfill their promise for widespread practical use, and that it is only a matter of time until these will be a new solution to the problem of drug-resistant bacteria.

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ABBREVIATIONS

ABPs = Cationic antibacterial peptides
BL = Total lipid extract from *E. coli*
BSA = Bovine serum albumin
CD14 = A 55-kD protein involved in the serum-dependent response of cells to bacterial lipopolysaccharide
CH = Cholesterol
CL = Cardiolipin
CSA = Cationic steroid antibiotics
DPPG = L-α-phosphatidylglycerol dipalmitoyl
FPRL1 = Formyl peptide receptor-like 1
IF-γ = Interferon gamma
LBP = LPS-binding protein
LPS = Bacteria lipopolysaccharide (endotoxin)
LTA = Lipoteichoic acid
MD2 = A small protein associated with TLR4 required for LPS signaling
NF-κB = Nuclear factor kappaB
PBP10 = Gelsolin-derived rhodamine B-QRLFQVKGRR peptide
PIP_2 = Phosphatidylinositol 4,5-bisphosphate
PPI = Phosphoinositides
PC = Phosphatidylcholine
PS = Phosphatidylserine
RBC = Red blood cells
TNF-α = Tumor necrosis factor alpha
TLR = Toll-like receptors

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