REVIEW ARTICLE

Drugs 2003; 63 (4): 389-406
0012-6667/03/0004-0389/$33.00/0

Antimicrobial Peptides
Current Status and Therapeutic Potential

Andreas R. Koczulla and *Robert Bals*

Department of Internal Medicine, Division of Pulmonary Medicine, Hospital of the University of Marburg, Philipps-University Marburg, Marburg, Germany

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Abstract

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system. A variety of AMPs have been isolated from species of all kingdoms and are classified based on their structure and amino acid motifs. AMPs have a broad antimicrobial spectrum and lyse microbial cells by interaction with biomembranes. Besides their direct antimicrobial function, they have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells influencing diverse processes such as cell proliferation, immune induction, wound healing, cytokine release, chemotaxis and protease-antiprotease balance. AMPs qualify as prototypes of innovative drugs that may be used as antimicrobials, anti-lipopolysaccharide drugs or modifiers of inflammation. Several strategies have been followed to identify lead candidates for drug development, to modify the peptides' structures, and to produce sufficient amounts for pre-clinical and clinical studies. This review summarises the current knowledge about the basic and applied biology of AMPs.

Publications on antimicrobial peptides (AMPs) increased significantly during recent years (figure 1). More than 700 antimicrobial peptides have been isolated and can be divided into two classes based on the mechanism of their cellular synthesis: non-ribosomally synthesised and ribosomally synthesised peptides.[1] The former are largely produced by bacteria and are discussed briefly in this article with emphasis on similarities with ribosomally synthesised peptides. The latter are produced by all species of life as an important component of their host defence. AMPs are effector molecules of innate immunity with direct antimicrobial and mediator function.[2-4] They have an important role in scenarios of host defence early during the course of infection. Two aspects have made AMPs a focus of scientific and commercial interest during the last years: (i) recent insight into the basic biology of AMPs showed that these molecules have various functions in host defence, inflammation and tissue regeneration, and are likely involved in the pathogenesis of several diseases; and (ii) AMPs are candidates for drug development. The evolution and rapid spread of resistant microorganisms are significant problems in nosocomial infections and are of increasing importance in community acquired diseases. It is the aim of this review article to summarise the current knowledge on the basic biology of AMPs and to describe attempts to develop these substances as innovative drugs.

1. Basic Biology of Antimicrobial Peptides (AMPs)

1.1 Nomenclature and Families of AMPs

The term 'antimicrobial peptide' (AMP) is descriptive for a peptide with antimicrobial properties. AMPs have been isolated from organisms of all kingdoms. A web based database can be viewed at URL: http://www.bbcm.univ.trieste.it/~tossi/antimic.html showing more than 700 entries of AMPs and other useful information. Most AMPs are cationic (polar) molecules with spatially separated hydrophobic and charged regions. These structural hallmarks are important for the proposed mechanisms of action of peptide antibiotics that are described below. AMP in a broader sense refers to all oligo- or polypeptides that kill microorganisms or inhibit their growth, including peptides that result from cleavage of larger proteins or peptides that are synthesised non-ribosomally.

Non-ribosomally synthesised AMPs are produced by a multiple-carrier thiotemplate mechanism by large, multifunctional peptide syn-

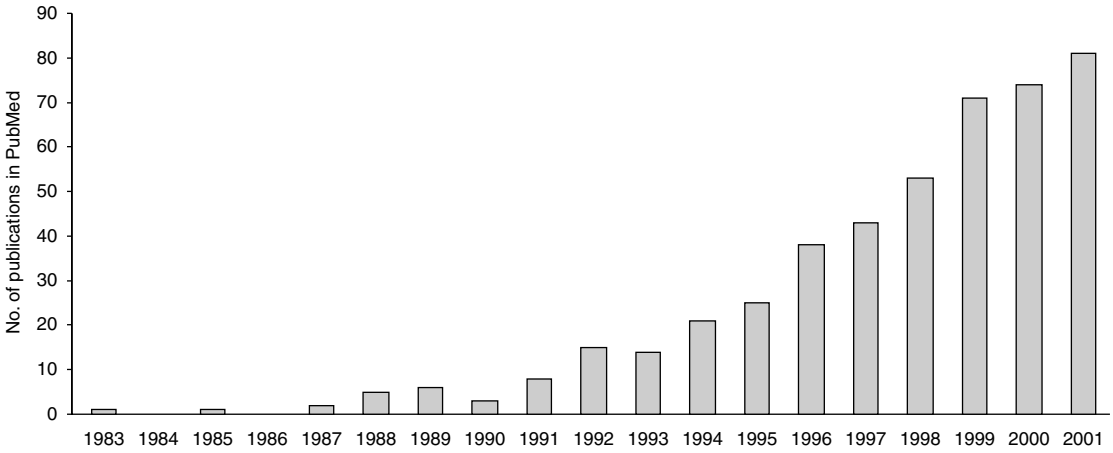

Fig. 1. Publications on antimicrobial peptides. Relevant publications were identified by WWW-based search in PubMed using the search term 'antimicrobial peptide'.

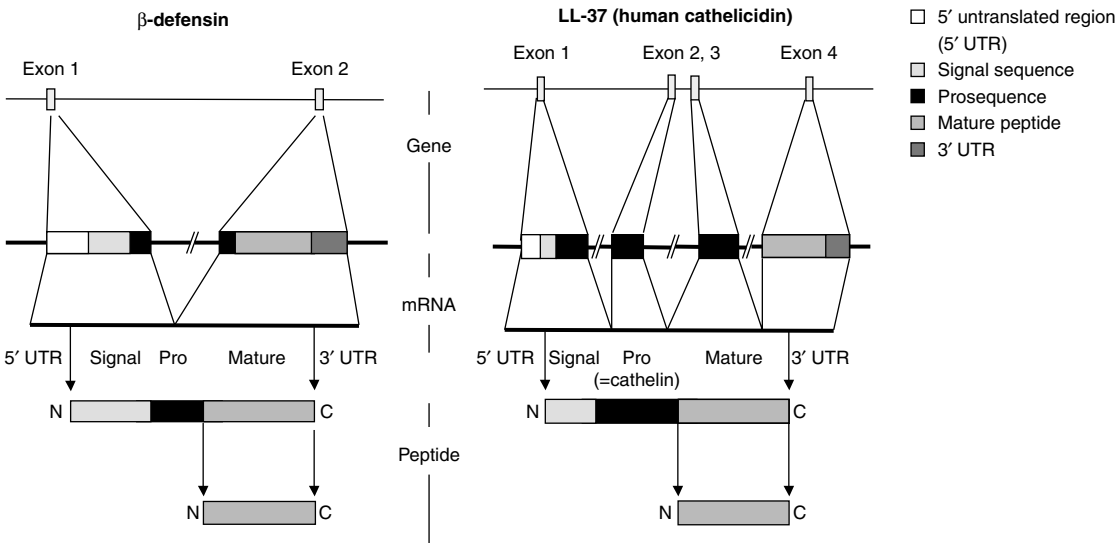

Fig. 2. Genes of vertebrate antimicrobial peptides and processing. Structure of prototypical genes and peptides of the defensin and cathelicidin families. Both genes have several exons the primary translational product of which is a pre-pro-peptide. The C-terminus represents the part of the molecule with antimicrobial activity. The genes are represented schematically.

thetases.[5] Produced mostly by bacteria, these substances are already used for clinical applications: polymyxin B and colistin (polymyxin E, colimycin) have been developed mainly for topical applications.[6,7] Cyclosporin is widely used for immunosuppression. The non-ribosomally synthesised tripeptide ACV is the precursor of penicillin and cephalosporins.[8,9]

AMP in a more narrow sense refers to ribosomally synthesised, gene-encoded peptides, meaning that one gene codes for one peptide. AMP genes of vertebrates have a characteristic intron-exon structure with regulatory elements in their promoter regions (figure 2). Families of AMP genes are located in clustered arrangements in the genome and map to syntenic chromosomal regions in different species, providing clues about their evolutionary development. For eukaryotes, the primary translational product is a prepropeptide consisting of an N-terminal signal sequence for targeting of the endoplasmic reticulum, a pro segment, and a C-terminal cationic peptide that has antimicrobial activity after cleavage (figure 2). The pro segment is often anionic in charge and may have several biological functions including the correct folding of the C-terminus, intracellular trafficking or the inhibition of the activity of the mature peptide. The propeptide is cleaved off during later stages of intracellular processing or after secretion. AMPs are stored in cells as propeptides or mature C-terminal peptides.

1.1.1 Regulation of Expression and Secretion

The expression of AMP genes is tightly regulated. AMPs are expressed in specific tissues that are predisposed for host defence reactions, such as epithelial tissues or inflammatory cells. Some peptides are produced constitutively, such as human β-defensin 1 (hBD-1) or mouse β-defensin 1 (mBD-1).[10] Others are upregulated by the contact of cells with microbial products or proinflammatory mediators. It has been shown that hBD-2, hBD-3, hBD-4, LL-37 and several other AMPs are induced *in vitro* by bacterial products and inflammatory mediators.[11-16] Several studies on patient material also showed increased concentrations of β-defensins in various body fluids during inflam-

Table I. Families and nomenclature of antimicrobial peptides (AMPs). AMPs are grouped according to characteristics of their molecular structure

Peptide	Species, organ	Activity
Group I: Linear, α-helical peptides without cysteines		
Bombinins	Frog, skin	Antimicrobial
Cecropins	Insects, haemocytes, sperm	Antimicrobial
LL-37	Human, neutrophils, epithelial cells	Antimicrobial, chemoattractant
Magainins	Frog, skin	Aantimicrobial
Styelins	Tunicates, haemocytes	Antimicrobial
Clavanins	Tunicates, haemocytes	Antimicrobial
Melittin	Bee, venom	Antimicrobial
Group II: β-sheet structures stabilised by two or three disulphide bridges		
Protegrin	Pig, intestine	Antimicrobial
Tachyplesins	Horseshoe, haemocytes	Antimicrobial
Defensins	Vertebrates, immune cells, epithelia	Antimicrobial, chemoattractant
Insect defensins	Insects, haemocytes	Antimicrobial
θ-Defensins	Monkeys, neutrophils	Antimicrobial
Plant defensins	Plants, seeds, leaves	Antimicrobial
Drosomycin	Insects, haemocytes	Antimicrobial
Group III: Peptides with a predominance of one or more amino acids		
PR-39	Pig, intestine, neutrophils	PR-39, angiogenesis, wound healing
Bac5, Bac7	Cow, neutrophils	Antimicrobial
Drosocin	Drosophila,[a] haemolymph	Antimicrobial
Metchnikowin	Drosophila,[a] haemolymph	Antimicrobial
Group IV: Peptides with loop structures		
Bactenecin	Cow, neutrophils	Antimicrobial
Ranalexin	Frog, skin	

a *Drosophila melanogaster*, the fruit fly.

matory or infectious diseases, such as pneumonia[17] or cystic fibrosis.[18] Cutaneous injury induces the release of cathelicidin AMPs active against group A streptococcus.[19] Mechanisms involved in the regulation of human β-defensins involve lipopolysaccharide (LPS) detection by CD14 and toll like receptor 2 and activation of the nuclear factor (NF)-κB cascade.[13,20] In *Drosophila melanogaster* (Drosophila; the fruit fly) several groups of AMPs have been identified.[21-23] These peptides are regulated by several signalling pathways including the immuno deficiency and toll pathways. The immune system of the fruit fly recognises different classes of microorganisms and responds with the production of the appropriate spectrum of peptide antibiotics.[22,24-28]

1.1.2 Classification and Nomenclature

AMPs can be grouped according to their size, conformational structure or predominant amino acid structure (table I); however, the diversity of these molecules is so great that it is difficult to categorise them in a generally accepted classification. On the basis of their gross composition and 3D structure, peptide antibiotics can be divided in four main classes.[29-31]

- Group I: linear peptides with an α-helical structure.
- Group II: β-sheet structures stabilised by disulphide bridges.
- Group III: peptides with predominance of one or more amino acids.
- Group IV: peptides with loop structures.

Table I lists several examples for these groups. On the basis of structural homology motifs, a different classification of AMP families can be generated. For example, cathelicidins are characterised by the conserved sequence of their pro-peptide and include mature peptides of various structures. In addition to positively charged AMPs, peptide antibiotics with a negative charge at neutral pH have also been identified.[32,33] Some of these peptides seem to originate as cleavage products from larger proteins. Individual organisms produce peptides of various families. Insects synthesise AMPs with diverse structures that belong to different families.[4,21,22,24] In some cases these families of insect AMPs have similar names to families of mammalian AMPs. However, these similar names do not necessarily reflect similarity of the amino acid structure or evolutionary homology.

AMPs are isolated using variations of classical methods of protein and peptide biochemistry combined with a variety of antimicrobial assays. For this 'bioscreening' approach large amounts of starting material are processed and fractionated using ion exchange or reverse phase high performance chromatography. Fractions are collected and assayed for the presence of antimicrobial activity. A variety of assays have been developed differing in sensitivity, reproducibility and scale. It is important to measure microbicidal activity in the correct fashion since AMPs tend to precipitate at high concentrations or bind to surfaces. Assays include radial diffusion assays, microbroth dilution assays, and tests based on luminescence or release of potassium from killed bacteria.[34-37] Polymerase chain reaction (PCR) cloning strategies based on the use of degenerated primer have been applied to identify additional members of families characterised by conserved sequences. Using this approach, novel defensins[38,39] and cathelicidins[40] have been cloned and subsequently characterised. Computer-based searches of databases containing information on sequences of genomes or expressed genes have recently been used to identify novel AMPs. Using this approach, candidate sequences are identified using tools of bioinformatics. Besides molecular biological analysis of the putative new AMPs, the peptides also have to be synthesised to assay for their biological activity. Two new human β-defensins, and several candidates of mouse and human peptides, have been identified using this approach.[41-44]

The next sections provide an overview on peptides of various families that, in the author's opinion, might be most relevant to the fields of basic biology and drug development. The selection is therefore intentionally limited and subjective.

Defensins

Mammalian defensins are cationic, relatively arginine-rich, nonglycosylated peptides with a molecular weight of 3.5 - 4.5 kDa and contain six cysteines that form three characteristic intramolecular disulfide bridges.[45] According to the spacing of the cysteines, the alignment of the disulfide bridges and the overall molecular structure, defensins can be divided into three classes: α-defensin, β-defensin and θ-defensins. AMPs of insects or plants have also been termed defensins, however, they have different structural features compared with vertebrate defensins.[21,46]

α-Defensins: α-defensins are 29-35 amino acids in length, contain three disulfide bridges in a 1-6, 2-4, 3-5 alignment and reveal a triple stranded β-sheet structure with a β-hairpin which contains cationic amino acids. The first human α-defensin was isolated from neutrophils in 1985.[47] At the present time, six α-defensins have been identified from humans. Human neutrophil peptides 1-4 (HNP 1–4) are localised in azurophilic granules of neutrophil granulocytes where they represent the principal protein and contribute to the oxygen-independent killing of phagocytosed microorganisms.[47,48] The two other α-defensins, human defensins 5 and 6 (HD 5–6), are primarily found in Paneth's cells of the small intestine. The genes of α- and β-defensins are located in a cluster on chromosome 8p23.[49] The gene for HNP-2 has not been localised at this location indicating that HNP-2 is a proteolytic product of HNP-1 or HNP-3. The disruption of the gene for matrilysin

(MMP7), a tissue metalloproteinase, in a knockout mouse model prevented the cleavage of the pro-peptide of cryptins (cathelicidins of the mouse) resulting in increased susceptibility of the animals to bacterial infection.[50] Cleavage of the pro-peptide takes place in the lumen of the intestinal crypts where AMPs are present in high concentrations together with the secreted MMP7.

β-*Defensins:* in 1991, an AMP from cow tongue called tracheal antimicrobial peptide (TAP)[51] was isolated. TAP contains six cysteines connected by three disulfide bridges, however, spaced in a different way compared with α-defensins. Therefore, this new family of AMPs was named β-defensins. β-defensins are 36–42 amino acids in length, reveal a disulfide alignment of 1–5, 2–4, 3–6, and have been isolated from several species. The first human β-defensin, called human β-defensin 1 (hBD-1), was originally isolated from large volumes of haemofiltrate,[52] and is expressed constitutively in epithelial cells of the urinary and respiratory tract.[53-55] Human β-defensin 2 (hBD-2) was isolated from psoriatic skin using an affinity chromatography procedure applying columns coated with components of *Escherichia coli.*[56] hBD-2 was found to be expressed in epithelia of the inner or outer surfaces of the human body, such as skin and the respiratory and gastrointestinal tract.[35,57] Both peptides have been detected in airway secretions and concentrations have been found in the μg/ml range.[35,57] hBD-3 was identified by a bioscreening approach and in parallel by screening of databases.[41,43,58] hBD-4 was identified solely by searches of genomic databases.[42] Recently, 28 new human and 43 new mouse β-defensin genes in five syntenic chromosomal regions were identified by screening of human and murine genome databases.[44] The processing of β-defensins is likely similar to α-defensins, however, no detailed analysis has been published until now.

θ-*Defensins:* a novel class of defensins has been isolated from rhesus monkey neutrophils and named θ-defensins according to their circular molecular structure.[59] The peptide rhesus theta-defensin 1 (rTD-1) is produced by the posttranslational ligation of two truncated α-defensins and demonstrated salt-independent antimicrobial activity. rTD-2 and rTD-3 are formed by tandem nonapeptide repeats derived from only one of the rTD-1 precursors.[60] No data about the presence of these molecules in different tissues or their regulation are available at this time.

Cathelicidins

Peptide antibiotics of the cathelicidin family contain a highly conserved signal sequence and pro-region ('cathelin' = cathepsin L inhibitor) but show substantial heterogeneity in the C-terminal domain that encodes the mature peptide which can range in size from 12 to 80 or more amino acids.[61,62] The only human cathelicidin, LL-37 or hCAP-18, was isolated from human bone marrow.[63-65] LL-37/hCAP-18 is expressed in myeloid cells where it resides in granules but is also found in inflamed skin. LL-37/hCAP-18 has been found to be regulated by inflammatory stimuli.[64,66] In the airways, the peptide is produced by the same cell types as the β-defensins and secreted into the airway surface fluid.[67] LL-37 has been detected in tissue culture supernatants of respiratory epithelial cells as well as in lung washings from patients.[67,68] At this time there are no details known about the processing of LL-37/hCAP-18 in epithelial cells. In neutrophils, where LL-37/hCAP-18 is localised to specific granules, the peptide is stored in its pro-peptide form and cleaved after secretion by the activity of protease 3.[69] The LL-37/hCAP-18 gene consists of four exons and is localised on chromosome 3. Cathelicidins have been isolated from mouse (CRAMP), rat (rCRAMP), pig (protegrin, PMAP-23, PR-39), monkey (rhLL-37,[38] RL-37[70]), rabbit (CAP-18) and sheep (SMAP 29, SMAP34). Mice deficient in CRAMP were found to be more susceptible to bacterial infections of the skin.[71]

Granulysin

Granulysin is an AMP produced by human cytolytic T lymphocytes and natural killer (NK) cells.[72] It is active against a broad range of microbes, including Gram-positive and Gram-negative bacteria, fungi and parasites. Although function-

ally related to other AMPs, defensins and cathelicidins, granulysin is structurally distinct. Like porcine NK lysin and amoebapores made by *Entamoeba histolytica*, granulysin is related to saposins, small lipid-associated proteins present in the central nervous system. The presence of this molecule indicates a broader and perhaps more significant role for T lymphocytes in both innate and acquired antimicrobial defences.[73,74]

Bacteriocins, Lantibiotics

The bacteriocins are a large group of AMPs that are ribosomally synthesised by micro-organisms belonging to different eubacterial taxonomic branches.[75] Bacterial-derived AMPs enjoy a large degree of structural and chemical diversity. Some of them are small cationic membrane-active compounds that form pores in the target cells, disrupting membrane potentials and causing cell death. The lantibiotic peptides (from 'lanthionine-containing antibiotic') are unique in that lactic acid bacteria produce them. They contain unusual amino acids, such as dehydrated and lanthionine residues.[76,77] This group of peptides has attracted much attention in recent years as a result of the success of the well characterised lantibiotic, nisin, as a food preservative.[78] Nisin combines the high affinity for the membrane-bound peptideoglycan precursor Lipid II with a pore-forming ability.[79,80] Numerous other lantibiotics have since been identified and can be divided into two groups on the basis of their structures, designated type-A and type-B based on their structural and functional features. In general, type-A lantibiotics are elongated, cationic peptides up to 34 residues in length that show similarities in the arrangement of their lanthionine bridges. These peptides primarily act by disrupting the membrane integrity of target organisms, and include nisin, subtilin and epidermin. Type-B peptides are globular, up to 19 residues in length, and act through disruption of enzyme function, for example, inhibition of cell wall biosynthesis.[77]

In addition to these families of AMPs, several novel peptides have recently been identified such as dermcidin from human skin.[81]

1.2 Functions of AMPs

1.2.1 AMPs as Effector Substances of the Innate Immune System

The innate immune system involves a broad functional spectrum of host defence measures against pathogenic microbes including the recognition of pathogen-associated molecular patterns (PAMPs), the stimulation of adaptive immunity and the secretion of host defence substances. Genes of innate immunity, including those of AMPs, are encoded in the germline and do not require the gene rearrangement essential to adaptive immune recognition. Innate immunity is an evolutionarily ancient part of the host defence system. Drosophila have served as model organisms for innate immune recognition and effector mechanisms. Several of the innate immune mechanisms found in the fruit fly have their homologous counterparts in vertebrates. The families of toll receptors (Drosophila) and toll-like receptors (TLRs, vertebrates) serve as prominent examples.[82] TLRs are examples of pattern recognition receptors (PRR) of vertebrates that are expressed in different cell types including epithelial, inflammatory and immune cells. It has been described for Drosophila that different classes of microorganisms activate specific receptors of innate immunity finally resulting in an host defence response aimed at the specific microorganism.[22,24,27,82] This intriguing hypothesis of an adaptive innate immune response with AMPs as effector molecules has yet to be proven for vertebrates.

1.2.2 Antimicrobial Activity of AMPs

The antimicrobial activity of peptide antibiotics was deduced from *in vitro* tests assaying purified substances against micro-organisms. AMPs have a broad spectrum activity against Gram-positive and Gram-negative bacteria as well as against fungi and enveloped viruses. The minimal inhibitory concentrations of the more effective peptides are in the range from 0.1–10 μg/ml. AMPs show synergistic activity with other host defence molecules, such as lysozyme and lactoferrin.

The antimicrobial activity is based on several mechanisms (figure 3). In most cases interactions

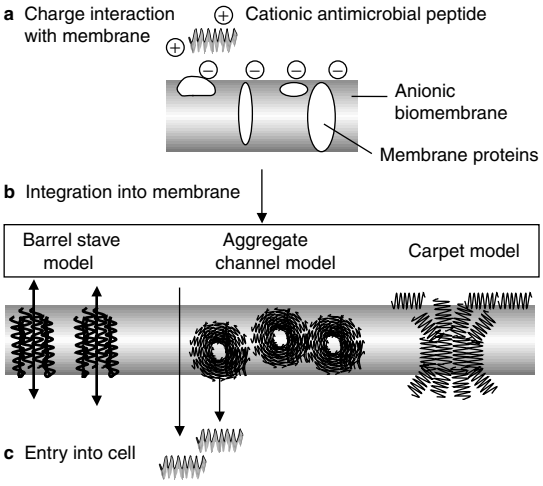

Fig. 3. Antimicrobial activity of antimicrobial peptides (AMPs). AMPs accumulate near microbial membranes as a result of electrostatic interactions between negatively charged membranes and positively charged peptides (**a**). In a next step, the peptides associate with the membranes leading to a destabilisation of the membrane and subsequent cell death of the microorganism (**b**). Several models of action may apply. Barrel stave model: α-helical, amphipathic peptides accumulate in the membrane as a barrel-like structure with non-polar molecule parts facing the membrane lipids and forming a hydrophilic pore spanning the membrane. Aggregate channel model: after binding to the membrane the peptides aggregate in clusters in the membrane with subsequent dynamic formation of pores for short time. AMPs can also enter the intracellular space through this mechanism. Carpet model: AMPs cover the microbial cell membrane in a carpet-like formation, which causes a collapse of the integrity of the membrane. In addition to membrane interaction, some AMPs enter the cell of the microorganisms and inhibit cellular processes such as RNA or protein synthesis (**c**).

between the peptide and surface membranes of the target organisms are thought to be responsible for the activity. The initial binding is thought to depend on electrostatic interactions between the positively charged peptides and the negatively charged molecules at the surface of the target cell. A secondary step results in the modification of the biophysical properties of the membrane caused by direct interactions with the peptide. The membrane-active properties of AMPs have been analysed by model systems demonstrating induction of leakage of artificial liposomes[83-86] and the formation of ion-permeable channels.[87,88]

Three main mechanisms have been suggested for peptide permeation of the membrane of the target cell:[29] (i) a barrel-stave mechanism involves the formation of transmembrane channels in a voltage-dependent manner with non-polar molecule parts facing the membrane lipids and forming a hydrophilic pore spanning the membrane;[89] (ii) the aggregate channel model involves aggregation of peptides in unstructured clusters in the membrane allowing the dynamic formation of pores for short time and the leakage of intracellular components. AMPs can also enter the intracellular space through this mechanism;[90] and (iii) a carpet-like mechanism describes the covering of the microbial cell membrane by a carpet of AMPs. The integrity of the membrane collapses by the formation of worm holes that form by the bending of the lipid layer back on itself.[91,92]

These interactions described in the three models are proposed to lead to loss of membrane function including breakdown of membrane potential, leakage of metabolites and ions and alteration of membrane permeability. The partial selectivity of AMPs for prokaryotic cells seems to depend on the different lipid composition associated with different amounts of negative charges of membranes of micro-organisms compared with eukaryotic cells. Other mechanisms of antimicrobial activity include the inhibition of protein- and RNA-synthesis by *Bac5* and *Bac7*[93] or the halting of protein production by PR-39.[94] The term 'self-promoted' uptake describes a mechanisms by which AMPs interact with LPS at the surface of Gram-negative bacteria to accumulate at the surface and to enter the microbial cell.[30] The antimicrobial spectra of individual peptides depend on their structure and amino acid sequence.

Functional studies on antimicrobial activity have primarily been restricted to *in vitro* experiments using purified components. Recently, several groups published results that provided proof of the host defence function of AMPs in living organisms. Indirect *in vivo* evidence for the host defence function of AMPs came from a study on mice with a disrupted gene for matrilysin, also called

metalloprotease 7. Mice with missing matrilysin were more susceptible to infections with enteropathogens.[50] Studies in a human bronchial xenograft model revealed decreased antimicrobial activity of airway surface fluid after inhibition of hBD-1 synthesis by antisense oligonucleotides.[54] Mice deficient in an AMP, mouse β-defensin-1 (mBD-1), revealed delayed clearance of *Haemophilus influenzae* from lung.[95] Mice with deleted CRAMP, the murine homologue of LL-37, showed more prominent infection after cutaneous inoculation of bacteria.[71] Drosophila mutants for both the immune deficiency gene (*imd*) and the toll (*Tl*) signalling pathways fail to express a significant extent of the antimicrobial genes, and rapidly succumb to either fungal or bacterial infections, indicating that these pathways, and likely the involved AMPs, are essential for antimicrobial resistance in insects.[96] In contrast, constitutive expression of a single AMP can restore wild-type resistance to infection in immunodeficient Drosophila *imd* and *spatzle* double mutants that do not express any known endogenous AMPs gene.[97] Also, the overexpression of LL-37 by viral gene transfer resulted in augmentation of innate host defence in an bronchial xenograft model of cystic fibrosis, and in murine animal models of pneumonia and septic shock.[98,99]

In addition to their antimicrobial activity, defensins and cathelicidins can bind to LPS and inactivate the biological functions of this endotoxin.[100] This property has been used to reduce LPS mortality in murine models of endotoxaemia by application of LL-37/hCAP-18 derived peptides.[101]

Taken together, AMPs have host defence functions by direct antimicrobial activity and represent effector molecules of the innate immune system.

Microbial Resistance Against AMPs

The development of microorganisms that are resistant to AMPs is a rare event.[102] Gonococcal susceptibility to the lethal action of protegrin 1, LL-37 and other AMPs is modulated by an energy-dependent efflux-system, a member of the resistance/nodulation/division efflux pump family.[103] Increase of the phosphocholine content of cell walls of *H. influenzae* decreased the susceptibility to LL-37.[104] Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other AMPs.[105] When exposed to the environment of the airways of patients with cystic fibrosis, *Pseudomonas aeruginosa* is able to modify the structure of the LPS attached to the outer membrane.[106] These changes of the endotoxin decrease the susceptibility of these bacteria to cationic AMPs. Infection or chronic inflammation seems also to be responsible for the transcriptional downregulation of AMPs. Gastrointestinal shigella infections in humans are associated with reduced expression of LL-37 in epithelial cells.[107] In general, mutants susceptible to AMPs are more efficiently inactivated by phagocytes and are virulence-attenuated, indicating that resistance to AMP may play a key role in bacterial infections. The reason why resistance to AMP seems to be relatively rare remains speculative. One reason might be that AMPs target structures or processes which are conserved features of the microorganisms and are important for survival and rapid growth.

1.2.3 Role of AMPs in Inflammation, Angiogenesis and Cell Function

AMPs have a variety of other biological effects besides their antimicrobial activity. On the basis of their membrane activity, AMPs have a concentration-dependent toxicity towards eukaryotic cells. High concentrations of α-defensins have been described in secretions of patients with cystic fibrosis[108] and chronic bronchitis,[109] where these substances probably contribute to the overwhelming inflammation. α-Defensins induce interleukin (IL)-8 production by lung epithelial cells.[110] The cellular damage by α-defensins is probably augmented by defensin-induced lysis of epithelial cells[111] or binding of α-defensins to protease inhibitors of the serpin family such as α-1-antitrypsin.[112]

Besides this unspecific toxicity, some AMPs bind to cellular receptors at low concentrations, activate intracellular signalling pathways and stimulate various cellular functions (table II). α-

Table II. Non-microbicidal activities of antimicrobial peptides mediated by specific receptors

Peptide	Receptor	Cell type	Function
hBD-1, hBD-2	CCR-6	Immature dendritic cells, memory T lymphocytes, mast cells	Chemoattraction, release of cytokines and histamine
hBD-3, hBD-4	?	Monocyte	Chemoattraction
mBD-2, mBD-3	CCR-6	Immature dendritic cells, memory T lymphocytes	Chemoattraction
LL-37	FPRL1	Monocyte, neutrophils, epithelial and endothelial cells	Chemoattraction, cell activation
α-defensins	?	Epithelial cells, T lymphocytes, immature dendritic cells, monocytes	Chemoattraction, cell activation, release of cytokines

CCR = CC chemokine receptor; **FPRL1** = formyl peptide receptor like 1.

Defensins are able to stimulate a variety of cells by mechanisms not yet identified. They attract human $CD4^+/CD45RA^+$ or $CD8^+$ T cells,[113,114] immature dendritic cells[115] and monocytes.[113] They also induce release of interferon (IFN)-γ, IL-6 and IL-10 from T cells.[116] α-Defensins also inhibit the adrenocorticotrophic hormone (ACTH)-stimulated cortisol production.[117]

hBD-1 and hBD-2 were found to bind to a chemokine receptor known as CCR-6.[118] This receptor is found on immature dendritic and memory T cells ($CD4^+/CD45RO^+$), and consequently these findings are interpreted as a link between innate and adaptive immune mechanisms mediated by defensins. hBD-3 and hBD-4 chemoattract monocytes by mechanisms that have not yet been clarified.[41,42]

In addition, LL-37 was found to bind to formyl peptide receptor like 1 (FPRL1), a promiscuous receptor expressed on a variety of cells including neutrophils, monocytes and lymphocytes.[115] By activation of this G-protein coupled receptor, LL-37 attracts neutrophils, monocytes and CD4+ T cells, and activates mast cells.[119] LL-37 also binds to apolipoprotein A-I.[120]

PR-39, a cathelicidin AMP from porcine neutrophils, reaches the intracellular space of host cells[121] where its binds to SH3 domains of the cytosolic component of the NADPH (nicotinamide adenine dinucleotide phosphate) complex protein $p47^{phox}$ and the signalling adapter protein $p130^{cas}$.[122] In recent months, it was realised that PR-39 stimulates angiogenesis by binding to the α7 subunit of the 26S proteasome and modulating the ubiquitin-proteasome pathway without affecting overall proteasome activity.[123] This selective inhibitory effect of the peptide in the proteasome pathway also results in anti-inflammatory activity by blocking the degradation of the NF-kB inhibitor IkBa.[124] Furthermore, PR-39 is chemoattractive for neutrophils in a calcium-dependent and pertussis toxin inhibitable reaction, and contributes to wound healing by stimulating the expression of syndecans, cell surface heparan sulfate proteoglycans.[121] These multiple biological activities of PR-39 have been applied to prevent post-ischaemic microvascular dysfunction, partly by the inhibition of neutrophil adhesion and oxidant production.[125,126]

Interestingly, it has been found that IFN-inducible ELR-CXC chemokines display defensin like antimicrobial activity highlighting a structure-function relationship between defensins and chemokines.[127]

Taken together, vertebrate AMPs have a variety of additional functions besides their microbicidal function (figure 4). The impact of these non-microbicidal functions on the pathogenesis of diseases is completely unknown. The non-microbicidal functions offer interesting opportunities to investigate the roles of AMPs in inflammatory diseases; however, they might also cause side effects when these peptides are used as therapeutics.

2. AMPs as Drugs

The broad spectrum of activity and the low incidence of bacterial resistance are attractive features of AMPs. The specific mode of action involving pore formation in biomembranes makes AMPs a new class of potential antibiotic drugs with a broad field of possible applications. Various at-

tempts have been made to develop AMPs as innovative antimicrobial or anti-LPS drugs; however, to design them rationally is difficult because of the complex interaction of the peptides with membranes and with each other. Table III summarises potential advantages and disadvantages of AMPs as innovative therapeutic antimicrobials.

2.1 Development of AMPs as Drugs

Identification of natural AMPs is one focus of basic scientists and also the starting point for drug development. Bioscreening, different cloning strategies and computer-based database searches have been used to identify novel AMPs as described above. Novel peptides for drug development are produced by modification of natural peptides. Several structural features have been identified to be relevant for the microbicidal function of AMPs: the size, the sequence, the charge, the degree of structuring (helicity), the overall hydrophobicity, the amphipathicity, and the angles subtended by hydrophobic and hydrophilic surfaces of the helical molecule.[128] Breaking the helix by insertion of proline residues resulted in slight decrease of antimicrobial activity associated with decreased haemolytic activity for histatin,[129] whereas other peptides such as magainins completely lost activity.[130,131] The concept that amphipathicity is a key feature of AMPs has initiated the *de novo* synthesis of simple peptides consisting of alternating sequences of hydrophobic and positively charged stretches.[29] High mean hydrophobicity has been correlated with increased cytotoxic activity against eukaryotic membranes.[29] There is no simple correlation between activity and charge. When the net charge becomes more positive the binding to negatively charged surfaces of microorganisms is increased but the formation of trans-membrane pores is inhibited.[132-134] Since all these structural features are strongly interrelated, it is difficult to predict the antimicrobial or cytotoxic activity from a given amino acid sequence. In general, peptides with a moderately high positive charge, a large hydrophobic moment and a small hydrophobic angle tend to have high activity against microbial membranes, low cytotoxicity and a preference for carpet-like mechanisms of action. In contrast, peptides with a low positive charge, a small amphipathic moment and high intrinsic hydrophobicity reveal high activity to microorganisms and to eukaryotic membranes and a preference to form barrel-like structures.[135-137] A variety of naturally occurring peptides has been modified to select the desired properties.

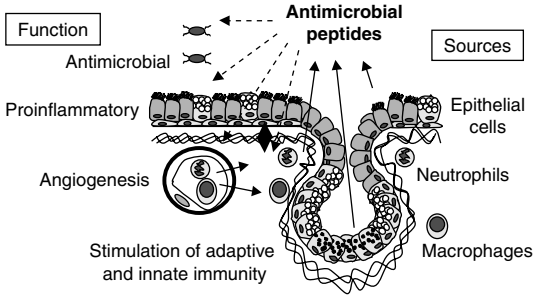

Fig. 4. Activities of antimicrobial peptides (AMPs) as effector molecules of the innate immune system. AMPs are produced by a variety of cells including epithelial, inflammatory and immune cells. Microorganisms or inflammatory mediators induce the expression of AMPs by interaction with specific receptors. AMPs have direct microbicidal activity. In addition, AMPs modulate inflammation, cell proliferation and cell activation.

Classical methods of combinatorial chemistry have been used to search for amino acid substitutions that result in higher antimicrobial activity or less cytotoxicity.[138-140] Caused by intrinsic methodological problems of combinatorial approaches the peptides can only reach relatively short length. Structural motifs identified by this approach usually share little similarity with those of naturally occurring peptides. Despite promising reports in the literature, no peptide identified by combinatorial chemistry has reached clinical studies so far.

Recently helical peptides were generated based on different strategies. Circularised α-defensins showed a salt-insensitive antibacterial activity.[141] Peptide rings consisting of six or eight alternating D- and L-amino acids were found to form tubes in

Table III. Theoretical advantages and disadvantages of antimicrobial peptides as potential innovative antimicrobials

Advantages	Disadvantages
Novel mechanism of action	Sensitivity to ion strength
Sterilising	Susceptibility to proteolysis
Other host defence functions	Relatively high concentrations necessary
Fast activity	Intrinsic side affects based on mediator function
	High production cost
	Toxicity
	Immunogenicity
	Slow tissue penetration due to charge

biomembranes making them permeable to molecules up to 10 kDa in size.[142] Variations of the amino acid composition resulted in antimicrobial activity against different bacteria. These peptides were found to effectively clear bacterial infections in murine animal models without significant adverse effects. A disadvantage of this approach is the necessity for chemical synthesis which is expensive compared with expression strategies.

A specific problem is the high cost of chemical synthesis for the production of AMPs. It is possible to chemically synthesise most AMPs including β-defensins with their complex disulfide bonds; however, the costs preclude most peptides from clinical development. Biological expression strategies have been developed to circumvent this problem. Several groups have developed recombinant baculovirus systems for expression of β-defensins and have obtained purified, correctly folded and active peptides in milligram quantities.[55,57] AMPs have been produced in transgenic plants[143-145] and overexpressed in bacteria.[146,147] Defensins have also been produced in the milk of transgenic animals.[148] After biological production, a thorough process of purification is necessary to avoid contamination with proteins of the host that would result in an immune response in the patient.

The coding sequence of AMPs can also be transferred into the target cells by means of gene transfer, as shown by the overexpression of LL-37 in animal models[98,99] or the transfer of histatin 3 to salivary glands.[149] Another interesting approach is to stimulate the expression of AMPs by small chemical compounds. The application of the essential amino acid L-isoleucine to airway epithelial cells upregulated the expression of epithelial defensins.[150]

2.2 Pre-Clinical and Clinical Studies

AMPs and their derivatives have been used in several approaches in animal or human studies to evaluate their usefulness for clinical applications. Animal studies used various disease models and application routes. Peptides of various classes were applied in models of pneumonia,[37] septic shock[101] and oral mucositis.[151] It has been demonstrated that the peptides MBI-27 and MBI-28 can protect against *P. aeruginosa* peritoneal infections and against endotoxaemia in mouse models.[152] Liposomal indolicidin protected mice against systemic fungal infections.[153] In addition to direct application of AMPs, we overexpressed LL-37 in airway epithelium by means of adenoviral gene transfer and found augmentation of the innate host defence in murine models of septic shock and pneumonia.[98,99] Other applications in animal models include fusion of mouse β-defensins to a nonimmunogenic tumour antigen, lymphoma idiotype, resulting in a protective immune response against the tumour in an animal model.[154] In a diagnostic approach AMPs were labelled radioactively, and were able to localise inflammation and to discriminate between bacterial infections and sterile inflammatory processes.[155-157] Taken together, animal studies provided first proof that AMPs can be used to modify the course of infectious and inflammatory diseases.

Small biotech companies in association with larger pharmaceutical companies forming strategic alliances carried out human studies using AMPs. Table IV gives an overview on the companies involved in the development of AMPs as drugs. Recently, large pharmaceutical companies have started research programmes related to AMPs. Despite several preclinical studies by small biotechnology companies on the host defence peptides, there are unanswered concerns about production

costs, lability to proteases *in vivo* and unknown toxicities. AMPs from the skin of frogs, called magainins, have been one of the first substances to go through a drug development process. The magainin derivative pexiganan (MSI-78; Magainin Pharmaceuticals Inc., Plymouth Meeting, PA, USA) was investigated in phase III trials of 926 patients and topical pexiganan has been found to show equivalence to oral ofloxacin against polymicrobic diabetic foot ulcer.[158] A previous phase III study of pexiganan against impetigo failed because of a very large placebo effect associated with merely washing the infected site. A US FDA panel recently rejected this drug, and the developing company subsequently changed its name and refocused its activities.

Both ambicin (also known as nisin, a lantibiotic cationic peptide produced by AMBI, Purchase, New York, USA) and iseganan (also known as IB-367, a protegrin-like cationic peptide produced by Intrabiotics, Mountain View, California, USA) have undergone phase I (safety) clinical trials successfully and entered further clinical evaluation. They have been considered for stomach ulcers caused by *Helicobacter pylori* (nisin) and oral mucositis (iseganan). A phase I safety trial of aerosolised iseganan has been initiated in healthy adults with the objective of using this peptide in cystic fibrosis patients with chronic *P. aeruginosa* lung infections. Micrologix Biotech Inc., Vancouver, British Columbia, Canada, recently entered two agents (MBI-594AN and MBI-226) into phase II or III clinical trials for catheter-associated infections and serious acne infections.

3. Conclusion

AMPs have emerged as effector substances of the innate immune system involving not only activities as endogenous antibiotics but also as mediators of inflammation. Several important topics will have to be addressed in the future: (i) identification of novel AMPs – it is likely that families of AMPs consist of multiple molecules with different functions; (ii) analysis of the biologically relevant functions of AMPs involving both antimicrobial activity and other functions – AMPs might contribute to the development of diseases not only as endogenous host defence substances but also as pro- or anti-inflammatory mediators; and (iii) development of AMPs as drugs involves optimised strategies for candidate identification, for modification of phamacodynamic and pharmacokinetic

Table IV. Companies involved in the development of antimicrobial peptides as drugs (based on the literature and a search of the WWW using common search engines)

Company name	Peptide name	Target disease and status of clinical development
Magainin Pharmaceuticals Inc. (renamed Genaera, Plymouth Meeting, PA, USA)	Pexiganan (MSI-78)	Infected diabetic food ulcers (III, local use, no US FDA approval)
Micrologix Biotech Inc. (Vancouver, Canada)	MBI 594AN	Acne (II, local use), finished
	MBI-226	Catheter sepsis (III, local use)
Intrabiotics (Mountain View, CA, USA)	Iseganan (IB-367; protegrin)	Mucositis (II, oral – topical use, failed); lung infection in cystic fibrosis (II, inhalative – local use)
Xoma Corp. (Berkeley, CA, USA)	Neuprex (recombinant BPI-derivative)	Meningitis (III, systemic use, no US FDA approval)
Demegen (Pittsburgh, PA, USA) (Periodontix acquired by Demengen)	P-113 (histatin analogue)	Oral candidiasis, mucositis (II, oral use); lung spray (Investigational New Drug Application with FDA)
	D2A21	Burn wounds, infected wounds (I, local use)
Cubist Pharmaceuticals (Lexington, MA, USA)	Daptomycin	Sepsis (III)
AM (Pharma, Bilthoven, The Netherlands)	Lactoferricin-B	Antifungal (preclinical, systemic use)
Entomed (Illkirch, France)	Heliomycin	Antibacterial (preclinical, systemic use)
Trimeris (Durham, NC, USA)	Enfuvirtide (T-20)	HIV (III completed)

profiles, and for production. Studying the biology of AMPs should allow the development of novel therapeutics for infectious or inflammatory diseases.

Acknowledgements

Studies in the Dr. Bals' laboratory related to innate immunity of the respiratory tract are supported by grants of the Deutsche Forschungsgemeinschaft (Ba 1641/1, Ba 1641/3-1) and the Mukoviszidose e.V. We thank Dr. D. J. Weiner (University of Pennsylvania, PA, USA) for helpful discussions. The authors declare no conflicts of interest relevant to the contents of this manuscript.

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Correspondence and offprints: Dr *Robert Bals*, Department of Internal Medicine, Division of Pulmonology, Hospital of the University of Marburg, Baldingerstrasse, 35043 Marburg, Germany.
E-mail: bals@mailer.uni-marburg.de