Journal of Peptide Science
J. Peptide Sci. **11**: 697–706 (2005)
Published online 1 August 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.698

JOURNAL OF PEPTIDE SCIENCE

Review

Antibacterial peptides and proteins with multiple cellular targets¶

LASZLO OTVOS, Jr.*

OLPE, LLC, 801 Mockingbird Lane, Audubon, PA 19403, USA

Received 26 April 2005; *Revised 25 May 2005*; *Accepted 29 May 2005*

Abstract: Native antimicrobial peptides and proteins represent bridges between innate and adaptive immunity in mammals. On the one hand they possess direct bacterial killing properties, partly by disintegrating bacterial membranes, and some also by inhibiting functions of intracellular biopolymers. On the other, native antimicrobial peptides and proteins upregulate the host defense as chemoattractants or by various additional immunostimulatory effects. Structure-activity relationship studies indicate that residues responsible for the activities on bacterial membranes or for the secondary functions do not perfectly overlap. In reality, in spite of the relatively short size (18–20 amino acid residues) of some of these molecules, the functional domains can frequently be separated, with the cell-penetrating fragments located at the *C*-termini and the protein binding domains found upstream. As a cumulative effect, multifunctional and target-specific (agonist or antagonist) antimicrobial peptides and proteins interfere with more than one bacterial function at low concentrations, eliminating toxicity concerns of the earlier generations of antibacterial peptides observed in the clinical setting.

Keywords: antimicrobial peptide; bacterial membrane; cell penetration; enzyme inhibition; host defense; immunostimulatory; protein folding

THE CONCEPT OF MULTIFUNCTIONAL ANTIMICROBIAL PEPTIDES

It is an undeniable fact that upon injury or bacterial infection higher organisms upregulate their production level of native antimicrobial peptides [1,2]. Peptide antibiotics represent major defense mediators in mammals [3] and are naturally considered viable alternatives to small molecules in antimicrobial drug therapy [4,5]. Major antibacterial classes in current clinical development are new generation β-lactams, oxazolidinones, tetracyclines, dihydrofolate reductase inhibitors, peptide deformylase inhibitors, nonclassical glycopeptides and peptide antibiotics [6]. Peptide-based antimicrobials offer decreased potential for resistance induction [7], but their parenteral use is hampered by low efficacy and inadequate safety margins (in addition to the usual rapid clearance of peptide-based drugs) leaving them suitable only for topical applications [6]. However, the most attractive features of peptide drugs in general are their high specificity and low toxicity [8]. Apparently our selection criteria for any particular peptide antibiotic for pharmaceutical development have to be reevaluated, and/or we have to look at the peptides' clinically relevant *in vitro* efficacy from a new point of view that takes into consideration the specific properties of cationic antimicrobial polyamides.

The differences between cationic antibacterial peptides and conventional antibiotics start with the measurement of the antimicrobial potency. In the microbiology literature, growth inhibition is assayed on vigorously replicating bacterial colonies [9], in a media composition that promotes dynamic bacterial growth. In our experience, when 25% serum is added to full-strength Muller–Hinton broth to mimic the media conditions in mammals, the bacterial growth rate is reduced, indicating that commonly used media may not ideally represent the environment of *in vivo* infections. Solvents with high salt content or dielectric constant deactivate many peptide antibiotics [10], likely via neutralizing the positive charges needed for initial interaction with bacterial membranes. Indeed, the *in vitro* antibacterial activity of peptides is improved in diluted Muller–Hinton broth. Peptide antibiotics are frequently considered nonstarters in drug development because they do not exhibit acceptable minimal inhibitory concentrations (MIC) in microbiology-approved media, a basic estimate of the expected *in vivo* therapeutic dose [11]. Yet, the same peptide derivatives do reduce the bacterial counts during experimental infection of mice to a clinically significant degree, indicating that the peptides do something else than just rapidly lyse bacterial colonies.

It was originally proposed that the bacterial target of cationic peptides is the cytoplasmic membrane [12]. Cationic peptides are generally able to interact electrostatically with the negatively charged bacterial

* Correspondence to: L. Otvos, OLPE, LLC, 801 Mockingbird Lane, Audubon, PA 19403, USA; e-mail: lotvos@comcast.net
¶ Selected paper presented at the 1st International Congress on Natural Peptides to Drugs, 30 November–3 December 2004, Zermatt, Switzerland.

phospholipids and then insert into model membranes, forming transient channels or pores [13,14]. In alternative mechanistic views, the antibacterial peptides kill bacteria either by the 'carpet' model in which the peptides cooperatively destroy the membrane barrier without channel formation [15] or by binding to the outer leaflet of model membranes and flipping inward, carrying lipids along the way and creating brief disruptions in permeability [16]. Of course, these processes would lead to immediate membrane lysis and would miss any interaction of the peptides with intracellular targets. Indeed, these arguments found plenty of support from the classical drug development dogma which claims that molecules over 1200 Da molecular weight – and almost all native antibacterial peptides fall in this category – are unable to traverse epithelial surfaces, i.e. enter cells, at least not by active transport, and thus are not suitable for interfering intracellular processes [17]. The situation rapidly changed with the realization that a series of cationic peptides can enter bacterial and mammalian cells by passive transport [18], and antibacterial peptides are no exceptions [19]. Actually the polycationic delivery module of native antibacterial peptides or their derivatives can deliver their own pharmacophore into cells, as well as foreign cargo [20]. These observations led to the realization that antibacterial peptides can disturb intracellular processes without killing bacteria and perhaps explain some of the controversies related to the mode of action. Combined with the pioneering theories on membrane disintegration, these findings guided the development of the concept of multifunctional antimicrobial peptides. It needs to be mentioned that recent results point to the existence of high- and low affinity receptors on endothelial cells for at least some native antimicrobial peptides [21]. A series of basic science review articles were published on the subject in recent years [22–24]. This account tries to highlight the major findings from a potential therapeutic point of view.

PEPTIDES THAT SIMULTANEOUSLY ATTACK MULTIPLE BACTERIAL FUNCTIONS

Buforin II is a 21mer antimicrobial peptide discovered in the stomach tissue of the Asian toad *Bufo bufo gargarizans*, a peptide that kills bacteria by binding to nucleic acids [25]. Buforin and its truncated analogs penetrate the cell membrane, but do not permeabilize it. The sequence of buforin includes a single proline in midchain position [26]. The peptide penetrates the bacterial cell membrane and accumulates in the cytoplasm, in contrast to the proline-free magainin that remains associated with the inner leaflet of the lipid bilayer after translocation of the artificial membrane. However, when buforin's proline residue is replaced with alanine, the mode of action changes from intracellular target inhibition to magainin-type membrane destruction [27]. Indeed, when prolines are inserted into the sequences of α-helical antimicrobial peptides, the peptides' ability to permeabilize the cytoplasmic membrane of *Escherichia coli* decreases substantially as a function of the number of proline residues incorporated [28]. With its primarily intracellular target inhibition and ability to move between various modes of action, metabolically stable buforin derivatives are ideal targets of antimicrobial drug development.

Thrombin-induced platelet microbicidal protein-1 and human neutrophil defensin-1 are small, cationic peptides exhibiting *in vitro* microbicidal activity against a broad spectrum of human pathogens. Similarly to buforin, these antimicrobial peptides kill bacteria by inhibiting protein and DNA synthesis, a slow process that augments early effects on the structure and function of the cytoplasmic membrane [29]. Experiments with the thrombin-induced platelet peptide and other peptides killing *Staphylococcus aureus*, demonstrates that permeabilization, *per se*, does not invariably result in bacterial death [30]. Although each of the test peptides interacts with the *S. aureus* cytoplasmic membrane, diversity exists in their mechanisms of action with respect to the relationship between membrane permeabilization and staphylocidal activity.

Staying with the theme of interfering with macromolecular assembly, the frog dermaseptin inhibits RNA synthesis in bacteria within 5 min at the minimal inhibitory concentration (MIC) or higher peptide concentrations [31]. At the MIC, dermaseptin inhibits *E. coli* growth in a broth dilution assay but does not cause bacterial death within 30 min. Consistent with this, at the MIC, the peptide translocates across lipid bilayers, but demonstrates only a weak ability to permeabilize membranes. Due to its activity on the membrane structure, at doses of 10 times the MIC, dermaseptin reduces bacterial viable counts by about 2 log orders of magnitude within 5 min. The proline-rich peptide apidaecin enters cells through a multistep pathway [32]. The proposed mechanism involves an initial, nonspecific encounter of peptide with an outer membrane component, followed by invasion of periplasmic space and by a specific and essentially irreversible engagement with a receptor/docking molecule that is probably membrane bound. In the final step, the peptide is translocated into the interior of the cell where it meets its ultimate target, perhaps one or more components of the protein synthesis machinery.

Our peptides act one step later: at the folding of already assembled proteins. We have shown earlier that pyrrhocoricin and drosocin, representatives of the short, proline-rich antimicrobial peptides, kill bacteria by inactivating the bacterial heat-shock protein DnaK and inhibiting chaperone-assisted protein folding [33,34]. The molecular architecture of these peptides features an *N*-terminal DnaK-binding

half and a *C*-terminal membrane-penetrating unit. To obtain drug leads with improved antimicrobial properties and possible utility as therapeutic agents, we synthesized chimeric dimers, in which pyrrhocoricin's potent DnaK-binding domain was connected to drosocin's superior cell penetrating module [35]. The new constructs not only exhibit enhanced *in vitro* antibacterial properties against the originally sensitive strains *E. coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*, but show activity against *S. aureus*, a pyrrhocoricin- and drosocin-resistant bacterium. While a novel mixed pyrrhocoricin–drosocin dimer and the purely pyrrhocoricin-based old dimer bind *E. coli* DnaK with an identical 4 μM K_d, the mixed dimers penetrate a significantly larger number of *E. coli* and *S. aureus* cells, and destroy a larger percentage of bacterial membrane structures. Clearly, the pyrrhocoricin–drosocin mixed dimers yielded antibacterial peptide derivatives acting with a multiple mode of action.

INHIBITION OF BACTERIAL OR EXHIBITION OF PROTECTIVE (BACTERIAL AND HOST) ENZYME FUNCTIONS

Upon DnaK binding, pyrrhocoricin inhibits the heat-shock protein's ATPase activity [34]. The *N*-terminal enzyme-inhibitory and the *C*-terminal membrane-active modules can be separated in a peptide as small as the 20mer pyrrhocoricin [36]. Apparently many antimicrobial peptides are interfering with bacterial enzymatic processes, as much as the question was asked whether these molecules are pore formers or metabolic inhibitors in bacteria [24]. A recent hypothesis suggests that in addition to membrane activities, a large variety of cationic peptides and proteins might also render bacteria nonviable by activating their autolytic wall enzymes – muramidases resulting in bacteriolysis [37]. This group of cationic polyamides includes: lysozyme, lactoferrin, neutrophil-derived permeability-increasing peptides, defensins, elastase, cathepsin G, and secretory phospholipase A2. In this respect, cationic peptides mimic the bactericidal/bacteriolytic effects exerted by β-lactam antibiotics. The lantibiotics nisin and epidermin – but not Pep5 or epilandin – form complexes with the cell wall peptidoglycan monomer, Lipid II, as a cell surface–docking molecule [38]. This is very significant for future therapeutics in the light of current clinical success with ramoplanin, a cyclic polyamide that also inhibits MurG via complex formation with Lipid I [39].

Antimicrobial peptides and proteins themselves frequently resemble enzymes or their functions. Angiogenins are members of the ribonuclease superfamily and they are implicated as mitogens for vesicular endothelial cells, immune modulators, activators of certain protease cascades and adhesion molecules [40]. Ang4, a recently characterized angiogenin, is produced by mouse Paneth cells, is secreted into the gut lumen and has bactericidal activity against intestinal microbes [41]. Ang4 expression is induced by *Bacteroides thetaiotaomicron*, a predominant member of the gut microflora, revealing a mechanism whereby intestinal commensal bacteria influence gut microbial ecology and shape innate immunity. Furthermore, mouse Ang1 and human angiogenin, circulating proteins induced during inflammation, exhibit microbicidal activity against systemic bacterial and fungal pathogens, suggesting that they contribute to systemic responses to infection. Lactoferrin is an 80-kDa iron-binding glycoprotein found in human milk and other glandular epithelium secretions [42]. The inherent antimicrobial properties of lactoferrin were originally thought to be exerted via strong chelation of iron required for microbial growth [43]. Curiously, human milk RNase is an isoform of human lactoferrin, sharing physical, chemical and some biological properties with the antimicrobial protein [44].

Nisin is currently used commercially as a food preservative in pasteurized process cheese to prevent clostridial growth [45]. The production of lantibiotics appears to be regulated at the transcriptional level in a cell-density-dependent manner in various bacteria [46]. This autotoxic phenomenon, called *quorum sensing*, has been studied in detail for the production of nisin by *Lactococcus lactis*, and the structurally similar subtilin by *Bacillus subtilis*. The autoinducing peptides have both signaling and antimicrobial activities [47]. In nisin, the induction capacity and the antimicrobial activity are mediated through different peptide domains [48]. Significantly, just like for pyrrhocoricin, the *C*-terminal domain is responsible for membrane interaction and pore forming, and the *N*-terminal region for the bacteria-specific recognition process.

EFFECTS ON HOST DEFENSE I. CATHELICIDINS AND DEFENSINS

Mammalian-born antimicrobial proteins with enzymatic and other eukaryotic activities lead us to antibacterial peptides that both kill bacteria *in vitro* and upregulate various aspects of the immune system of the host. While innate immunity is the only defense mechanism of small, short-lived animals such as insects, antimicrobial peptides modulate both innate and adaptive immune systems in larger animals, thus providing a link between the various mechanisms that result in host protection [49]. Remarkably, these peptides can serve as templates for rational drug development efforts. From this family, the cathelicidins and the defensins enjoy the largest literature and therefore these peptide families will be discussed separately.

Peptide antibiotics of the cathelicidin family range from 12 to 80 amino acid residues and are characterized

by a highly conserved cathepsin L inhibitor *N*-terminal signal sequence or proregion and a heterogeneous *C*-terminus that exhibits the bacterial killing properties [50]. Their biological roles unrelated to the antimicrobial function include mediation of inflammation influencing diverse processes such as cell proliferation and migration, immune modulation, wound healing, angiogenesis and the release of cytokines and histamine [51]. It was noted that despite their small size (the cathelicidin peptides LL-37 and PR-39, for instance, are, respectively, 37–and 39-amino acid residues) and their relatively simple structures, these peptides contain all the necessary information for exerting multiple and diverse actions, which in most cases require specific molecular interaction [52]. Peptide LL-37 shows chemotactic effects *in vitro*, inducing selective migration of human peripheral blood monocytes, neutrophils, and CD4 T cells [53]. LL-37 plays roles in infected epithelia as a direct antimicrobial effector and as a mediator of positive amplification loops of innate and adaptive immune responses. Analysis of a synthetic peptide library derived from LL-37 shows that antimicrobial activity against bacterial, fungal and viral skin pathogens resides within specific domains of the parent peptide, but the antimicrobial activity does not directly map to the same residues as the ability to stimulate IL-8 production in keratinocytes [54]. The pig analog PR-39 stimulates angiogenesis by inhibiting the ubiquitin–proteasome-dependent degradation of hypoxia-inducible factor 1a protein [55]. PR-39 could have been also discussed in the section dealing with multiple antibacterial properties, as its *N*-terminal 1–26 protein-binding fragment induces filamentation of *S. typhimurium* [56], while disruption of the *C*-terminal part abolishes the direct antimicrobial function [57].

Inhibition of cell division appears to be a common theme in antimicrobial peptide mode of action, as both indolicidin and microcin 25 induce long aseptate filaments in Enterobactericeae strains [58,59]. *In vivo*, peptide LL-37 protects mice against lethal endotoxemia [60]. In animal models of pancreatitis and acute myocardial infarction, PR39 administration blocks the stimulation of NFκB-dependent gene expression relatively selectively (and without visible toxic effects), therefore the peptide may have important implications as a potential therapeutic approach to management of a number of disease states, including acute inflammation, immune and autoimmune responses and ischemia-reperfusion injury [61].

Animal models indicate that host defense peptides are crucial for both prevention and clearance of infection [62]. Although our knowledge about the direct role of defensins in the innate immune response is still fragmentary, it is proposed that human β-defensin 2 has a direct killing effect on invading gram-negative microorganisms [63] as well as an immune modulating effect in recruiting macrophages and dendritic cells to the site of the infection [64]. Human β-defensin 2 is another antibacterial peptide that appears to have a cell surface receptor, namely, CCR6, for dendritic- and T-cell migration [65], although this receptor is not functional for the peptide on mast cells, known to accumulate at the sites of inflammation in response to chemoattractants [66]. Intranasal or systemic administration of α-defensins along with immunogens enhances the cellular and humoral immune responses to the antigens [67,68]. In general, the use of multiple cellular receptors endows defensins with the capacity to marshal adaptive host defenses against a series of microbial invaders *in vivo* [69].

EFFECTS ON HOST DEFENSE II. OTHER PEPTIDES

β-Defensins conjugated with lymphoma epitopes not only generate potent humoral immune responses in mice against an otherwise nonimmunogenic B-cell lymphoma antigen, but also develop antitumor immunity [70]. The functional overlap between defensins and chemokines is reinforced by reports that some chemokines also have antimicrobial activities [71]. However, the defensins are not the only antimicrobial peptides that exhibit antitumor activities. Gaegurin 6 is a 24-residue antimicrobial peptide with two lysines and without an arginine in the sequence [72]. Gaegurin and its shorter designer analogs featuring an increased number of positive charges show equivalent cytotoxicity against drug-sensitive and multidrug-resistant tumor cell lines, but once again residues responsible for the antitumor activity are not mapped to those responsible for the antimicrobial potency [73]. While little or no toxicity is observed against dendritic cells or red blood cells, plasma membrane blebbing and DNA fragmentation of peptide-treated tumor cells indicate that the gaegurin-derived peptides can induce apoptosis in tumor cells. Similarly, a cecropin–magainin 2 chimera displays strong bactericidal and tumoricidal activity without inducing hemolysis [74]. Interestingly the cation-providing residue in this model peptide is lysine and not the more strongly positively charged arginine. All these results suggest that antimicrobial peptide derivatives can be developed as new anticancer agents and may provide a new strategy for overcoming multidrug resistance.

Returning to the lactoferrin, this protein is able to inhibit the growth of solid tumors and the development of metastases in mice [75]. Lactoferrin is released by neutrophils in response to inflammatory stimuli [76], and its involvement in many aspects of host defense includes antiviral properties [77]. Similarly to other antimicrobial proteins, a cleaved peptide fragment (lactoferricin) shows a wider antibacterial spectrum than the full-sized protein [78].

Azurocidin, a 225-residue protein from the azurophil granule, a specialized lysosome of human neutrophils,

exhibits strong structural homology to serine proteases but is proteolytically inactive [79]. The ability to cleave a peptide bond is lost due to replacement of two of the three residues from the conserved catalytic triad characteristic for serine proteinases [80]. Azurocidin has a broad-spectrum antimicrobial activity, mainly against gram-negative bacteria. It is also recognized as a multifunctional inflammatory mediator for its contracting effects on endothelial cells causing an increase of vascular permeability, capacity to bind endotoxin and ability to attract monocytes to the sites of inflammation [81]. The eight basic residues that are altered in a Loop 3/Loop 4 mutant contribute to the ability of wild-type azurocidin to bind heparin and to kill *E. coli* and *Candida albicans* [82]. Because binding to the bovine pancreatic trypsin inhibitor is comparable in wild-type and Loop 3/Loop 4 mutant azurocidins, it was concluded that the protein-binding site is distinct from the site involved in heparin-binding and antimicrobial activity [82].

Adrenomedullin is yet another large antimicrobial peptide playing roles in communicating between the innate and adaptive immune systems [83]. Both the full-sized 52mer gene product and the proadrenomedullin *N*-terminal 20 peptide show strong antibacterial activity against *E. coli* [84]. Among its many biological functions, the 1–20 peptide, and even a shorter 12-residue fragment, regulate catecholamine release and synthesis by interfering with nicotinic cholinergic receptors in chromaffin cells [85]. The 20mer peptide inhibits Ca^{2+}-dependent, agonist-stimulated aldosterone and catecholamine secretion, acting via specific receptors. The 12–20 fragment acts as a weak antagonist, suggesting that the entire 20mer is required for the antisecretagogue action on the human adrenal gland [86]. While adrenomedullin has a gross positive charge and shows some functional similarities to the defensins, the positive charges are concentrated at the calcitonin-analog *C*-terminal region [87] and are unlikely to be correlated with the bacterial killing functions. Indeed, the antimicrobial mode of action of adrenomedullin is currently unknown [88].

Temporin A, a short, frog-derived antimicrobial peptide induces the migration of human monocytes, neutrophils and macrophages with a bell-shaped response curve in a pertussis toxin-sensitive manner, activates p44/42 MAPK, and stimulates Ca^{2+} flux in monocytes, suggesting that temporin is capable of chemoattracting phagocytic leukocytes by the use of a $G_{i\alpha}$ protein-coupled receptor [89]. Comparison of the chemotactic and antimicrobial activities of several synthetic analogs suggests that these functions are likely mediated by different structural characteristics. The data suggest that two types of potential, clinically applicable antibiotics can be developed based on temporins: analogs with antimicrobial activities but no effect on phagocytic leukocytes and derivatives maintaining both antimicrobial and phagocyte-activating activities [89].

SYNERGY BETWEEN ANTIMICROBIAL PEPTIDES AND BETWEEN PEPTIDES AND CONVENTIONAL ANTIBIOTICS

One would expect that antibiotics, peptides or nonpeptides, acting by different modes, can synergize each other even when the components are not covalently linked [90]. The *in vitro* activity of three polycationic peptides, cecropin A, melittin and a cecropin A–melittin hybrid peptide alone and in combination with various clinically used antimicrobial agents was investigated against 32 nosocomial isolates of *Acinetobacter baumannii* [91]. While these test peptides disintegrate the bacterial membrane structure, synergy was observed when the hybrid or melittin were combined with β-lactam antibiotics [91] that prevent cell wall assembly [92]. Synthetic peptides corresponding to *N*- or *C*-terminal histone regions are inactive in antimicrobial assays, but they potentiate the antimicrobial activities of the flounder-originated antibacterial peptide pleurocidin, lysozyme and crude lysozyme-containing extracts from coho salmon [93]. While the histone peptides bind specifically to anionic lipid monolayers, synergy with pleurocidin does not appear to occur at the cell membrane level. The synergistic activities of inducible histone peptides indicate that they play an important role in the first line of salmon defenses against infectious pathogens and that while some histone fragments may have direct antimicrobial effects, others improve existing defenses [93].

The antibacterial activity of a prodomain fragment of the *Drosophila* antibacterial protein attacin C is potentiated when the peptide is used in combination with cecropin A, another *Drosophila* antimicrobial peptide [94]. The synergistic action observed between these two molecules, killing microbes via different mechanisms, suggests that actually Nature designed complementary and jointly active antimicrobial peptides to protect higher organisms from bacterial infection. When the combined action of six antimicrobial peptides/proteins found in the airway surface liquid was examined, paired combinations of lysozyme–lactoferrin, lysozyme–secretory leukocyte protease inhibitor (SLPI), and lactoferrin–SLPI were found to be synergistic [95]. Unfortunately, as the ionic strength of the test media increased (a situation in this specific case characteristic for cystic fibrosis), the synergistic interactions were lost. This finding may indicate only a limited therapeutic potential of cationic antimicrobial peptides, not only alone but also in combination with other antimicrobial agents, a treatment option many biotech companies working on peptide antibiotics consider, for market entry.

The antibacterial peptides and proteins discussed in this review and their suggested activities on bacterial and host functions are shown in Table 1.

Table 1 Antibacterial peptides and proteins and their suggested activities on bacterial and host functions

Antibacterial peptide or protein	Activity on bacterial membranes	Other bacterial targets	Molecular function in the host	Additional cellular or tissue host functions	Reference
Buforin II	–	Nucleic acids			25
Ala11-Buforin II	+				27
tPMP-1	+	30S, 50S ribosomal subunits DNA gyrase?			29
HNP-1	+	50S ribosomal subunit DNA gyrase?			29
Dermaseptin	–(low dose)	RNA synthesis			31
Apidaecin 1a	–	Protein synthesis			32
Pyrrhocoricin	–	DnaK (ATPase, protein folding)			33,34
Pyrrhocoricin–drosocin chimeras	+	DnaK			35
Lysozyme	+	Cell wall assembly			37
Lactoferrin	+	Cell wall assembly, Chelation of iron	RNase?	Antitumor, antiviral?	37, 43, 44, 75, 77
Cathepsin G	+	Cell wall assembly			37
Nisin, epidermin	+	Lipid II, quorum sensing			38, 47
Ramoplanin		Lipid I			39
Angiogenins			RNase	Adhesion molecules, mitogens	40
LL-37				Chemotaxis, IL-8 production, antiendotoxemic	53, 54, 60
PR-39		Filamentation		Ubiquitin-proteasome inhibition, blockage of gene expression	55, 56, 57
Indolicin, microcin	–	Inhibition of cell division			58, 59
β-Defensin 2			CCR6 ligand	Chemoattractant, immune modulator, Antitumor	63, 64, 65, 70
Gaegurin				Antitumor	73
Cecropin–magainin chimera	+			Antitumor	74
Azurocidin		Heparin binding	Serine-protease analog	Inflammatory mediator, chemoattractant	79, 80, 81, 82
Pro-adrenomedullin				Inhibition of nicotinic cholinergic receptors	85
Temporin A				Monocyte migration and calcium flux, kinase activation	89
RNA III inhibitor-dermaseptin chimera	+	RNA inhibition			102

CONCLUSIONS

Many antimicrobial peptides are multifunctional molecules. In addition to the direct interaction with bacterial membranes, some of the antibacterial peptides exhibit inhibitory properties to target bacterial biopolymers. Yet others, such as cathelicidins or defensins, upregulate the immune system of the host

organism. In spite of recent clinical failures on mainly homofunctional antimicrobial peptides, a new generation of antibacterial peptide derivatives appears to emerge, peptides in which certain residues are modified to improve the activity profile or pharmacological parameters [96]. Improved stability may result in unwanted accumulation in nontarget tissues such as the nervous system. Cationic peptides not only bind to cellular surfaces, but indeed undergo an enhanced cellular uptake also via passive transport. *In vitro* and *in vivo* studies with a dynorphin analog as well as an analog of adrenocorticotropic hormone show pharmacokinetic characteristics of brain, typical for absorptive-mediated transcytosis [97,98]. *In vivo*, approximately 15% of cationized bovine serum albumin taken up was transcytosed into postcapillary extracellular space. Actually cationized rat serum albumin accumulated very well after bolus intravenous injection in the kidney and the brain, less significantly in the liver, and not at all in the myocardium and the lungs [99]. Conversely to all these earlier findings, in our experience pyrrhocoricin or its stabilized analogs were unable to deliver cargo into the brain. Moreover, peptide antibiotics undergo fast renal clearance with the urinary tract, the site of infection being the preferred tissue distribution sites [100].

The multimodal function and improved efficacy reduce the required therapeutic dose and provide analogs with acceptable, if not good, safety margins. In our most recent studies, a completely statistical, short, proline-rich peptide derivative kills fluoroquinolone-resistant clinical *E. coli* and *K. pneumoniae* isolates with a median MIC of 12 μg/ml in full-strength Muller–Hinton broth with virtually no toxicity to COS cells (Otvos *et al.*, submitted). We believe the mode of action of the A3-APO analog is a combination of DnaK inhibition and bacterial membrane disintegration. This peptide is currently under rigorous *in vitro* efficacy testing and awaits detailed *in vivo* evaluation in various systemic and local infection models. A more developed molecule, a hybrid peptide made of an RNA III-inhibiting heptapeptide and a 13-residue dermaseptin derivative, is highly potent in totally preventing staphylococcal graft infections at a dose of 20 μg/ml [102]. In addition, the hybrid peptide acts synergistically with conventional antibiotics. The data indicate that the two independent peptide fragments of the chimera act in synergy by attacking bacteria simultaneously by two different mechanisms, RNA inhibition and membrane disruption. Such a chimeric peptide may be useful for coating medical devices to prevent drug-resistant staphylococcal infections [102]. Since bacteria have to modify their genetic composition to each individual mode of action to develop resistance to any given antibiotic, such a multifrontal attack on bacterial growth and infectivity in general will be extremely useful in fighting antibiotic resistance on the long run [35].

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