Antimicrobial peptides: broad-spectrum antibiotics from nature

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THE CATIONIC PEPTIDES OF NATURE

No therapeutically useful new classes of antibiotics have been identified in the past quarter of a century. This has become of major consequence in the face of the increase in the incidence of significant antibiotic resistance in the past decade. Indeed, strains of several important pathogens have been identified for which very few, or even no, possibilities for antibiotic therapy exist. Although several broad-spectrum improved variants of existing antibiotic classes have been produced (e.g. new fluoroquinolones, imipenem), it seems that bacteria can relatively easily become resistant to these by modulation of known resistance mechanisms. For this reason there is great interest in identifying novel antibiotic classes with potential in human therapeutics.

Human beings and other species are continually exposed to numerous bacteria. Even in the absence of an immune response, they rarely become infected because of the potency of their so-called non-specific defenses. Thus, it is worth looking to these defenses as a potential source of new antimicrobial strategies. In the past decade it has become apparent that many organisms use cationic peptides as a major non-specific defense against infections (Table 1). Indeed, more than 140 cationic peptides are known, having been identified in nearly every species of life [1]. In plants (e.g. thionins) and insects (e.g. cecropins and insect defensins), cationic peptides provide the predominant mechanism of defense against bacteria and fungi. In insects, such peptides are inducible by mechanisms that closely resemble immune response regulation [2]. Among amphibians, cationic peptides (e.g. magainins) constitute the major protection for the skins of frogs and toads, which almost never become infected, despite their exposure to enormous numbers of potential pathogens. In higher animals, including humans, cationic peptides (e.g. defensins) are the major protein species (~8-15% of total proteins) in neutrophils, which are the most important cells in the non-specific defense of the body against invasive pathogens. In addition, other cationic peptides are found in high concentrations at mucosal surfaces; for example, lingual antimicrobial peptide, tracheal antimicrobial peptide and cecropin P1 are considered by some to contribute significantly to mucosal immunity [3]. Cationic peptides are also produced by bacteria, fungi and crustaceans. Thus they can truly be described ubiquitous. This brief review presents an overview of the cationic peptides. For more details, the reader is referred to our more extensive review [1].

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THE NATURE OF CATIONIC PEPTIDES

Cationic peptides are perhaps the finest example of convergent evolution, in which diverse molecules have

Table 1 Examples of antimicrobial peptides found in nature

Source	Example	Structural motifs
Mammalian neutrophils	Defensins	3 β-strands 3 disulphides
Amphibian skin	Magainins	Amphipathic α-helix
Insect hemolymph	Cecropins	Amphipathic α-helix
Crustaceans	Tachyplesins	2 β-strands 2 disulphides
Plants	Thionins	3 disulphides Structure unknown

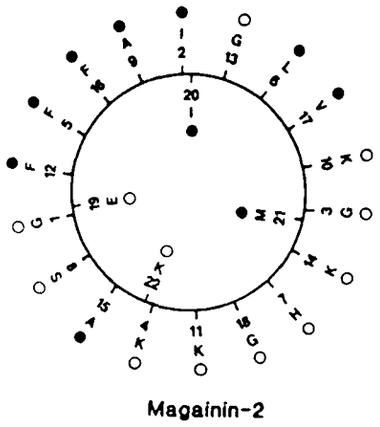

Figure 1 Helical wheel diagram (axial projection) for magainin-2, demonstrating the amphipathic nature of the α-helix. This demonstrates that one side of the α-helix is quite hydrophobic (solid circles) whereas the other contains most of the hydrophilic residues, including the five clustered lysine (K) residues (open circles). A single-letter code is used for amino acids. Reproduced by copyright permission from Kini and Evans [23]© International Journal of Peptide and Protein Research.

evolved towards a common function: the ability to kill infectious microbes. However, it is clear that the differences between the peptides with this common function are as profound as the similarities. All cationic peptides consist of sequences of between 12 and about 40 amino acids. These sequences usually contain two to six positively charged amino acids (lysine or arginine) and rarely more than one negatively charged amino acid. Another similarity is that such molecules can fold in three dimensions, under the appropriate conditions, which usually involve incorporation in a membrane, to present a hydrophobic face and a hydrophilic face (Figure 1), the latter of which contains the charged amino acids. Within this common structure, however, many variations exist. For example, the uncharged amino acids are usually restricted to a subset of amino acids, but the identity of the specific amino acids from this subset varies substantially from species to species. In addition, unusual amino acids (e.g. lanthionine) are often found in bacterial cationic peptides (e.g. nisin), and modified C- and N-terminal amino acids are also quite common. More importantly, the secondary and tertiary structures of the peptides can vary, including β-structured peptides with strands stabilized by disulfide bridges [4,5], α-helices [6,7] and more extended structures (Hancock and Falla, unpublished). The four general classes of cationic peptides recognized in the literature are β-structured peptides, α-helical peptides, loop structures created by a cysteine disulfide bridge and peptides with a predominance of a single amino acid (Trp, Pro or His).

ANTIMICROBIAL ACTIVITIES

The spectrum of antimicrobial activity of cationic peptides varies, to include broad-spectrum antibacterial activity, selectivity for either Gram-negative or Gram-positive bacteria, and antifungal action [1]. Table 2 presents the minimal inhibitory concentrations (MICs) of selected cationic peptide variants, compared with their parent peptides from natural sources and with some conventional antibiotics. While not as potent as the classical antibiotics against the most susceptible microorganisms, these cationic peptides have certain apparent advantages. First, they have quite consistent activities against both antibiotic-susceptible and clinically antibiotic-resistant variants of a given antimicrobial species (e.g. methicillin-resistant *Staphylococcus aureus,* multiresistant *Pseudomonas aeruginosa* and *Enterobacter cloacae*). Second, they do not seem to select resistant mutants even after multiple passages on media containing the peptide at a concentration of half the MIC. Third, they kill very rapidly at the MIC, with more than 99.9% of bacteria killed within 20 min [8] and probably within 2 min (Falla and Hancock, unpublished data), whereas most conventional antibiotics kill less than 90% of bacteria within 60 min at the MIC. Studies on intraperitoneal *P. aeruginosa* infections of neutropenic mice have clearly demonstrated a therapeutic effect of cationic peptide application, even when only a single dose is applied [9]. Furthermore, the company Applied Microbiology Inc. has reported briefly on a protective effect against infections by *Helicobacter* spp. in mice.

It must be stated that the above MICs are increased in the presence of high concentrations of divalent cations. Although this may reflect, in part, competition for bacterial surface binding sites, our recent experiments indicate that there may be a more trivial explanation for this phenomenon, and we are currently attempting to define this more fully.

Table 2 MICs of selected cationic peptide variants and antibiotics data selected from Piers et al [8]

	MIC (mg/L)				
Species	Melittin	CEME	CEMA	PX[a]	GM[a]
Escherichia coli	8	2.4	2.8	0.5	1
Pseudomonas aeruginosa	8	2.4	2.8	0.5	1
Salmonella typhimurium	16	2.4	5.6	1	4
Enterobacter cloacae	8	2.4	2.8	0.5	0.5

[a]PX = polymyxin B; GM = gentamicin.

In addition to direct antimicrobial activity, cationic peptides have two other useful activities against bacteria. First, they can act as 'enhancers' of conventional antibiotics by showing synergy in vitro with such antibiotics. The cationic peptide polymyxin B nonapeptide has been well characterized with respect to this property [10]. Also, clear synergy has been demonstrated between the peptide magainin and cefpirome in a mouse infection model, even though the magainin was ineffective by itself [11]. Second, unlike some conventional antibiotics which release endotoxin from bacteria and contribute to the development of endotoxemia, cationic peptides actually bind endotoxin and prevent it from inducing tumor necrosis factor, and they substantially reduce endotoxic shock in galactosamine-sensitized mice [9].

OTHER ACTIVITIES OF CATIONIC PEPTIDES

Numerous other activities have been demonstrated for individual cationic peptides. These include activity against pathogenic protozoa [12,13] and enveloped viruses [14], anticancer activity [15], antiprotease activity [16], a role in insect development [17], and an ability to promote re-epithelialization of damaged tissues.

PRODUCTION

Despite the relatively high levels of cationic peptides within specific tissues, it is impractical to use such natural sources for high-level production. More commonly protein chemical methods are used for synthesis. However, even using the most refined new methods of peptide synthesis (solution-phase chemistry), it is quite expensive to produce peptides by this route, and this may limit potential clinical usage. An interesting possibility is provided by recombinant DNA technology, in which the peptides are produced as parts of fusion proteins encoded by plasmids. This method [18] can be used to produce virtually any cationic peptide sequence in quite high yields (approximately 2% of bacterial cell biomass) using the classical methodology of bacterial fermentation. We believe that this method will yield peptides in large enough amounts, and inexpensively enough, to make clinical use practicable.

MODE OF ACTION

The actual mechanism of bacterial killing by cationic peptides involves the formation of channels in the cytoplasmic membranes of the target bacteria [1]. Model membrane studies have suggested that such channels are of two types, multistate channels, in which different numbers of peptides participate in forming individual channels (more subunits = bigger channels), and channels of defined size, which presumably involve some energetically favored number of subunits in a fixed arrangement [19,20]. For example, two molecular models for channel formation by cecropin have been proposed by Durell et al [20]. These contain the arrangement of six dimers in star and circular conformations, producing channel sizes consistent with two experimental conductance increments observed by Christensen et al [21]. Bacterial cells are then killed because of the leakage of ions and essential chemicals from the cell. Some features of channel formation suggest the basis for peptide selectivity, in that channel formation requires a high transmembrane potential of greater than -80 mV and is inhibited by the presence of cholesterol in membranes. Thus bacterial cytoplasmic membranes, being cholesterol-free and having a high transmembrane electrical potential gradient (of -140 mV), are favored for peptide insertion, whereas eukaryotic cell membranes having a low gradient (-15 mV or so) and including cholesterol are not.

The above process occurs in both Gram-positive and Gram-negative bacteria. However, an additional interaction must occur in Gram-negative bacteria, i.e. with the outer membrane. We have provided substantial data [8] to indicate that the mechanism utilized by cationic peptides to cross the outer membrane is self-promoted uptake. In this mechanism the cationic peptides physically interact with divalent-cation-binding sites on surface lipopolysaccharide and, being bulkier than the divalent cations they displace, cause a perturbation of the outer membrane through which uptake of other cationic peptide molecules is promoted. This mechanism explains two of the other antibacterial activities of cationic peptides, namely enhancer and anti-endotoxin activities. Presumably those peptides that are selective for Gram-negative bacteria (e.g. cecropins) have their activities actually enhanced by this primary interaction.

CLINICAL CONSIDERATIONS

Every antibiotic introduced into clinical use faces three major issues besides efficacy, namely formulation, toxicity and stability. To date only topical usage of these peptides has been attempted. The first clinical trials of MSI-78 against impetigo failed, probably because of poor clinical trial design (75% cure in the mock-treated controls), but the second clinical trial against diabetic foot ulcer has been claimed by the company Magainin Sciences to demonstrate equivalent efficacy to quinolones. Clearly, the successful passage of peptides into

phase III clinical trials suggests that the three major concerns described above have been solved for topical usage. However, such usage does not seem to play to the strengths of cationic peptide antibiotics and it is more interesting to consider systemic or organ-specific therapy. With regard to formulation, not much is known. The few published animal trials have largely involved delivery of the cationic peptides in saline [9,11]. An alternative approach is liposomal formulation, which has been shown to improve the bioavailability and activity in vivo of the peptide indolicidin [22].

The issue of toxicity is a serious one, since there are known cationic peptides which are potent toxins (e.g. melittin, the major toxic component of bee venom). There is very little information about the known cationic peptides, although our own experiences to date show no toxic effects on macrophage tissue-culture cells, and no obvious toxicity at the highest doses in our animal models. Stability is a major issue, too, since proteases abound in the body. Despite these considerations, we feel that cationic peptides offer exciting prospects as clinical agents and real potential as the first breakthrough class of antibiotics in 25 years.

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