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Abstract: Discusses work done to create peptide antibiotics as a response to bacterial resistance to the classic antibiotics. How peptide antibiotics, specifically cationic peptides, work against bacteria; The decreasing efficacy of antibiotics; How bacteria can mutate to resist antibiotics; Information about cationic peptide antimicrobials; Minimum inhibitory concentrations for cationic peptides; Mode of action; Therapeutic considerations; Outlook. INSETS: Panel 1: Structural classes of cationic peptides.;Panel 2: Therapeutic status of cationic peptide antimicrobials..

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PEPTIDE ANTIBIOTICS

The era of the "classical antibiotic" may be over. The emergence of resistance has seen to that. Yet no truly novel class of antibacterial agent has come on the market in the past 30 years. Currently there is great interest in peptide antibiotics, especially the cationic peptides. Thousands of such molecules have been synthesised and just a few are entering clinical trials. Because they kill bacteria quickly by the physical disruption of cell membranes, peptide antibiotics may not face the rapid emergence of resistance.

The past 50 years have been dubbed the "antibiotic era" in which natural, semisynthetic, or synthetic antibacterial chemicals have been used with great success against life-threatening infections. However, that era may be coming to an end; at best antibiotics are progressively demonstrating decreased efficacy.[1] In 1994 the World Health Organization's Scientific Working Group on Antibiotic Resistance and Surveillance stated that resistance to antibacterial agents was already a serious public health problem in developed and developing countries alike. Levels of resistance had been increasing at an alarming rate and were "expected to increase at a similar or even greater rate in the future as antibacterial agents lose their effectiveness". This concern has been widely publicised in newspapers and on television and has been discussed on the floor of the United Nations.

Antibiotic resistance has always been a fact of life for the clinician. The short doubling-times and genetic plasticity of bacteria permit these microorganisms to rapidly "test" specific mutations for their ability to enhance growth in challenging environments. A mutation conferring resistance will help bacteria to survive attack from antibiotics used therapeutically. However, two factors have accelerated the development of resistance. One is the accumulation of mutations over time so that, for a given bacterial pathogen, entire classes of antibiotics have been rendered inactive. The other is the absence of a new class of antibiotics in recent years. Nalidixic acid, representing the most recent new class, was introduced over 30 years ago. The third and fourth generation

cephalosporins, imipenem, and ciprofloxacin are merely permutations on previous classes, and bacterial resistance to these antibiotic variants is rapidly increasing too.

Two new candidates have emerged. The carbohydrate agents were reviewed in The Lancet in 1995.[2] In this review, I will look at the peptide antimicrobials, with an emphasis on cationic peptides.

Peptides as antibiotics

Two groups of peptide antibiotics were discovered some time ago and have been used extensively for topical therapy--namely, the gramicidins and the polymyxins (including polymyxin B and colistin, which are really lipopeptides). Both are cationic peptides. They tend to be cytotoxic and this, together with the availability of alternative agents against pseudomonads, has limited their usage as injectables, although colistin has been used in the clinic in aerosol formulation.[3, 4]

Glycopeptide antibiotics such as vancomycin and teicoplanin are used as injectables. They carry net negative charges on their sugars, but if the sugars are removed, a net positive charge of +2 must be introduced to give reasonable activity.[5]

The antibacterial cationic peptides are at an early stage of drug development, and the limited success of other naturally occurring peptides such as gramicidin and, more recently, daptomycin, compels one to be cautious about their future potential. Nonetheless, as I shall show, antibacterial cationic peptides do have several very desirable properties. For further details and for more references readers should turn to in-depth reviews in specialist publications.[6-9]

Cationic peptide antimicrobials: basic information

Structure

Cationic peptides have two distinguishing features.[7] They have a net positive charge of at least +2 (and usually 4, 5, or 6) by virtue of their possession of the aminoacids arginine and lysine (and of modified aminoacid residues for the bacterially-produced lantibiotics and polymyxins). These aminoacids are positively charged at neutral pH. These peptides are also folded in three dimensions so that they have both a hydrophobic face, comprising non-polar aminoacid side-chains, and a hydrophilic face of polar and positively charged residues--ie, these molecules are amphipathic. Despite these two similarities the molecules vary considerably in length, aminoacid sequence, and secondary structure. More than 140 natural cationic peptides fit the general definition of being between 11 and 50 aminoacids long and having a net positive charge of +2 or more (with at most a single negatively charged aminoacid). These fit into four major classes--namely, beta-sheet structures stabilised by two or three disulphide bridges, alpha-helices, extended helices (polyprohelices) with a predominance of one or more aminoacids,[10] and loop structures (figure 1, panel 1). These natural peptides apart, thousands of cationic peptides, especially those belonging to the alpha-helix class, have been synthesised in an attempt to optimise activity.

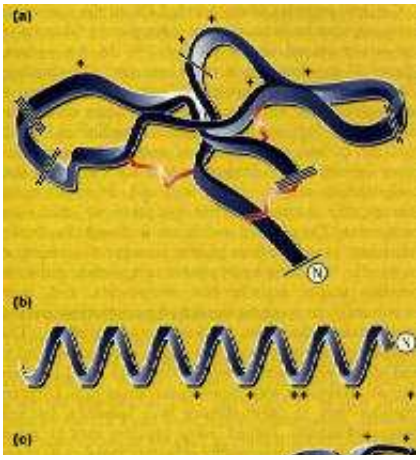

Figure 1: Examples of structures of four classes of peptide antibiotic (see panel 1) (a) b-stranded human defensin-1, (b) a-helical cecropin-melitlin hybrid, (c) extended coil indolicidin, (d) loop bactenecin."Backbones" of structures shown with positive changes (+), amino termini (N), and disulphide bridges in (a) and (d). In (a) there are three b-strands; their beginnings and ends are indicated by pairs of one, two, or three lines on the backbone

Function and isolation from nature

Cationic peptides are important components of the natural defences of most living organisms against microbial infection[8, 9] (panel 1). In man and other mammals such peptides (eg, defensins) constitute the major proteinaceous species (10-18% of total protein) in neutrophils, which are the most important cells involved in immediate defence against microbes as well as in acute inflammatory reactions. Many other cell types also produce them (panel 1). They are found in high concentrations on damaged mucosal surfaces, including the tongue, trachea, and intestine, and may be an important (but poorly recognised) component of mucosal defences. Their involvement in local defences carries over into the amphibians. Indeed, the magainins were discovered when scientists wished to explain the remarkable resistance of frogs and toads to infection after external injury, despite the contaminated environments in which these animals live. Both magainins and the peptides of insects (eg, cecropins) are induced by injury, and this induction of peptides may be a primitive equivalent of the immune response. The transcription of genes for certain insect peptides are controlled by DNA sequences which also regulate human antibody expression. Even primitive organisms such as bacteria and fungi sometimes use cationic peptides (eg, lantibiotics and bacteriocins) as selective antimicrobials. Cationic peptides have been found in all forms of life from bacteria to man and are probably the most conserved theme in nature's struggle to control aggressive microorganisms.

Production

With one exception, natural sources have not proved to be economically viable for peptide production in large quantities. The exception is nisin, a lantibiotic,[11] which is a natural fermentation product of Lactococcus lactis. (Polymyxins and gramicidins are also natural fermentation products.) The two production methods being used are protein chemistry and recombinant DNA technology. The chemical building of sequences of aminoacids from the N- to the C- terminus can be done by automated peptide synthesis.[12, 13] This method has the advantage that non-natural (eg, chemically modified or D-isomer) aminoacids can be simply introduced to generate diversity. However, chemical synthesis is expensive. Recombinant DNA production by fusion protein technology[14] is much less expensive, and the final step is a fermentation procedure as used for other antibiotics.

Activity in vitro Cationic peptides never have the exceptionally high in vitro antimicrobial activities seen with some conventional antibiotics against selected bacteria.[15, 16] However, they do have minimum inhibitory concentrations (MIC) in the range 1-8 MU g/mL that are competitive with those found for even the most potent antibiotics against resistant organisms (eg, Pseudomonas

aeruginosa, Acinetobacter baumanii, and Stenotrophomonas maltophilia) and with highly antibiotic-resistant strains such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococci, extended spectrum beta-lactamase-producing Escherichia coli, and multiple-antibiotic-resistant gram-negative bacteria. The MICs against clinically antibiotic resistant and clinically susceptible strains of a given species do not vary greatly, and cationic peptides can include both gram positive and gram negative bacteria as well as fungi in their spectrum of activity.

Most cationic peptides, in my laboratory's experience, do not induce resistant mutants even after as many as 20 passages on an antibiotic concentration close to the MIC.

These peptides, at concentrations around the MIC, kill bacteria much more quickly than conventional antibiotics do, an observation that has been ascribed to their physical mechanism of action (see below). Generally speaking, so long as the peptides are formulated correctly, they are little affected by physiological divalent or monovalent cation concentrations. Some naturally resistant bacteria exist. Two examples are Burkholderia cepacia and Serratia marcescens which may be resistant to cationic peptides by virtue of a non-interactive outer membrane and production of specific proteases, respectively.

Cationic peptides have two important activities that arise from their interaction with the self-promoted uptake system (see below). These peptides bind to lipopolysaccharide (LPS) and so have antiendotoxin activity,[15, 16] in contrast to other antibiotics, which induce endotoxinaemia. They also possess "enhancer" activity[7, 17] (ie, synergy with classical antibiotics); the more resistant an isolate is to a given antibiotic, the more profound is the enhancement of activity by an appropriate cationic peptide. Thus cationic peptides have the ability to serve as antiresistance compounds.

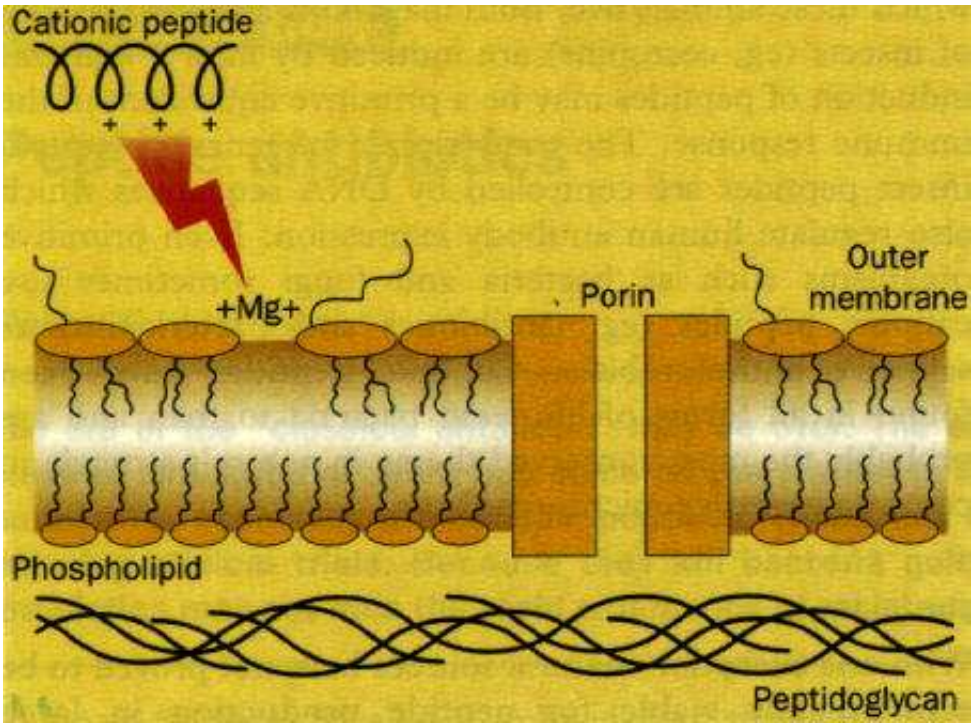

Figure 2: Self-promoted uptake of cationic peptides across outer membranes of gram-negative bacteriaPositively charged peptide interacts with negatively charged divalent-cation-binding sites on surface LPS, disrupting these sites and leading to enhanced uptake of cationic peptides across outer membrane. Disruption of outer membrane can also lead to promotion of uptake of conventional antibiotics across outer membrane, leading to "enhancement" (see text); binding to LPS (endotoxin) explains antiendotoxin properties of these peptides

Mode of action and selectivity

The activities and selectivity of cationic peptides are determined by their mode of interaction with bacterial cell-membranes. For gram-positive bacteria, only a single (cytoplasmic) membrane is involved, so I will describe the more complex dual-membrane (outer and cytoplasmic) interaction with gram-negative bacteria.

Cationic peptides interact with LPS on the surfaces of gram-negative bacteria, and are subsequently taken up via the self-promoted uptake pathway.[7, 10, 15] In this pathway (figure 2) the first step is the interaction of polycations (including such molecules as cationic peptides or aminoglycosides) with divalent cation binding sites on cell surface LPS. Because the peptides have affinities for LPS that are at least three orders of magnitude higher than those for the native divalent cations Ca^{2+} or Mg^{2+}, they competitively displace these ions and, being so bulky, disrupt the normal barrier property of the outer membrane. The affected membrane is thought to develop transient "cracks" which permit passage of a variety of molecules, including hydrophobic compounds and small proteins and/or antimicrobial compounds, and, more importantly, promote the uptake of the perturbing peptide itself (hence the term "self-promoted uptake"). This mechanism explains both how cationic peptides bind to and inhibit endotoxin (a form of released LPS) and how they act in synergy with conventional antibiotics. Different peptides vary in their efficacy as a substrate for self-promoted uptake. Indeed, some peptides may be gram-positive-selective, presumably reflecting inability to access the self-promoted uptake pathway needed for gram-negative activity. Our data with several classes of peptides, including gramicidin S, suggests that most cationic peptides are active against gram-negative bacteria if the appropriate assay is used--ie, broth or agarose dilution rather than agar dilution.[19] For certain peptides in which outer membrane uptake is rate-limiting, such as the indolicidins, my colleague T Falla and I (unpublished) have found a correlation between the affinity constant for outer-membrane interaction and the MIC. The strength of this interaction also determines how effective the cationic peptide is as an antiendotoxin or enhancer agent.

The killing event, for both gram positive and gram negative bacteria, is the formation of channels in the cytoplasmic membrane (figure 3).[7, 10] Typically the positively charged residues of the peptides interact with the negatively charged membranes. During their subsequent electrophoresis, under the influence of the large electrical potential of the bacterial cytoplasmic membrane, the peptides undergo a transition from an unstructured to a structured form (see table 1). They then aggregate into clusters with their hydrophobic faces directed towards the membrane interior and their hydrophilic faces pointing inwards to form a channel. Membrane integrity is destroyed and the bacterial cell dies. The ability to form channels is favoured by the large transmembrane potentials, high content of negatively charged lipids, and lack of cationic lipids and cholesterol that are characteristic of bacteria. Eukaryotic cells, on the other hand, have low membrane potentials, high levels of cholesterol, and modest anionic lipid contents--hence the selectivity of these peptides for bacteria.

In vivo activity and clinical status

In vivo antimicrobial activity

Very few in vivo studies of cationic peptide action have been published. We can assume that such data do exist to justify the granting of IND (investigation of new drug) status by the US Food and Drug Administration, allowing these compounds to be used in clinical trials. Darveau et al,[20] working with magainins, failed to show stand-alone in vivo activity, although they did demonstrate synergy with the b-lactam cefpirome in a mouse model. The lipopeptide colistin (polymyxin E) has been demonstrated to be effective in a variety of infections[4] whereas the related peptide polymyxin B will protect animals from the toxic effects of endotoxin.[17] We have demonstrated that the a-helical cationic peptides MBI-27 and MBI-28 can protect against P aeruginosa peritoneal infections and against endotoxaemia in mouse models.[16] Ahmad et al[21] showed that liposomal indolicidin could protect mice against systemic fungal infections. There have also been claims from those involved in the commercial development of these compounds. The efficacy of

nisin against Helicobacter infections in mice has been announced by Applied Microbiology; Micrologix Biotech has reported 80-100% protection by two lead candidates in their MBI 10 series against systemic S aureus infections of mice; Intrabiotics has reported up to 100% systemic protection against intraperitoneal infections by P aeruginosa, S aureus, and methicillin-resistant S aureus with a single intravenous dose of protegrin PG-1;[22] and Xoma has indicated that their BPI-derived peptide Mycoprex is active in vivo against systemic candidiasis. Despite limited data, it does seem that cationic peptides can show efficacy in animal models.

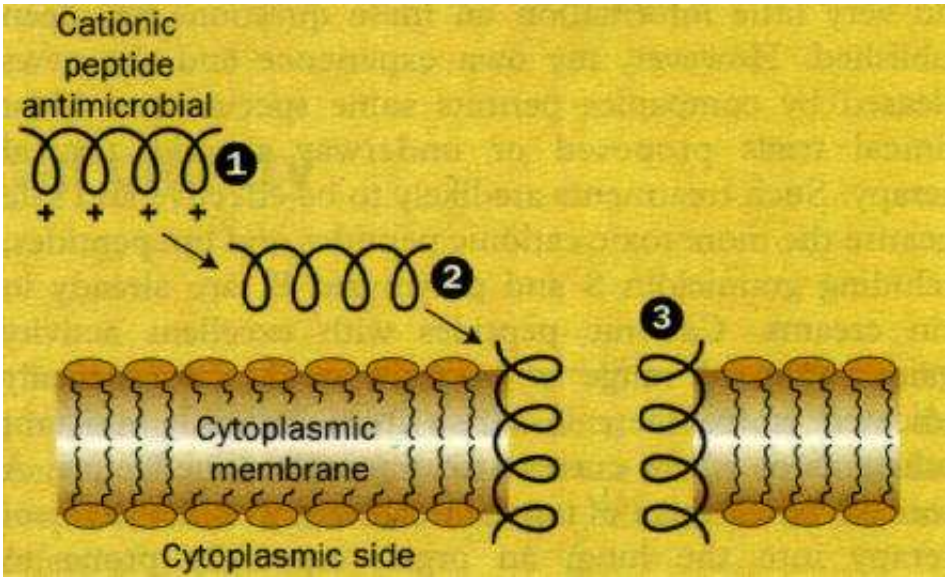

Figure 3: Mechanism of bacteria killing by cationic peptidesPositively charged peptides (1) bind to external surface of negatively charged phospholipid bilayer (cytoplasmic membrane [2]) leading to localised thinning of bilayer. Under influence of membrane potential peptides insert into membrane and form channels (3) leading to leakage of cytoplasmic molecules and cell death

Antiendotoxin and other activities

Most antibiotics promote endotoxinaemia by releasing LPS during cell killing and/or lysis. In contrast, at least some cationic peptides neutralise LPS and prevent endotoxinaemia. For example, the alpha-helical peptide MBI-28 at 8 mg/kg intraperitoneally protected 78% of mice against lethal endotoxinaemia.[16] The protective mechanism appears to be the binding of LPS[15] in such a way that it fails to induce tumour necrosis factor production in either macrophage cell lines or mice. This ability to prevent endotoxinaemia, which not all cationic peptides possess, is a great advantage for these peptides over other antibacterial agents.

Other activities in animal models include anti-cancer activity and the promotion of re-epithelialisation of damaged eyes (Magainin Sciences). Antiviral activity against enveloped viruses has been reported in vitro but in vivo antiviral activity is unlikely because most cationic peptides cannot cross eukaryotic cell membranes.

Therapeutic considerations

The considerations that will determine any clinical use of cationic peptides include toxicity, stability, immunogenicity, route of application, and formulation, and very little information on these questions has been published. However, my own experience and the news released by companies permits some speculation. Most clinical trials proposed or underway involve topical therapy. Such treatments are likely to be effective and safe because the more toxic cationic peptides and lipopeptides, including gramicidin S and polymyxin B, are already in skin creams. Cationic peptides with excellent activity against a broad range of bacteria would be especially indicated where there is a risk from seriously resistant pathogens or where current treatments are ineffective. A more

advanced form of topical treatment would be aerosol therapy into the lung, an organ especially prone to problems with resistant microorganisms. Aerosol treatment is already used successfully for the polycationic trisaccharides gentamicin and tobramycin and the polycationic lipopeptide colistin in treatment of P aeruginosa lung infections in patients with cystic fibrosis.[3, 23] These antibiotics are usually given in liposomal formulation and show little toxicity and reasonable efficacy long term.

Oral therapy may be possible for gastrointestinal infections; nisin is being developed through to clinical trial in Helicobacter pylori infection. At least two companies are developing parenteral therapies (panel 2). There is, however, need for more input from academic clinicians. For example, the data of Ahmad et al[21] and our own experience suggest that formulation is a major issue; another question to be addressed is the stability of cationic peptides to proteases in the body; and, although acute toxicity does not appear to be a problem, more subtle toxicities must be searched for.

Cationic peptides are at a watershed. Several indications are being tested (panel 2) but most clinical studies are of topical treatments, which in my opinion do not address either the areas of greatest need or the real strengths of cationic peptides. Trials for topical therapy will not provide a good indicator of success with parenteral formulations. The failure of a phase III trial in impetigo--largely due to a 75% efficacy of simply washing the infected area so that any effect of peptide treatment could not be measured--has led to no reduction of efforts in this field. The success of the same compound (MSI-78) in a trial of therapy of diabetic foot ulcers where the peptide was as effective as ofloxacin and with better side-effect profile, is encouraging.

Future of cationic peptides

Cationic peptides have several assets that make them excellent prospects as novel antimicrobial agents. The large pharmaceutical companies have traditionally mistrusted peptide drugs but several other peptides (eg, hormones) are starting to have an impact and we clearly do need new antibiotics. I think that the major concerns surrounding cationic peptides will prove resolvable and that these agents will, over the next decade, see substantial clinical usage.

My cationic peptide research was initiated with funding from the Canadian Bacterial Diseases Network and the Medical Research Council of Canada, and is currently also sponsored by the Canadian Cystic Fibrosis Foundation's SPARx program and by Micrologix Biotech Inc.

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