Molecular Biology of Pseudomonads
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Chapter 38

Cationic Peptides: a Class of Antibiotics Able To Access the Self-Promoted Uptake Pathway across the *Pseudomonas aeruginosa* Outer Membrane

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Pseudomonas aeruginosa has been a major target for antibiotic drug discovery programs in large pharmaceutical companies. However, as each succeeding "antipseudomonal" antibiotic has hit the market, it has been rapidly followed by the development of serious resistance. For example, the antipseudomonal penicillins and third-generation cephalosporins have been plagued by resistance due to depressed chromosomal β-lactamases; the carbapenem β-lactam, imipenem, rapidly induced mutations lacking the imipenem-specific porin OprD; and the use of the fluoroquinolones has been restricted by DNA gyrase and multiple-antibiotic resistance (efflux) mutations. Each of these newer groups of antibiotics is a variation on a theme, a compound that has a structure and mode of action that are related to previously introduced antibiotics. Thus bacteria can adapt existing mechanisms (through mutation) to develop resistance to these variant antibiotics. This is an even greater problem with *P. aeruginosa*, which has high intrinsic resistance to antibiotics due to a large extent to the low permeability of its outer membrane (Hancock and Bell, 1989). Thus a relatively small decrease in susceptibility to a given antibiotic is sufficient to make this organism clinically untreatable by this antibiotic. For this reason there is considerable rationale for investigating novel classes of antimicrobial agents.

One of the more reliable classes of antipseudomonal antibiotics is the aminoglycosides. Resistance rates have been fairly steady at around 10% of isolates over the past decade. In the course of studying the mechanisms of uptake of antibiotics across the *P. aeruginosa* outer membrane, we discovered that the aminoglycosides utilized a fundamentally different mechanism (Hancock, 1984; Hancock and Bell, 1989; Hancock et al., 1981) compared to the β-lactams, which in general pass through the water-filled channels of pro-

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teins termed porins. We termed this mechanism self-promoted uptake (Hancock, 1984). The property of aminoglycosides that permits them to use this pathway is their positively charged nature, with three to five positive charges appended to a trisaccharide backbone. Subsequently we demonstrated that self-promoted uptake occurred in *Escherichia coli* (Hancock and Farmer, 1993) and *Enterobacter cloacae* (Piers et al., 1994) and was the major uptake mechanism for the polymyxins (Hancock, 1984; Hancock et al., 1981), azithromycin (Farmer et al., 1992), and deglucoteichoplanins (Hancock and Farmer, 1993). The latter two represented interesting situations in which traditional classes of gram-positive specific antibiotics were derivatized to give them a charge of +2 such that they acquired useful activity against gram-negative bacteria due to their ability to access self-promoted uptake across the outer membrane (which excluded the parent antibiotics).

For this reason, we started to look for polycationic molecules that would have the ability to utilize self-promoted uptake. The cationic peptides, one of nature's most common answers to microbial infections, represent such a class.

SELF-PROMOTED UPTAKE

Self-promoted uptake involves the interaction of polycationic antibiotics with the divalent cation (Ca^{2+}/Mg^{2+})-binding sites on cell surface lipopolysaccharide (LPS) (Fig. 1). These cationic antibiotics have an affinity for such sites that is two to four orders of magnitude higher than that of the divalent cations that normally occupy them. Therefore they competitively displace the divalent cations in a highly cooperative manner. This results in an increase in the surface area of the outer membrane (see, e.g., Sawyer et al.,

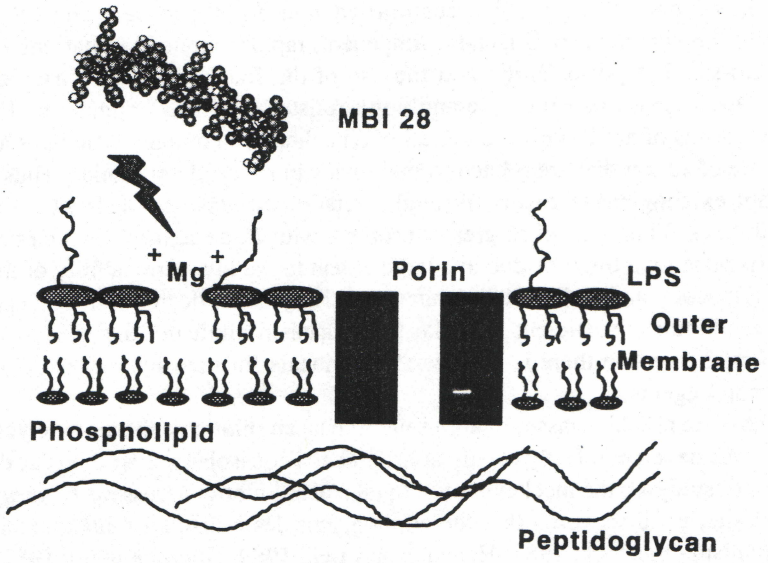

Figure 1. Diagram of self-promoted uptake across the outer membrane. See text for explanation. MBI 28 (formerly termed CEMA [Piers et al., 1994]) has the sequence KWKLFKKIGIGAVLKVLTTGLPALKLTK.

1988), since the antibiotics are much bulkier than the divalent cations they displace, and a dramatic change in its physiochemical and electrostatic properties. Biochemically, we have observed cooperative permeabilization of the outer membrane to such probe molecules as the small basic protein lysozyme, the chromogenic β-lactam nitrocefin, and the hydrophobic fluorophor 1-*N*-phenyl naphthylamine (NPN). Thus, we have proposed that the outer membrane becomes more permeable to the cationic antibiotic itself. The relevance of self-promoted uptake is evidenced by aminoglycoside-supersusceptible mutants of *P. aeruginosa* which have LPS changes resulting in enhanced affinity of these aminoglycosides for LPS binding and outer membrane permeabilization (Rivera et al., 1988), as well as polymyxin-resistant mutants of *P. aeruginosa* and *E. coli* for which the inverse is true (Hancock et al., 1991; Young et al., 1992). Thus we can say that self-promoted uptake leads directly to cell killing.

There is, however, considerable heterogeneity in the ways such cationic antibiotics interact with the outer membrane. For example, some cationic antibiotics permeabilize outer membranes to probe molecules at concentrations well below the MIC, whereas others are only effective at the MIC. This clearly depends on the killing potency of the antibiotic, since, e.g., polymyxin B only permeabilizes at the MIC (0.5 μg/ml) and is able at this concentration to cause membrane perturbations (which would equate to loss of cytoplasmic membrane integrity in cells). In contrast, deacylated polymyxin B can permeabilize outer membranes at a similar concentration (Vaara and Vaara, 1983) but is ineffective in perturbing phospholipid bilayer membranes (Schroder et al., 1992).

A second indication of heterogeneity resides in the differential effects of mutants. Thus, a *tolA* mutant (Rivera et al., 1988) of *P. aeruginosa* has a dramatic (16- to 32-fold) effect on susceptibility to aminoglycosides but a minimal influence on polymyxin susceptibility (around 2-fold). Similarly, a *pmx* mutant of *E. coli* induces a 64-fold increase in polymyxin resistance but only a 2- to 4-fold increase in gentamicin and azithromycin resistance. These data may reflect differences in the preferred interaction sites of polymyxin and the aminoglycosides (possibly due to LPS heterogeneity [Hancock et al., 1994] and differences in the structures of these molecules). Consistent with this idea, the number of binding sites per LPS molecule is different for polymyxin and the aminoglycosides.

CATIONIC PEPTIDES FROM NATURE

Cationic peptide antimicrobial agents have been discovered in many living things. They are produced by bacteria, fungi, plants, insects, crustaceans, amphibians, mammals, and humans (Boman et al., 1991; Hancock et al., 1995; Lehrer et al., 1993; Zasloff, 1987). In each case they are a major contributor to defense against microbes. For example, when insects acquire a bacterial infection, they induce the production of one or several peptides which are antibacterial in nature. The skin of frogs virtually never becomes infected even when damaged in the presence of highly contaminated water because cationic peptides are induced to destroy any bacteria introduced. Similarly, the most abundant proteinaceous molecules in the neutrophil, a dedicated microbe-killing cell in humans and mammals, are the defensins, which are antimicrobial cationic peptides present at a level of 5 to 18% of neutrophil protein (Lehrer et al., 1993). Other abundant species of cationic peptides are found at mucosal membrane surfaces in the

mammalian upper intestine and trachea (Hancock et al., 1995). Indeed, some feel that such cationic peptides represent the "missing link" in muscosal immunity. (Of interest to *Pseudomonas* sp. researchers is the possibility that the prevalence of *P. aeruginosa* infections in cystic fibrosis patients could possibly result from a defect in secretion across the lung mucosa of as-yet-unidentified antimicrobial cationic peptides.)

Cationic peptides show marked structural variations despite thematic similarities. Thus there are at least four classes of cationic peptides (Hancock et al., 1995), including β-stranded peptides with two or three antiparallel β-strands connected by disulfides (e.g., neutrophil defensins, crab tachyplesins); α-helical peptides that may contain a kink or turn in the middle of two α-helical regions (e.g., insect cecropins); loops which are stabilized by a single disulfide (e.g., neutrophil bactenicins); and peptides of unknown structure with predominating amino acid such as proline, tryptothan, or histidine (e.g., indolicidins). These peptides all have two strongly preserved properties. First, they contain multiple positive charges due to a number of lysines and/or arginines. Second, the peptides are amphipathic, with a hydrophilic face (containing the positive charges) and a hydrophobic face. In light of these strongly conserved properties and functions, despite variant secondary structure, we consider this to be the finest argument for convergent evolution, in which a variety of peptides have converged to acquire a common function.

ANTIPSEUDOMONAL ACTIVITIES OF CATIONIC PEPTIDES

Cationic peptides in nature vary from gram-positive selective to gram-negative selective to broad spectrum. For example, insect cecropins are considered to be preferentially active against gram-negative bacteria, nisin is selective for gram-positive bacteria, and magainins are effective against both classes. In addition, selected peptides can have antifungal, antiviral (against enveloped viruses), or antiparasite (Hancock et al., 1995; Lehrer et al., 1993) activities.

There are no known peptides that are specific for *P. aeruginosa*, but the gram-negative selective peptides can include *Pseudomonas* spp. as part of their antibacterial spectrum. Studies on cecropin variants have indicated that the precise determinants of antipseudomonal activity are different from the determinants of activity against other gram-negative bacteria. For example, a Lys-6→Leu-6 mutation of cecropin A virtually abolished activity against *P. aeruginosa* while not affecting activity against *E. coli* (Andreu et al., 1985).

Table 1 shows the comparative activities of several cationic peptides against *P. aeruginosa* compared with activities against *E. coli* and *Staphylococcus aureus*. These MICs are never exceptionally low. However, they are competitive with MICs of many antibiotics against these organisms. Furthermore, these MICs do not appear to vary much between different strains of a given species, and they appear unaffected by most clinically important antibiotic resistance mechanisms (Piers et al., 1994; R. E. W. Hancock, unpublished data). Furthermore, attempts to isolate mutants resistant to cationic peptides have been unsuccessful to date.

The *P. aeruginosa* supersusceptible mutant Z61 has a defect in its barrier properties due to multiple mutations (at least three) which affect antibiotic efflux (Li et al., 1994) and possibly also outer membrane permeability (Angus et al., 1987). This mutant has enhanced susceptibility to all classes of antibiotic, with MICs that are 4-fold to 10,000-

Table 1. Activity of selected cationic peptides against *P. aeruginosa*, *E. coli*, and *S. aureus*

Peptide or antibiotic	Class	MIC (μg/ml)[a]							
		P. aeruginosa			*E. coli*			*S. aureus*	
		SS	S	R	SS	S	R	S	R
Melittin	α-Helical	8.0	8.0	8.0	4.0	8.0	8.0	8.0	8.0
CEME	α-Helical	1.2	2.4	4.8	0.6	1.2	2.4	9.6	9.6
MBI-28	α-Helical	1.4	2.8	2.8	0.7	1.4	2.8	>64.0	>64.0
Bactenecin	Loop	2.0	32.0		4.0	64.0		>64.0	>64.0
Polymyxin B	Cationic lipopeptide	0.06	0.5	0.5	0.06	0.5	32.0	>64.0	>64.0
Gentamicin	Aminoglycoside	0.25	1.0	200	0.5	1.0	4.0	2.0	>64.0
Ceftazidime	Cephalosporin	0.03	0.5	200	0.06	0.5	0.5	8.0	>64.0

[a] All MICs were determined in Luria broth (0.8% tryptone, 0.5% yeast extract) using the microtiter broth dilution assay as described by Piers et al. (1994) or in unpublished laboratory data. The Mg^{2+} content of this medium is recognized to be lower than that of serum. Abbreviations: SS, supersusceptible mutant (*P. aeruginosa* Z61, *E. coli* DC2); S, normally susceptible strain (*P. aeruginosa* H103, *E. coli* UB1005); R, resistant variant (*P. aeruginosa* H103/RP1, *E. coli* SC9252, *S. aureus* SAP0017).

fold lower than those of the parent strain K799. Interestingly, the decrease in MIC for the cationic peptides appeared minimal for the more potent α-helical peptides (Table 1; Piers and Hancock, 1994), indicating that these efflux systems are irrelevant for this class of peptides.

An important feature of these cationic peptides is their rapid killing of bacteria (Piers and Hancock, 1994) as compared with other antibiotics at the MIC. This is probably related to their ability to selectively form channels in bacterial membranes (Hancock et al., 1995; Skerlavaj et al., 1990).

RECOMBINANT PRODUCTION OF CATIONIC PEPTIDES

It is extremely difficult to produce large quantities of cationic peptides from nature. For example, for the rabbit defensins, only 200 mg of purified peptide can be obtained from a single animal. An alternative methodology involves protein chemical synthesis using a peptide synthesizer. However, this is relatively expensive and difficult to scale up. Magainin Sciences Inc. have had some success using solution-phase chemical synthesis, but this still tends to be relatively expensive.

With this in mind, we set out to devise a recombinant DNA methodology for production of cationic peptides in bacteria. It soon became apparent that the two challenges we were facing were (i) protecting the producing bacterium from the antibacterial peptide and (ii) protecting the peptide from bacterial proteases during synthesis. We will not discuss all of the approaches that did not work (Piers et al., 1993), but rather the general approach that did. The cationic peptides were produced as a four-component fusion protein comprising, from the N terminus, an affinity binding segment (to permit downstream affinity purification), an anionic protective segment (to bind to the cationic peptide protein and protect it from proteases), a methionine (to permit removal of the peptide by cyanogen bromide), and a cationic peptide antibiotic segment. Two basic variations

were devised. For *S. aureus* expression, the affinity binding and anionic protective segments were fused as a protein A binding domain, resulting in secretion of the fusion protein. In *E. coli* a separate anionic binding domain had to be included, and the resultant protein was produced cytoplasmically as inclusion bodies. However, a broad range of affinity binding regions and cationic peptide regions could be synthesized, and especially in the latter case, since the peptide expressing region was synthesized on a DNA synthesizer, virtually any sequence could be produced. The resultant peptide, after purification, was indistinguishable from chemically synthesized peptide (Piers et al., 1993; Piers and Hancock, 1994). This recombinant DNA technology provides a basic platform for isolation of variant peptides through screening of combinatorial libraries.

INTERACTION OF CATIONIC PEPTIDES WITH THE OUTER MEMBRANE

Cationic peptides interact with cells in a manner consistent with their being taken up by self-promoted uptake (Fig. 1). Dansyl polymyxin displacement experiments have indicated that they interact both with purified LPS (Fig. 2) and with LPS in the context of intact cells (Piers et al., 1994; Piers and Hancock, 1994). The affinities of MBI-28 (formerly CEMA), polymyxin B, rabbit defensin 1 (MCP-1), CEME, and gentamicin for *P. aeruginosa* LPS were, respectively, 1,214-, 914-, 800-, 654-, and 71-fold higher than the affinity for Mg^{2+} (see Piers et al. [1994] for the structures of CEME and CEMA). The actual I_{50} values (concentration resulting in 50% displacement of dansyl polymyxin from the LPS) were as low as 0.7 μM, indicating an interaction with an affinity as high as that of most transport systems in *P. aeruginosa*.

Interaction of the cationic peptides with intact *P. aeruginosa* cells resulted in permeabilization of these cells to lysozyme and NPN (Table 2). Lysozyme is a 14,000-Da basic protein which is able to destroy the peptidoglycan and lyse cells. However, it is normally denied access to the periplasmic peptidoglycan by the outer membrane. Thus one can follow outer membrane permeabilization by monitoring enhanced lysis as a function of cationic peptide concentration and derive an EC_{50} as the concentration leading to 50% of maximal lysis by lysozyme. In contrast, NPN is a small (219-Da) hydrophobic probe which fluo-

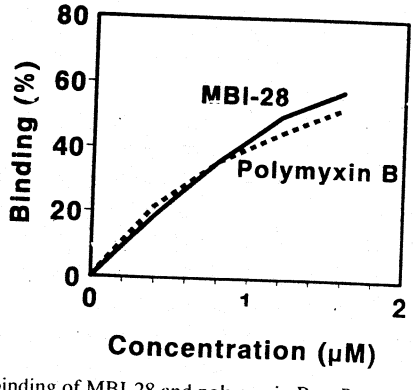

Figure 2. Level of binding of MBI-28 and polymyxin B to *P. aeruginosa* LPS, assessed as the percentage of dansyl polymyxin displaced by the given compound.

Table 2. I_{50} values for permeabilization of intact *P. aeruginosa* cells to lysozyme and NPN

Peptide	EC_{50} (μg/ml)[a]	
	Lysozyme	NPN
MCP-1	ND	15
CEME	0.7	0.7
MBI-28	0.3	0.2
Polymyxin B	1.5	0.2
Melittin	5.5	0.9

[a]EC_{50}, concentration of peptide leading to 50% maximal cell lysis (in the case of lysozyme) or 50% maximum uptake of the fluorophor NPN (as revealed by enhanced fluorescence as the NPN enters the cell membrane interior). Data are excerpted and rearranged from Piers et al. (1994). In the absence of peptide, no lysis was seen and only background fluorescence was observed since neither lysozyme nor NPN is taken up. The experiments were performed in 5 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5) with 1 mM KCN to permit observation of rapid kinetics. Divalent cations were highly antagonistic. ND, not done.

resces strongly in a hydrophobic environment (e.g., a membrane interior) but weakly in a hydrophilic environment (the extracellular solution). The intact outer membrane of wild-type cells normally excludes NPN and other hydrophobic probes. However, when cells are permeabilized by cationic peptides they take up NPN proportionally with the extent of outer membrane damage. It should be noted that there is an energized efflux system for NPN (Loh et al., 1984), and energy inhibitors must be added to permit NPN to remain in the cell, where the cellular concentration can be assessed by enhanced fluorescence.

It is of interest that there is a substantial difference (eightfold) in the concentration of polymyxin B required to enhance uptake of NPN as opposed to lysozyme, an observation that presumably reflects the relatively small size and/or hydrophobicity of the former. In contrast, CEME and MBI-28 cause enhanced permeability to both at similar concentrations, suggesting that these cationic peptides cause more extreme perturbations of the outer membrane at their minimal effective concentrations than does polymyxin B. Other results indicated that peptides can be designed for enhanced access to self-promoted uptake, since MBI-28, which differs from CEME by the addition of two extra positively charged amino acids at the carboxyl terminus, is a more effective outer membrane permeabilizer.

In contrast to many cationic antibiotics, the cationic peptides are unaffected by polymyxin resistance mutations in *E. coli* (Piers et al., 1994) and *P. aeruginosa* (unpublished data). However, they are more effective against an *S. typhimurium phoP/phoQ* mutant than against its parent strain, a result consistent with data for other cationic peptides including defensins (Piers et al., 1994). Furthermore, their activities were antagonized by addition of 1 mM Mg^{2+} but not by 80 mM Na^{+}, a result consistent with competition for Mg^{2+} binding sites on LPS (Piers et al., 1994; Piers and Hancock, 1994). Thus these data are generally consistent with the utilization of the self-promoted uptake pathway by the cationic peptides.

CONSEQUENCES OF SELF-PROMOTED UPTAKE

One of the immediate consequences of accessing the self-promoted uptake pathway is that cationic peptides bind to LPS (Fig. 3). We have recently demonstrated

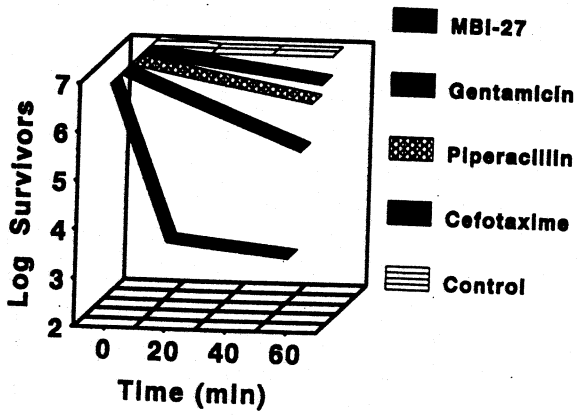

Figure 3. Kinetics of killing of *P. aeruginosa* by the cationic peptide MBI-27 (=CEME) and by other antipseudomonal antibiotics at the MIC. Data are from Piers et al. (1993) and the literature.

that cationic peptides neutralize the ability of LPS (endotoxin) to induce tumor necrosis factor in macrophage cell lines and in intact animals, and they prevent death from endotoxic shock in galactosamine-sensitized mice (N. M. Kelly, M. Gough, and R. E. W. Hancock, unpublished). Thus these peptides actually prevent endotoxic shock, whereas other antibiotics promote LPS release (Goto and Nakamura, 1980) and consequent endotoxemia.

A second consequence is that cationic peptides increase the permeability of the outer membrane (Table 2). Thus, since the outer membrane barrier of *P. aeruginosa* limits uptake of conventional antibiotics, the cationic peptides have the clear potential to work in synergy with these antibiotics. One caveat should be observed when the outer-membrane-permeabilizing and inner-membrane-perturbing concentrations of these molecules are quite similar. Under these circumstance, one would not necessarily expect synergy. However, we have been able to design peptides with lower or no antibiotic activity which are still competent outer membrane permeabilizers and which do show synergy with conventional antibiotics (data not shown).

CONCLUSIONS

There have been no new classes of antibiotics in the past 23 years since the introduction of the quinolones. We believe that the cationic peptides represent a breakthrough in this respect. While there are many barriers to overcome prior to demonstration of clinical efficacy, the good antibiotic activities, apparent lack of resistance problems, potential for synergy with conventional antibiotics, and antiendotoxic activity of cationic peptides, as well as their use as natural antibiotics throughout nature, indicate that they are worthy of consideration. Our ability to make these peptides by recombinant DNA technology makes these the first recombinant antibiotics.

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