Salt-Resistant Alpha-Helical Cationic Antimicrobial Peptides

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Antimicrobial cationic peptides are ubiquitous in nature and are thought to be components of the first line of defense against infectious agents (16). There are four structural classes of cationic peptides: the disulfide-bonded b-sheet peptides, including the defensins; the amphipathic a-helical peptides, such as the cecropins and melittins; extended peptides which often have a single amino acid predominating, e.g., indolicidin; and the loop-structured peptides, like bactenecin (16). Initial interactions of some cationic peptides with gram-negative bacteria are thought to involve binding to surface lipopolysaccharide (LPS) (28, 31). The peptides displace divalent cations that are essential for outer membrane integrity and consequently distort the outer membrane bilayer (26). This allows access to the cytoplasmic membrane, where peptide channel formation has been proposed to occur (21). It is increasingly disputed as to whether peptide channel formation leads to dissolution of the proton motive force and the leakage of essential molecules (9, 19, 37) or whether it is an intermediate step in the uptake of peptide into the cytoplasm, where it inhibits essential functions, e.g., by binding to polyanionic DNA (38).

Cecropins were originally isolated from the immune hemolymph of the North American silk moth Hyalophora cecropia (17). Cecropins have been well studied and characterized with respect to structure and function (12). Based on model membrane studies, the broad spectrum of antimicrobial activity of cecropins has been attributed to its ability to form large pores in bacterial cell membranes (8). A series of hybrid peptides were created, consisting of the amphipathic a-helical N-terminal region of cecropin A and the hydrophobic N-terminal a-helix of the bee venom peptide melittin (35). These hybrids form ion-permeable channels in model lipid membranes (36). To understand the structure-function relationships of these peptides, analogues based on the cecropin (1-8)-melittin (1-18) hybrid (CEME) were studied. The general conclusions from these studies were that the analogues should have a hydrophilic domain and a hydrophobic domain linked by a hinge region (7). A hinge region provides conformational flexibility due to the presence of glycine and proline residues (4). The aromatic residue at position 2 and the a-helical region in the first 11 amino acids have been found to be necessary for antimicrobial action (3). Piers et al. (27) further modified the hybrid peptide CEME (also called MBI-27 [14]) by adding two extra positively charged residues to the C terminus in order to assess the role of ionic charges in interactions with bacteria. The resulting peptide, CEMA (also called MBI-28 [14]), had MICs similar to those of CEME but had an increased ability to permeabilize the outer membranes of gram-negative bacteria and an increased affinity for LPS.

Pseudomonas aeruginosa is a pathogen that is known to colonize the lungs of cystic fibrosis patients. It is believed that the increased salinity of the bronchopulmonary fluids in these patients decreases the efficacy of endogenous cationic peptides of epithelial surfaces, thereby allowing colonization by these bacteria (13, 34). It is because of this that there is an interest in salt-resistant cationic peptides. Lee et al. (20) reported that clavanins, a-helical peptides that derive their cationicity from histidine residues, function in environments with normal and elevated levels of NaCl. This is in contrast to magainin, an a-helical peptide that is susceptible to the presence of 100 mM NaCl (20). In this study we looked at the resistance to the antagonistic effects of salt, the antimicrobial activity, and the mechanism of action of a family of related CEME variants designed here to be slightly different in charge, length, and hydrophobicity and to conform to Edmundson helical-wheel projections (32).
**MATERIALS AND METHODS**

Materials and bacterial strains. All peptides were synthesized by N-(9-fluorenylethoxycarbonyl) chemistry at the Nucleic Acid/Protein Service unit at the University of British Columbia. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoeholeryl (POPG) were purchased from Northern Lipids Inc. (Vancouver, British Columbia, Canada). α-Nitrophenyl-β-D-galactosidase (ONPG) and carbonyl cyanide-3-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Bovine serum albumin fraction V (VH) was purchased from Boehringer Mannheim (Mannheim, Germany).

Strains used for determining antimicrobial activity included *P. aeruginosa* PAO1 (15) as well as some antibiotic-resistant strains. *P. aeruginosa* K385 and 1008OCR01 (rfaB and rafB mutants, respectively) are strains that overproduce α-lactamase and chloroamphenicol, respectively. *P. aeruginosa* PAO963 has the nalA phenotype due to a mutation in the locus encoding a DNA gyrase subunit, resulting in nalidixic acid resistance (18, 30). A *P. aeruginosa* PA-83-48 (nalA) or 1008OCR01 (nalA) or 12.8 mg/ml in the presence of POPC-POPG (7:3) liposomes but rather in the so-called membrane-mimicking environment provided by the addition of sodium dodecyl sulfate (SDS) and trifluoroethanol (TFE; also considered a helix-inducing solvent). The concentrations of peptide and lipid in the buffer were 50 μM and 2 mM, respectively. In buffer, all peptides exhibited spectra characteristic of unordered structure. The spectra of CP26, CEME, CEMA, CP29, and CP201 in the presence of liposomes showed the typical appearance of α-helical secondary structures. In general, all peptides had only slight differences in charge, length, and hydrophobicity (Table 1).

**TABLE 1. Amino acid sequences of antimicrobial cationic peptides used in this study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Length (no. of amino acids)</th>
<th>Charge</th>
<th>% Hydrophobic amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP26</td>
<td>KKKFSIKLTTAKVKLVTGLPALIS</td>
<td>26</td>
<td>+7</td>
<td>46</td>
</tr>
<tr>
<td>CP29</td>
<td>KKKFSIKLTTAVKVLVTGLPALIS</td>
<td>26</td>
<td>+6</td>
<td>56</td>
</tr>
<tr>
<td>CP201</td>
<td>KKKFSIKLTTAVKVLVTGLPALIS</td>
<td>26</td>
<td>+5</td>
<td>42</td>
</tr>
<tr>
<td>CP208</td>
<td>KKKFSIKLTTAKVKLVTGLPALIS</td>
<td>26</td>
<td>+6</td>
<td>42</td>
</tr>
<tr>
<td>CEME</td>
<td>KKLFKGGIGAVKLVTGLPALIS</td>
<td>26</td>
<td>+5</td>
<td>69</td>
</tr>
<tr>
<td>CEMA</td>
<td>KKLFKGGGIGAVKLVTGLPALRK</td>
<td>28</td>
<td>+6</td>
<td>64</td>
</tr>
</tbody>
</table>

**RESULTS**

**Peptide design and secondary structure.** In previous studies we had examined the cepacin-mellitin hybrid CEMA and its variant CEMA. The CEMA sequence was modified to increase the α-helical content in the first 14 amino acids, resulting in CP29, using the Edmundson helical-wheel projection (32) to design this and the other peptides described here. This involved substituting hydrophilic amino acids for residues 4, 8, 10, 11, and 14, while residues 6 and 9 were replaced with hydrophobic amino acids. The maintenance of cationic charge was achieved by placing lysine residues at positions 8 and 14. Another related peptide, CP26, had the same first 10 residues as CP29, but the C terminus was predicted to be more hydrophilic because of the addition of an extra positively charged lysine. CP201 was similar to CP29, with the exception of the flexible hinge region consisting of two glycines in the center of the peptide. CP208 was similar to CP26, with four amino acid replacements, including the replacement of the tryptophan at position 2 with a lysine. In general, these peptides had only slight differences in charge, length, and hydrophobicity.

**REFERENCES**


revealed that the peptides were α-helical, but the relative α-helicitics varied among different peptides. For example, in SDS, CP208 was as α-helical as CP29. This stresses the importance of the microenvironment in peptide structure formation.

**Antimicrobial activity.** The MICs of the peptides for selected gram-negative bacteria are shown in Table 3. The MIC was taken as the lowest peptide concentration at which growth was inhibited in a broth dilution assay. CP26 and CP29 were similar in activity to CEMA and CEME. It was of interest to determine if mutations influencing susceptibility to conventional antibiotics had an effect on peptide MICs, especially given the recent observation by Shafer et al. (33) that peptides were influenced by multidrug efflux pathways in Neisseria. Neither nalB nor rfsB, which result in multidrug resistance due to derepression of the MexA MexB OprM and MexC MexD OprF efflux pathways, respectively, had much effect on peptide MICs. Similarly, little effect was observed for mutants representing the relatively common clinical mutations in DNA gyrase (nalA) or derepression of β-lactamase or the laboratory-derived mutations in Z61 which make the strain susceptible to virtually all conventional antibiotics due to outer membrane permeability and efflux defects. Of the other peptides, CP201 had intermediate activity and CP208 had little to no activity against the bacteria tested.

The best peptides demonstrated modest antimicrobial activity in an animal model. Neutropenic mice (8 to 12 per group) were injected intraperitoneally with 200 to 300 P. aeruginosa M2 cells, leading to 8% survival in control (saline-injected) animals. Intrapерitoneal injection, 30 min after the injection of bacteria, of a single dose of 200 µg of CP26 or CP29 led to identical (37%) survival rates after 4 days (P < 0.05 by Fisher’s exact test), results comparable to those achieved by CEME (26.7%) and CEMA (43.3%) (14).

**Antimicrobial activity in the presence of salts.** The MICs of the most active peptides for P. aeruginosa were determined in the presence of NaCl, MgCl₂, and sodium alginate (Table 4). There was no significant increase in the MICs of CP29, CEME, or CEMA in the presence of 300 mM NaCl, whereas CP26 showed a 16-fold increase in MIC under those conditions. However, CP26 was still resistant (less than a twofold increase in MIC; data not shown) to NaCl antagonism at a NaCl concentration up to 160 mM. The concentration of NaCl in the epithelial cell secretions of a cystic fibrosis patient is about 120 mM (13), and at this concentration all peptides maintained good anti-Pseudomonas activity.

The antibacterial activities of CP26, CP29, and CEME were more affected by the presence of divalent cations. At 3 mM MgCl₂ (modeling the serum divalent-cation concentrations of 1 mM Mg²⁺ and 2 mM Ca²⁺) (1), there was a four- to eight-fold increase in MICs. Since the concentration of added Cl⁻ in this case was only 6 mM, and 100 mM Cl⁻ had virtually no effect on MIC in the form of NaCl, it was concluded that the increase in MICs was due to the divalent cation Mg²⁺. In contrast to these three peptides, CEMA appeared to be relatively resistant to serum divalent-cation concentrations of 3 mM, but not 5 mM, Mg²⁺. Sodium alginate, a polyanionic

### Table 2. α-Helicity in various environments as assessed by CD spectroscopy interpreted according to the K2D algorithm (2)

<table>
<thead>
<tr>
<th>Condition</th>
<th>CP26 % α-Helix</th>
<th>CP29 % α-Helix</th>
<th>CP201 % α-Helix</th>
<th>CEMA % α-Helix</th>
<th>CEME % α-Helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Liposomes</td>
<td>26, 35</td>
<td>50, 57</td>
<td>20</td>
<td>8</td>
<td>17, 33</td>
</tr>
<tr>
<td>SDS (40:1)</td>
<td>42</td>
<td>23</td>
<td>13</td>
<td>26</td>
<td>50</td>
</tr>
<tr>
<td>50% TFE</td>
<td>30</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
<td>32</td>
</tr>
</tbody>
</table>

- The first number is for 90-nm liposomes, and the second is for 60-nm liposomes. ND, not determined.

### Table 3. Broth dilution MICs of cationic peptides against various gram-negative bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Relevant phenotype or genotype</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CP26</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>PA01</td>
<td>Wild type</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>K385</td>
<td>nalB</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1008OCR01</td>
<td>nalB</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PA0965</td>
<td>nalA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PA-83-48</td>
<td>β-Lactamase derepressed</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Z61</td>
<td>Antibiotic sensitive</td>
<td>2</td>
</tr>
<tr>
<td>E. coli</td>
<td>UBI1005</td>
<td>Wild type</td>
<td>0.5</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>14028s</td>
<td>Parent of MS7955s</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MS7955s</td>
<td>Defensin sensitive</td>
<td>0.5</td>
</tr>
</tbody>
</table>
polysaccharide related to the mucous exopolysaccharide of *P. aeruginosa* and intended to be representative of polyanions and polysaccharides present in vivo, antagonized the activities of CEME and CEMA more than those of CP26 and CP29. This effect was probably due to the alginate anion, not the sodium cation. CEMA, which was the most resistant to divalent cations, was the most sensitive to alginate.

**Killing assays.** We assessed the ability of the peptides at four times their MICs to kill logarithmic- and stationary-phase *E. coli* UB1005 in MH medium (Fig. 2A and B, respectively). The peptides killed logarithmic-phase *E. coli* rapidly by 3 to 5 log orders within 5 min. After 20 min, CP29 and CEME had reduced the number of log-phase bacteria by a total of 6 log orders, whereas CP26 and CEMA showed little reduction after the initial 3-log reduction, although all four peptides had similar MICs for *E. coli*. In contrast, the conventional antibiotics cationic aminoglycoside gentamicin and β-lactam ceftazidime induced only 2 and 1 log order of killing, respectively. Stationary-phase bacteria in MH broth were killed in a manner similar to log-phase bacteria, with the exception that the kinetics of killing was considerably slower (and for CP29 appeared biphasic) except for CEMA. CP26 (not shown for clarity) was only as efficient at killing as ceftazidime, but the other peptides were more effective than the conventional antibiotics.

**Cytoplasmic membrane permeabilization assay.** The extent of cytoplasmic membrane permeabilization by peptides (at fourfold their MICs) is indicated by the hydrolysis of the chromogenic substrate ONPG by the cytoplasmic enzyme β-galactosidase. The hydrolysis of ONPG was measured spectrophotometrically (Fig. 3). For CEME, CEMA, and CP29, permeabilization was rapid and reached a maximal rate within 1 min. CP26, however, had a considerably lower rate of permeabilization and took 14 min to reach the same level of ONPG hydrolysis as the other three peptides reached in 4 min. CP201 (at threefold its MIC) permeabilized the membrane at a low rate similar to that of another cationic antimicrobial, the lipopeptide polymyxin B, whereas CP208 (at 12.8 μg/ml) demonstrated little to no permeabilizing ability.

The influence of various factors on cytoplasmic membrane permeabilization was tested for CEME, CP26, and CP29 (Fig. 4). The peptides maintained their abilities to permeabilize the cytoplasmic membrane of *E. coli* in the presence of NaCl, although CP29 was somewhat more effective. However, in the presence of 5 mM Mg丝, all peptides were affected, and the most salt-sensitive peptide, CP26, lost its ability to permeabilize the cytoplasmic membrane. The peptides were not highly affected by the presence of the uncoupler CCCP at high concentrations, indicating that these peptides can still exert their effects in the absence of a membrane potential, in contrast to, e.g., the indolicidins (10).

**DISCUSSION**

A combination of appropriate chain length, amino acid composition, and positioning of apolar and positively charged residues is required for the antibacterial activity of cationic antimicrobial peptides; however, the exact nature of this com-

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**TABLE 4. Influence of NaCl, MgCl<sub>2</sub>, and polyanionic alginate on MICs of cationic antimicrobial peptides for *P. aeruginosa* PAO1**

<table>
<thead>
<tr>
<th>Additive</th>
<th>Conc</th>
<th>CP26</th>
<th>CP29</th>
<th>CEME</th>
<th>CEMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 mM</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1 mM</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>&gt;32</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>0.01%</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.05%</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

* These are the average values of three experiments.
bination is still under study. Pathak et al. (24) hypothesized that the amphiphilicity of antimicrobial peptides is the most important factor governing activity, above mean hydrophobicity and α-helix content. In another study it was reported that the antimicrobial activity of the cecropin-melittin hybrid peptides depends on their helical nature (3). Here, we used the already-established cecropin-melittin hybrid peptide, CEMA, and CEMA, a variant peptide with two extra amino acids and positive charges in the C terminus, as the templates for further design. Piers et al. (27) showed that CEMA was more efficient than CP29 (solid), CEME (stippled), and CP26 (striped), all of which were more helical than CP29. The increase in the α-helicity of CP29 did not make the peptide more active in vitro. The MICs of CP26 were also similar, indicating either that the altered bend region and extra helix were the main reason of the activity of CP29. However, CP26, CEME, CEMA, and CP29 had the same number of amino acids but had charges ranging from +5 to +7, hydrophobic amino acid contents from 46 to 69%, and α-helix contents ranging from 17 to 57% in lipid environments. It was thus of interest to note the similarities and differences between these peptides. All had similar and good activities against the gram-negative bacteria tested and were able to rapidly kill logarithmic-phase bacteria. They also demonstrated similar MICs for antibiotic-resistant mutants of P. aeruginosa, indicating that the mode of action of these α-helical peptides differs from those of conventional antibiotics and that these antibiotics are not effluxed in P. aeruginosa (cf. Neisseria gonorrhoeae [33]). The increase in the α-helicity of CP29 did not make the peptide more active in vitro. The MICs of CP26 were also similar, indicating that the altered bend region and extra helix did not affect its MIC or that it has a different killing mechanism. CP201, which fell within the ranges of most of the physical properties of the above four peptides, was not a good antibacterial agent, possibly due to the combination of two detrimental factors, namely, lower hydrophobicity (42%) and lower charge (+5). CP208, which also had physical properties similar to those of the four active peptides, had virtually no antibacterial activity, possibly because it lacked a tryptophan residue required for the insertion of the peptide into the lipid membrane. This was supported by the observation that although CP208 was designed to be α-helical, it was unable to form an α-helix upon interaction with liposomes but was able to in the presence of SDS.

The ability to resist salt (NaCl is the most predominant salt in vivo) is important for cationic peptides to function under physiological conditions. In the case of cystic fibrosis, it has been suggested that the susceptibility of epithelial antimicro-
bial peptides (presumably β-structured defensins) to salt antagonism explains the persistence of chronic P. aeruginosa infections in the lungs of patients with this disease (13). Lee et al. (20) reported the NaCl resistance of the tunicate cationic peptide clavanin in contrast to the α-helical peptides magainin 1 and cecropin P1. It has been observed that extended indolicidins, β-sheet gramicidins, and looped and linear bacteriocins are all quite salt (KCl) sensitive (37). Thus, it was of interest to examine the effects of salts on the activities of our α-helical peptides. There were no significant changes in the MICs of CEME, CEMA, and CP29 in the presence of up to 300 mM NaCl, whereas CP26 appeared to be resistant to NaCl only at concentrations up to 160 mM. An NaCl concentration of 120 mM has been reported to be present in the environment of the epithelial cells of a cystic fibrosis patient, which is 30 mM higher than the level of NaCl that antagonizes the activity of epithelial cationic peptides (13). Therefore, even CP26 can be described as NaCl resistant. The differences in activity between CP26 and the other three peptides are presumably related to differences in flexibility and/or hydrophobicity. Interestingly, all peptides were relatively more susceptible to the presence of Mg²⁺ ions, with a 16-fold increase in their MICs in the presence of 5 mM Mg²⁺, although CEMA was clearly more resistant to physiologically meaningful (3 mM) divalent cation concentrations, although CP26 tended to be less affected by this polyvalent NaCl solution, whereas 100-fold-lower Mg²⁺ concentrations had the same effect as 300 mM Na⁺. Presumably, these different effects can be explained by differential affinities for a binding site on cells, which we propose here to be on cell surface LPS. Mg²⁺ has a much higher affinity for binding to LPS than does Na⁺ (25). The differential effects of divalent and monovalent cations were also demonstrated by the results of the cytoplasmic membrane permeabilization experiments (Fig. 4). However, since interaction with the outer membrane precedes interaction with the cytoplasmic membrane, it is likely that it was at this earlier stage that the peptides were being antagonized. We also examined the antibacterial activities of these peptides in the presence of alginante, a model polysaccharide intended to represent those found in vivo. This polyanion reduced the activity substantially at modest concentrations, although CP26 tended to be less affected by this polyanion. In addition to in vitro killing, these peptides demonstrated an ability to work in neutrophilic mice.

We have demonstrated here that α-helical peptides with the same general physical properties, but with small differences in hydrophobicity, amphipathicity, charge, and degree of α-helicity, can vary substantially in activity, salt resistance, and permeabilizing ability. CP26 and CP29, which differed by only seven amino acids (four of which were substitutions of one hydrophobic residue for another), had similar MICs and in vivo activities but substantial differences in their resistance to salt antagonism and ability to permeabilize the cytoplasmic membrane. Thus, modest alterations in sequence, and presumably in three-dimensional structure, can result in substantial alterations in a peptide’s properties.

The best α-helical peptides studied here had good activities and were resistant to physiological concentrations of salt. These characteristics may prove to be useful in the design of future therapeutic antibiotic drugs.

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