Antimicrobial activity of cecropins

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The lytic peptides, cecropins, were originally isolated from the haemolymph of the giant silk moth, Hyalophora cecropia and possess antibacterial and anticancer activity in vitro. This study investigated the antimicrobial activity of these peptides against human pathogens using standardised assay techniques, and the activity of cecropin B on outer and inner bacterial membranes. From a panel of 15 organisms, Gram-negative bacteria were generally more sensitive to cecropins than Gram-positive organisms, especially the lipopolysaccharide defective mutant, Escherichia coli BUE55. Cecropins B and P, shared similar MIC values whereas Shiva-1, a cecropin B analogue, was less active. Through combination studies with hydrophobic antibiotics and electron microscopy, cecropin B was shown to disrupt the bacterial outer membrane. Protoplasts of Staphylococcus aureus and Staphylococcus epidermidis were resistant to cecropin B, suggesting that the cytoplasmic membranes of Gram-positive organisms were inherently more resistant to the peptide.

Introduction

There is increasing evidence that lytic peptides are involved in the antimicrobial defence system of a number of animal species: including insects (Hultmark et al., 1980); crustaceans (Nakamura et al., 1988); amphibians (Zasloff, 1987) and mammals (Ganz et al., 1985). Cecropins are one family of these peptides which were first isolated from the haemolymph of the giant silk moth, Hyalophora cecropia (Hultmark et al., 1980). The principle insect cecropins (A, B and D) are 35 to 37 residues (Hultmark et al., 1982), devoid of cysteine, with a strongly basic N-terminal linked to a neutral C-terminal by a flexible glycine-proline link (Steiner et al., 1981; van Hofsten et al., 1985). The overall structure deduced by NMR for cecropin A is two near perfect amphipathic segments joined by a Gly-Pro hinge (Holak et al., 1988; Steiner, 1982). A cecropin-like 31 residue peptide (cecropin P₁) has been isolated from the small intestine of a pig (Lee et al., 1989), suggesting that the cecropins may be widespread throughout the animal kingdom.

Shiva-1, an analogue which shares 46% sequence homology and maintains the same charge distribution and hydrophobicity as cecropin B, has been shown to lyse leukaemia and lymphoma cell lines in vitro (Jaynes et al., 1989). Cecropin B was also cytotoxic.
to a range of mammalian cancer and non-cancer cell lines in vitro (Moore et al., 1994), yet inactive against sheep erythrocytes in agar (Wade et al., 1992).

The continual emergence of bacterial resistance to drugs has meant that there is a need to develop novel agents that are not susceptible to existing mechanisms of resistance and cecropins may be possible candidates. Antibacterial activity has been reported for cecropins A, B and P, against Gram-positive and Gram-negative organisms (Hultmark et al., 1982; Lee et al., 1989). However, it is difficult to fully assess their potential as antimicrobial agents on the basis of these studies because investigations tended to concentrate on insect and plant rather than human pathogens, and techniques which are not immediately comparable with standard MIC determinations were used to measure their activity.

The mechanism of action of cecropins is thought to involve channel formation in membranes and subsequent lysis (Christensen et al., 1988). Whilst the mechanism of action has been studied using artificial membrane systems, very little has been done using whole bacterial cells. In this study optimum assay conditions were established to evaluate the antimicrobial activity of cecropin B against a range of clinically relevant organisms and to compare the activities of cecropins B, P, and Shiva-1 (Figure 1). Effects of cecropin B on outer and cytoplasmic bacterial membranes were assessed by permeability, electron microscope and spheroplast and protoplast studies.

Materials and methods
Peptides and antibiotics
Stock solutions (1 mM) of cecropin B (Proteus Molecular Design Ltd, Macclesfield, UK), cecropin P, (Peninsula Laboratories Europe Ltd, Merseyside, UK) and Shiva-1 (Bachem, California, USA) were made in 0.1 M phosphate buffer pH 6.4 and stored at −20°C. Stock solutions (500 mg/L) of erythromycin, fusidic acid, rifampicin, clindamycin and novobiocin (Sigma Chemical Co., Poole, UK) were kept at −20°C. Sodium benzylpenicillin (Glaxo Laboratories Ltd, Greenford, UK) was dissolved in water (60 mg/mL) and stored at 4°C for up to 5 days.

Organisms and culture conditions
Bacillus cereus NCTC2599, Bacteriodes fragilis NCTC943, Clostridium perfringens NCTC8237, Escherichia coli NCTC11954, Klebsiella pneumoniae NCTC418, Proteus mirabilis NCTC11938, Pseudomonas aeruginosa NCTC10332, Burkholderia cepacia NCTC10744, Staphylococcus aureus NCTC8532, Staphylococcus epidermidis NCTC11047, Staphylococcus haemolyticus NCTC11042, Enterococcus faecalis NCTC775, Streptococcus mutans NCTC10449 and Streptococcus pneumoniae NCTC7465 were acquired from the National Collection of Type Cultures, UK. Escherichia coli BUE55, a lipopolysaccharide (LPS) deficient mutant, was supplied by Dr R. Dixon (University of Bradford). Candida albicans NCYC1363 was acquired from

Cecropin B KWKVF KKIEK MGRNIRNGIVK AGPAI AVLGEAKAL-NH₂
Cecropin P₁ SWLSTAKKLENSAKKRISEGIAIAIQGGR
Shiva-1 MPRWRLFRRIDRVGQKQGILRAGPAIALVGDA RAVG

Figure 1. Amino acid sequences of cecropins B, P₁ and Shiva-1.
the National Collection of Yeast Cultures, UK. All cultures were stored long-term at 
—80°C on cryostat beads (Technical Service Consultants Ltd, Bury, UK). Aerobic and faculative bacteria and C. albicans were also stored at 4°C on IsoSensitest (IS) agar (CM471, Oxoid Ltd, Basingstoke, UK) slopes. These organisms were grown in IS broth (Oxoid CM473) at 37°C, for bacteria, and at 30°C, for yeast. B. fragilis and C. perfringens were grown in Wilkins-Chalgren (WC) broth (Oxoid CM643) or on WC agar (Oxoid CM619) at 37°C under an atmosphere of 10% CO₂, 10% H₂ and 80% N₂ in a Compact Anaerobic Work Station (Don Whitley Scientific Ltd, Shipley, UK).

**Agar diffusion assay of antimicrobial activity**

An overnight broth culture was diluted to 4 × 10⁶ cfu/mL and 100 μL was added to 12 mL of IS or WC molten agar. Inoculated media were poured into 9 cm plastic petri dishes and left to solidify for 30 min. Swarming of P. mirabilis in agar was reduced by the addition of 120 μL of 5 mg/mL p-nitrophenylglycerol (Sigma Chemical Co.) into the molten medium. Aliquots (2 μL) of serial doubling dilutions of cecropins (maximum concentration 1000 or 100 μM), Shiva-1 (maximum concentration 100 μM) or phosphate buffer were added to 1.5 mm wells in the agar. Plates were incubated 16–18 h at 30°C for aerobic and facultative organisms and at 37°C for anaerobes. After incubation inhibition zones were measured and in this assay organisms were considered to be sensitive if the diameter of the inhibition zone, produced by a peptide, was 2.5 mm or greater. The lowest concentration of peptide producing inhibition zones of 2.5 mm or greater was recorded. Assays were carried out at least in triplicate. The effect of allowing pre-diffusion of the peptide through the agar was assessed by holding plates inoculated with E. coli NCTC11954, and with wells containing a range of concentrations of cecropin B, at 4°C for 4 h prior to incubating at 30°C.

**Broth microdilution assay of antimicrobial activity**

Broth assays were performed in 96-well round bottom microtitre plates (Corning Laboratory Sciences Co., Halstead, UK). Doubling dilutions of peptides were made in IS or WC broth to a final volume of 100 μL per well. Aliquots (50 μL) of exponentially growing organisms (2 × 10⁵ cfu/mL) were added to each well (3 × 10⁵ cfu per well). The maximum final concentration of peptide in a well was 66.7 μM. Plates were incubated 16–18 h at 30°C for aerobic and facultative organisms and at 37°C for anaerobes. After incubation the MIC values were determined and organisms were considered to be sensitive if growth was prevented in any well containing a peptide concentration of 66.7 μM or less.

To determine the combined activities of cecropin B and antibiotics, doubling dilutions of antibiotics (10 μL) plus 10 μL of a sub-inhibitory concentration of cecropin B (previously determined) or phosphate buffer were added to 80 μL of IS broth in a row of wells in a microtitre plate. In the wells the maximum concentration of hydrophobic antibiotics was 33.3 mg/L and of benzylpenicillin was 4000 mg/L. Aliquots (50 μL) of exponentially growing organisms (2 × 10⁵ cfu/mL) were added to each well and the microtitre plates were incubated 16–18 h at 30°C. The MIC of the antibiotic alone and in the presence of cecropin B was recorded. Bacteria were considered to be sensitive if they were inhibited at the maximum concentration of antibiotic or less.
**Rate of killing of E. coli by cecropin B**

An aliquot (500 μL) of exponentially growing *E. coli* NCTC11954 (2 × 10^7 cfu/mL) was added to 900 μL of IS broth and exposed to 100 μL of 100 μM cecropin B (final concentration 6.7 μM) for 0, 0.5, 1 and 2 h at 30°C. After these times serial ten-fold dilutions were made in 0.9% saline and bacteria were enumerated by the pour plate method using IS agar.

**Generation of spheroplasts and protoplasts**

Exponentially growing organisms were centrifuged at 1000g for 20 min and washed twice in 10 mM TRIS buffer. Pellets were resuspended in 9 mL of 0.1 M Tris buffer (containing 20% w/v sucrose) plus 1 mL of 2.5, 4 or 5 mg/mL lysozyme for *E. coli* NCTC11954, *S. aureus* and *S. epidermidis*, respectively. *E. coli* was incubated with lysozyme at 37°C for 12 min before the slow addition of 0.9 mL disodium EDTA and a further 15 min incubation. The Gram-positive organisms were incubated at 37°C with lysozyme for 2 h. Greater than 80% of bacteria formed spheroplasts or protoplasts as assessed by phase contrast microscopy, and this was confirmed by regeneration studies in sodium dodecyl sulphate (SDS). For regeneration, preparations of spheroplasts or protoplasts were treated with 0.1 mL 1% (w/v) SDS or TRIS buffer containing 20% sucrose. After streaking 50 μL of each suspension on to IS agar and incubating plates at 37°C overnight, colonies were counted and the percentage regeneration calculated.

**Assay of activity of cecropins against spheroplasts and protoplasts**

Spheroplasts and protoplasts were washed twice and resuspended in 0.1 M TRIS buffer (containing 20% w/v sucrose) to give an optical density at 600 nm (OD_600) of approximately one. Dilutions of cecropin B or phosphate buffer (100 μL) were added to 1 mL of spheroplasts or protoplasts in a cuvette and incubated at 37°C. OD_600 readings were taken immediately and every 5 min.

**Human erythrocyte lysis**

Aliquots (100 μL) of doubling dilutions of cecropin B in Alsevers solution, Alsevers solution alone (negative control) or distilled water (positive control) were added to a 96-well round bottom microtitre plate. Alsevers solution contained 20.5 g D-glucose, 8 g tri-sodium citrate, 4.2 g sodium chloride, 0.55 g citric acid in one litre of distilled water. Aliquots (50 μL) of human erythrocytes in Alsevers solution 5% (v/v) were added to the wells and plates were incubated at 30°C for 30 min with continuous shaking. Plates were centrifuged at 2000g for 10 min and the supernatant was transferred to equivalent wells in another plate and the OD_600 was read.

**Electron microscopy**

Volumes of 500 μL of exponentially growing *E. coli* NCTC11954 (2 × 10^7 cfu/mL) were added to 900 μL quantities of IS broth and 100 μL of cecropin B (final concentration 3.3 μM), polymyxin B (final concentration 4.3 mg/L; Sigma Chemical
Table I. Antimicrobial activity of cecropin B in the agar diffusion and broth microdilution assays

<table>
<thead>
<tr>
<th>Organism</th>
<th>Concentration of cecropin B producing inhibition in assay (µM)</th>
<th>Concentration of cecropin B producing inhibition in assay (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>agar diffusion</td>
<td>broth microdilution</td>
</tr>
<tr>
<td>E. coli BUE55</td>
<td>12.5</td>
<td>1.7</td>
</tr>
<tr>
<td>E. coli NCTC11954</td>
<td>25</td>
<td>3.3</td>
</tr>
<tr>
<td>K. pneumoniae NCTC418</td>
<td>25</td>
<td>ND</td>
</tr>
<tr>
<td>B. fragilis NCTC943</td>
<td>50</td>
<td>1.7</td>
</tr>
<tr>
<td>P. aeruginosa NCTC10332</td>
<td>50</td>
<td>16.7</td>
</tr>
<tr>
<td>P. mirabilis NCTC11938</td>
<td>100</td>
<td>R'</td>
</tr>
<tr>
<td>B. cepacia NCTC10744</td>
<td>R''</td>
<td>ND</td>
</tr>
<tr>
<td>S. haemolyticus NCTC11042</td>
<td>50</td>
<td>R''</td>
</tr>
<tr>
<td>S. epidermidis NCTC11047</td>
<td>100</td>
<td>33.3</td>
</tr>
<tr>
<td>E. faecalis NCTC775</td>
<td>100</td>
<td>R'</td>
</tr>
<tr>
<td>B. cereus NCTC2599</td>
<td>R'</td>
<td>ND</td>
</tr>
<tr>
<td>C. perfringens NCTC8237</td>
<td>R'</td>
<td>R''</td>
</tr>
<tr>
<td>S. mutans NCTC10449</td>
<td>R''</td>
<td>ND</td>
</tr>
<tr>
<td>S. pneumoniae NCTC7465</td>
<td>R'</td>
<td>ND</td>
</tr>
<tr>
<td>S. aureus NCTC8532</td>
<td>R''</td>
<td>R''</td>
</tr>
<tr>
<td>C. albicansNCYC1363</td>
<td>R''</td>
<td>ND</td>
</tr>
</tbody>
</table>

Resistant at R' = 66.7 µM, R'' = 100 µM and R''' = 1000 µM.
ND, Not determined.

Co.) or phosphate buffer and incubated for 30 min at 30°C. Samples were centrifuged at 13000 rpm for 2 min (Biofuge 13, Heraeus Equipment Ltd, Brentwood, UK) and pellets were resuspended and washed twice in distilled water. A drop of suspension and 0.5% methylamine tungstate (Agar Scientific Ltd, Stansted, UK) were added to Formvar/ carbon (200 mesh) 3 mm grid (Agar Scientific Ltd) that had previously been plasma-glowed in an Edwards SB150B sputter coater (Edwards High Vacuum Co., York, UK). The grid was examined and micrographs taken using a JEOL 100CX transmission electron microscope (JEOL Ltd, Welwyn Garden City, UK).

Results

Antimicrobial activity of peptides

Preliminary experiments compared the activity of cecropin B in various media and at different incubation temperatures. In Diagnostic Sensitivity Test (Oxoid CM261) and Mueller-Hinton agar (Oxoid CM337) peptide activity was less than in IS medium. Optimum activity was in IS medium after a 16–18 h incubation at 30°C compared with 25°C and 37°C. In agar all inhibition zones were small, up to a maximum of 10 mm, but the edges were very clear and well defined. When inoculated with E. coli BUE55 there was a further area of lighter growth surrounding the clear zone of total inhibition.

Gram-negative bacteria were generally more sensitive to the peptides than Gram-positive organisms in the agar diffusion and broth microdilution assays (Tables I and II). In the agar diffusion assay the minimum concentration of peptide to which
the organisms were sensitive produced inhibition zones of 2.5–4.0 mm in diameter. Pre-diffusion of cecropin B at 4°C did not result in larger zones of inhibition of *E. coli* NCTC11954. Cecropin B showed a range of activities against the Gram-negative organisms and the LPS defective mutant, *E. coli* BUE55, was the most susceptible bacterium. Some Gram-positive bacteria were sensitive towards the maximum concentrations of cecropin B used. *C. albicans* was resistant to 100 µM cecropin B in the agar diffusion assay. In agar cecropin P, was less active than cecropin B, however in the broth assay their activities were similar. Shiva-1 had much lower activity than cecropin B; in agar at 100 µM and in broth at 66.7 µM *E. coli* BUE55 was sensitive to the peptide whilst *E. coli* NCTC11954, *P. aeruginosa* and *P. mirabilis* were resistant.

The activity of cecropin B against *E. coli* NCTC11954 was complete after a 30 min exposure period. The survival of organisms after 30 min was below 0.5%.

**Effects of cecropin B on sensitivity of bacteria to other antibiotics**

*S. epidermidis* was sensitive to all antibiotics tested alone and *E. coli* BUE55 was sensitive to all except clindamycin and fusidic acid. The remaining three Gram-negative bacteria were resistant to most of the antibiotics (Table III). When sub-inhibitory concentrations of cecropin B were added with the antibiotics, *E. coli* BUE55, *E. coli* NCTC11954 and *P. aeruginosa* were more sensitive to all of them, apart from clindamycin (activity was only increased against *E. coli* BUE55). *P. mirabilis* was resistant to 33.3 mg/L clindamycin, erythromycin and fusidic acid in the presence of 66.7 µM cecropin B and became sensitive to 4.2 mg/L novobiocin. A sub-inhibitory concentration of cecropin B increased the sensitivity of *S. epidermidis* to benzylpenicillin, fusidic acid and novobiocin.

**Cecropin B activity against spheroplasts, protoplasts and human erythrocytes**

Spheroplasts of *E. coli* NCTC11954 were sensitive to cecropin B and there was a rapid decrease in OD600 even at 0.4 µM of peptide. *S. epidermidis* and *S. aureus* protoplasts were relatively resistant after exposure to 100 µM cecropin B for 10 min (Figure 2). After 35 min there was a 30% decrease in OD600 of *S. epidermidis* protoplasts at 100 µM

**Table II. Antimicrobial activity of cecropin P, in the agar diffusion and broth microdilution assays**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Concentration of cecropin P, producing inhibition in assay (µM)</th>
<th>Concentration of cecropin P, producing inhibition in assay (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BUE55</td>
<td>25</td>
<td>3.3</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC11954</td>
<td>50</td>
<td>3.3</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NCTC10332</td>
<td>500</td>
<td>8.3</td>
</tr>
<tr>
<td><em>P. mirabilis</em> NCTC11938</td>
<td>1000</td>
<td>R'</td>
</tr>
<tr>
<td><em>S. epidermidis</em> NCTC11047</td>
<td>ND</td>
<td>R'</td>
</tr>
<tr>
<td><em>E. faecalis</em> NCTC775</td>
<td>ND</td>
<td>R'</td>
</tr>
<tr>
<td><em>S. aureus</em> NCTC8532</td>
<td>R'</td>
<td>R'</td>
</tr>
</tbody>
</table>

Resistant at R' = 66.7 µM, R' = 1000 µM.

ND, Not determined.
Table III. Antibacterial activity of antibiotics alone and in combination with a sub-inhibitory concentration of cecropin B

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Addition of cecropin B*</th>
<th>E. coli BUE55</th>
<th>E. coli NCTC11954</th>
<th>MIC (mg/L) against P. aeruginosa NCTC10332</th>
<th>P. mirabilis NCTC11938</th>
<th>S. epidermidis NCTC11047</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>–</td>
<td>0.1</td>
<td>4000</td>
<td>4000</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>+</td>
<td>0.05</td>
<td>1000</td>
<td>500</td>
<td>ND</td>
<td>0.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>–</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>+</td>
<td>8.3</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>0.21</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>–</td>
<td>16.7</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>0.42</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>+</td>
<td>2.1</td>
<td>33.3</td>
<td>33.3</td>
<td>R</td>
<td>0.42</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>–</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>0.11</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>+</td>
<td>16.7</td>
<td>8.3</td>
<td>33.3</td>
<td>R</td>
<td>0.21</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>–</td>
<td>33.3</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>+</td>
<td>16.7</td>
<td>16.7</td>
<td>33.3</td>
<td>R</td>
<td>0.11</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>–</td>
<td>4.2</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>+</td>
<td>2.1</td>
<td>4.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Cecropin B concentrations for E. coli BUE55 = 0.83 μM, E coli NCTC11954 = 1.7 μM, P. aeruginosa = 4.2 μM, P. mirabilis = 66.7 μM, S. epidermidis = 8.3 μM.

R, Resistant at 33.3 μg mL⁻¹ of antibiotic ± cecropin B.

ND, Not determined.
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Cecropin B, however *S. aureus* protoplasts remained resistant. At 500 µM cecropin B there was no lysis of human erythrocytes compared with the negative control.

Electron microscopy

*E. coli* NCTC11954 treated with phosphate buffer had an intact outer membrane and fimbriae could be seen around the perimeter (Figure 3(a)). There was a general loss of large amounts of outer membrane material from the surface of *E. coli* NCTC11954 that had been exposed to 3.3 µM cecropin B for 30 min (Figure 3(b)). *E. coli* NCTC11954 treated with 4.3 mg/L polymyxin B for 30 min showed blebbing or discrete vesicles of the outer membrane around the perimeter of the organism (Figure 3(c)).

Discussion

This study describes the antimicrobial activity of cecropins and Shiva-1. Gram-negative organisms were generally more sensitive than Gram-positive bacteria to cecropins B and P1, while *C. albicans* was resistant. Among the Gram-positive organisms, sensitivity was only seen in *E. faecalis*, *S. epidermidis* and *S. haemolyticus* at relatively high peptide concentrations. Other studies of the antimicrobial activity of cecropins have shown a similar trend in activity against Gram-positive and negative bacteria (Hultmark et al., 1982; Lee et al., 1989). However, it is difficult to directly compare results because previous studies have not expressed peptide activity as a MIC or concentrated on bacteria of relevance to human infections. Cecropin activity has been expressed as

![Figure 2](https://academic.oup.com/jac/article-abstract/37/6/1077/863260)

**Figure 2.** Effect of cecropin B on the OD600 of *E. coli* (○) spheroplasts and *S. aureus* (●) and *S. epidermidis* (▽) protoplasts after 10 min. Results are expressed as the mean (±S.D.) of three OD600 readings.
a lethal concentration (LC), which was calculated from results of the agar diffusion assay using an equation incorporating the diameter of the zone of inhibition and agar depth. Zones of inhibition produced by peptides were very small and were not suitable for calculating a LC (Hultmark et al., 1982). Additionally, the small volume of agar used by Hultmark et al. (1982) were not suitable for providing accurate zone sizes since the depth of agar across the plate was inconsistent. For comparison with other
antimicrobial agents a MIC value is much more useful, but the cost of the peptide is limiting in many assays.

Trends in activity were the same in agar and broth, except for cecropin P, which was comparable to cecropin B in broth but less active in agar. In agar the activity of cecropin depends not only on the sensitivity of the organism but also on the ability of the relatively large and basic peptide to diffuse through the agar medium; pre-diffusion experiments indicated that this movement was poor, thus accounting for the small zone sizes.

Treatment of *E. coli* BUE55 with cecropin B showed a zone of lighter growth around the clear zone of inhibition. This may have represented a region of delayed growth or a sub-population within the strain that had a reduced susceptibility to the peptide.

The predicted site for the lethal action of the peptides is the cytoplasmic membrane (Christensen *et al.*, 1988) and therefore these molecules must initially be able to cross the outer membrane of Gram-negative organisms. The influence of the outer membrane in cecropin activity has not received much attention. Electron microscopy showed that there was disruption to the outer membrane of *E. coli* NCTC11954 after treatment with cecropin B. Other studies have stated that treatment of *E. coli* with polymyxin B (Storm, Rosenthal & Swanson, 1977) and defensins (Lehrer *et al.*, 1989) produce discrete vesicles of outer membrane fragments. While such discrete vesicles were not seen after treatment with cecropin B, further detailed work is needed to determine if the mechanisms of disruption caused by these agents are distinct. Vaara (1992) proposed that other strongly cationic drugs achieve penetration of the outer membrane by displacing the cations that form cross-bridges between adjacent LPS molecules in the outer membrane.

Significant permeabilization of the outer membrane was also indicated by experiments using cecropins in combination with other antibiotics. Large hydrophobic antibiotic molecules, which inhibit protein and nucleic acid synthesis, are usually ineffective against Gram-negative bacteria because they cannot diffuse across the outer membrane (Hancock & Wong, 1984). In the present study a sub-inhibitory concentration of cecropin B increased the activity of most of the antibiotics against Gram-negative bacteria by increasing permeability of the outer membrane. Since most of the Gram-negative bacteria were resistant at the maximum concentration of hydrophobic antibiotic tested (33.3 mg/L), synergism could not be determined. Vaara & Vaara (1994) did not categorise the combined activity of cecropin B and hydrophobic antibiotics as synergistic. Most Gram-negative bacteria remained resistant to clindamycin but this is most probably due to an inherent resistance of their ribosomes. Cecropin B may also have increased the access of fusidic acid and novobiocin across the cytoplasmic membrane of *S. epidermidis*. Activity of benzylpenicillin against Gram-negative bacteria was increased, presumably through an increase in permeability of the outer membrane: *E. coli* NCTC11954 and *P. aeruginosa* were more resistant to benzylpenicillin than *S. epidermidis* due to poor access of the drug through porins in the outer membrane. The enhanced activity of cecropin B against *S. epidermidis* would be expected, resulting from increased access of peptides to the cytoplasmic membrane following breakdown of the peptidoglycan by benzylpenicillin.

There was a range of sensitivities of Gram-negative organisms to cecropins and this may in part reflect differences in LPS composition of the outer membrane. Rana *et al.*
Antimicrobial activity of cecropins

(1991) suggested that susceptibility of the outer membrane to disruption caused by magainins (a similar group of cationic peptides) was probably determined by several factors, including charge and concentration of LPS and the presence or absence of the O-antigen side chain. The proposed mechanism of action of cecropin involves the initial binding of the peptide through electrostatic attraction. This may be important in the binding of cecropins to Gram-negative outer membranes, but may be absent or reduced in the generally less susceptible Gram-positive bacteria and C. albicans.

Clearly, binding to the outer surface of the bacterial cell is not the only determinant of susceptibility to cecropins. Results of binding studies by Steiner, Andreu & Merrifield (1988) suggested that different bacterial species could resist different amounts of bound cecropin before lysis. The lethal event which occurs at the cytoplasmic membrane is not fully understood. The interaction between the peptide and membrane is thought to be determined by factors such as the lipid composition of the membrane, its surface charge density and by the presence of an electrochemical potential across the membrane. In-vitro studies showing that acidic phospholipid vesicles were more susceptible to the peptide than those composed of zwitterionic phospholipids (Gazit et al., 1994) support the contention that lipid composition is important. That differences in the intrinsic properties of bacterial cytoplasmic membranes, and not merely access of the peptide to this membrane, play a large part in determining sensitivity is supported by the findings presented in this study. E. coli NCTC11954 spheroplasts were extremely sensitive to cecropin B, whereas S. aureus and S. epidermidis protoplasts were resistant to 100 mM cecropin B after exposure for ten minutes. After 35 min S. epidermidis protoplasts became sensitive to 100 mM cecropin B, but S. aureus protoplasts remained resistant and this corresponded to differences in their sensitivity when whole cells were used in agar diffusion or broth microdilution assays.

Cecropin B has a range of activities against different cancer and non-cancer cell lines (Moore et al., 1994). The resistance of human erythrocytes to the peptide may reflect properties of erythrocyte membranes, including lack of acidic lipids in the outer monolayer and abundant presence of cholesterol. The cytotoxicity of cecropin B was almost complete within one hour (Moore et al., 1994) and in this study rapid antimicrobial activity was also demonstrated, with killing of E. coli NCTC11954 being complete within a 30 min exposure to 6.7 mM cecropin B at 30°C. Studying more strains may show some which are able to withstand lethal effects longer. The effect of growth phase on killing by cecropin B should be examined. However, the activity of cecropin P, has been shown to be the same against growing and non-growing bacteria (Boman, Agerbeth & Boman, 1993).

This study has shown that this group of peptides, particularly cecropin B, possess a broad spectrum of antibacterial activity, especially against clinically relevant Gram-negative organisms. Human erythrocytes were not lysed at 500 M cecropin B and therefore the peptide would appear to show specificity towards some bacteria.

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References


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