Improved Activity of a Synthetic Indolicidin Analog

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A novel cationic peptide, CP-11, based on the structure of the bovine neutrophil peptide indolicidin, was designed to increase the number of positively charged residues, maintain the short length (13 amino acids), and enhance the amphipathicity relative to those of indolicidin. CP-11, and especially its carbomethoxymethylated derivative, CP-11C, demonstrated improved activity against gram-negative bacteria and Candida albicans, while it maintained the activity of indolicidin against staphylococci and demonstrated a reduced ability to lyse erythrocytes. In Escherichia coli, CP-11 was better able than indolicidin to permeabilize both the outer membrane, as indicated by the enhancement of uptake of 1-N-phenylnaphthylamine, and the inner membrane, as determined by the unmasking of cytoplasmic β-galactosidase, providing an explanation for its improved activity.

During the past decade many antimicrobial cationic peptides have been isolated from a wide range of animal, plant, and bacterial species (14). Indolicidin is a 13-amino-acid antimicrobial peptide present in the cytoplasmic granules of bovine neutrophils (24). As a naturally occurring peptide, indolicidin has a unique composition consisting of 39% tryptophan and 23% proline (ILPWKWPWWPRR), and in nature the peptide is amidated at the C terminus. Indolicidin has activity against gram-negative and -positive bacteria (9, 24), fungi (2), and protozoa (3). In addition, the peptide is cytotoxic to rat and human T lymphocytes (23) and lyases erythrocytes (2).

Due to its relatively small size and broad spectrum of antimicrobial activity, indolicidin has been suggested as a possible candidate for therapeutic use and in a liposomal formulation has been used successfully in a mouse antifungal infection model (2). However, due to its toxicity and only moderate antimicrobial activity, the development and characterization of indolicidin analogs may greatly improve the prospect of a clinical application. The mechanism of action of indolicidin against gram-negative bacteria has been established (9). The peptide binds to surface lipopolysaccharide (LPS) with a high affinity, resulting in self-promoted uptake across the outer membrane and subsequent channel formation in the cytoplasmic membrane, resulting in cell death. Modification of indolicidin to increase any or all of the factors involved in its mechanism of action may lead to the development of molecules with increased antimicrobial activity. The C-terminal methyl esterification of indolicidin was shown to increase the antimicrobial activity of this peptide due, in part, to increased LPS binding and increased outer membrane permeabilization. Such increased activity due to modification of the C terminus may be a reason for the amidation of indolicidin in nature.

The modification of cationic antimicrobial peptides to determine structure-function relationships and/or produce less toxic molecules with increased activity has been performed primarily on α-helical and β-structured peptides (5, 7, 8, 19, 25). The conclusions from these and other studies is that important factors in the activity of antimicrobial cationic peptides are the position and nature of positively charged residues, the formation of specific secondary structures, and the creation of a hydrophobic face on the molecule.

Indolicidin is a member of the cathelicidin family of antimicrobial peptides isolated from mammalian myeloid cells (26). Such peptides can be grouped according to composition and secondary structure. The most closely related peptides to indolicidin are the proline- and arginine-rich peptides Bac5, Bac7, and PR-39 (1, 11). Although these peptides are much larger than indolicidin (39 residues and larger) and contain higher proline contents (46, 47, and 49%, respectively) than indolicidin (23%), they may share a related secondary structure when inserted into membranes, that of a poly-L-proline II-like (extended) helix (9), and all contain a high percentage of hydrophobic residues. In the case of Bac5, Bac7, and PR-39, these are primarily phenylalanine, and in the case of indolicidin these are tryptophans. However, sequence modification has not been performed on any of the proline-rich peptides. Here we describe the development and characterization of an indolicidin analog developed on the basis of rational design and its mechanism of action.

MATERIALS AND METHODS

Materials and bacterial strains. Indolicidin peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry at the University of Victoria, Victoria, British Columbia, Canada. Polymyxin B, lysozyme, 1-N-phenylnaphthylamine (NPN), and o-nitrophenyl-β-D-galactosidase (ONPG) were purchased from Sigma Chemical Co., St. Louis, Mo. Dansyl polymyxin B was prepared as described previously (18).

The bacterial strains used for antimicrobial activity testing included Escherichia coli UBI1005 and its antibiotic-supersusceptible derivative DC2 (21), Pseudomonas aeruginosa PA01 H103 (12), P. aeruginosa K799 and its antibiotic-supersusceptible derivative Z61 (6), Salmonella typhimurium 14028s and its defensin-supersusceptible derivative MS7053s (10), Staphylococcus aureus ATCC 29533, and Staphylococcus epidermidis (clinical isolate obtained from A. Chow, Vancouver General Hospital). Relevant phenotypic descriptions are listed in Table 1. E. coli ML-35, a lactose permease-deficient strain with constitutive cytoplasmic β-galactosidase activity (lacI and lacY mutations, lacZ−), was obtained from E. Ruby, University of Southern California, Los Angeles.

C-terminal esterification of peptides. The boron trifluoride-methanol method was used to form methyl esters of the carboxy terminus (15). Between 3 and 10 μg of peptide was dried and dissolved in methanol (0.5 ml) under nitrogen. Boron trifluoride-methanol reagent (200 μl) was added, and the mixture was stirred at room temperature for 18 h. The solvents were removed by rotary evaporation. The residue was dissolved in 0.5 ml of 1% acetic acid and was passed through a column (28 cm by 8 mm) of Bio-Gel P4 with a column buffer of 1% acetic acid. The fractions containing the modified peptide, as determined by the dinitrophenol assay (19) for free amino groups, were pooled and lyophi-
assay, 600 μl of cells was mixed with 50 μg of chicken egg white lysozyme per ml and various concentrations of peptide. Cell lysis due to permeabilization of the outer membranes to lysozyme was measured as a decrease in the OD₄₀₀.

**Inner membrane permeability assay.** Inner membrane permeability was determined by the ability of the test organism to efflux the fluorescein analog 7-amino-4-methylcoumarin (AMC) in the presence of ethylenediaminetetraacetic acid (EDTA) and the antibiotic vancomycin. The decrease in fluorescence due to partitioning of AMC into the outer membrane was measured as a decrease in the OD₄₀₀.

**Indole assay.** The concentration of indolicidin was determined by the ability of the test organism to reduce nitrophenyl-β-D-galactoside (ONPG) to nitrophenol-β-D-galactoside. The decrease in fluorescence due to partitioning of ONPG into the outer membrane was measured as a decrease in the OD₄₀₀.

**RESULTS**

**Peptide design.** The amino acid sequences for CP-11 and indolicidin are presented in Fig. 1. The C-terminal methyl ester of each was formed, producing CP-11C and indolicidin-C, respectively. The design of the CP-11 sequence was based on increasing the number of positively charged amino acids, in specific positions, while maintaining the length and somewhat of the overall secondary structure of the parent compound, which has been shown to form a poly-L-proline II helix upon interaction with lipid membranes. Modeling of indolicidin as a poly-L-proline II helix with InsightII software (Biosym Technologies Inc., San Diego, Calif.) revealed that the molecule had a hydrophobic face consisting of Trp⁶, Trp⁸, and Trp¹¹. However, the orientation of Trp⁰ in this model did not position it in the hydrophobic plane. It has been shown that cationic peptides tend to adopt a amphipathic configuration in three-dimensional space, with distinct hydrophobic and hydrophilic, positively charged faces. The substitution of Pro⁴ and Trp⁸ with Lys⁵ in CP-11 produced a model in which all tryptophans were aligned in a single plane, and the positive nature of the N-terminal region was increased. The addition of Lys⁸ decreased the number of positively charged amino acids at the C terminus and maintained the overall length of the peptide, estimated to be approximately 40 Å.

**Antimicrobial activity.** CP-11 had MICs that were fourfold lower than those previously described for the parent compound indolicidin against wild-type *E. coli*, *P. aeruginosa*, and *S. typhimurium*. Also of significance was the reduction in the MIC for the medically significant fungal pathogen *Candida albicans* (indolicidin MIC, >64 μg/ml; CP-11 MIC, 8 μg/ml). In contrast, such increases in activity were not observed for gram-positive bacteria. Modification of the C terminus by methyl esterification to produce CP-11C resulted in improved activity against almost all gram-negative and gram-positive bacteria tested, with MICs of between 1 and 8 μg/ml. Killing by CP-11 was shown to be very rapid and resulted in log orders of cell death within minutes of peptide addition.

**TABLE 1. MICs of indolicidins**

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (μg/ml)</th>
<th>CP-11</th>
<th>CP-11C</th>
<th>Indolicidin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Indolicidin-C&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Polymyxin B</th>
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<tr>
<td>E. coli UB1005</td>
<td>WT</td>
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<td>64</td>
<td>64</td>
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<tr>
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<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
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<td>14028s</td>
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<td>32</td>
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<tr>
<td></td>
<td>MS7953s</td>
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<td>8</td>
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</tr>
<tr>
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<td>8</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild-type strain; SS, antibiotic-supersusceptible strain; DSS, defensin-supersusceptible strain.

<sup>b</sup> Previously reported data.

**Fig. 1.** Amino acid sequences of the peptides used in this study. Positively charged residues are in boldface type. The designation – C indicates C-terminal methyl esterification of the peptide.
Effect of peptides on erythrocytes. Indolicidin has previously been demonstrated to lyse erythrocytes at 50 μg/ml (2), a factor which is of obvious clinical significance. Therefore, in the development of peptide analogs with increased antimicrobial activity, this toxicity requires analysis. Lysis of human erythrocytes was observed for indolicidin-C (which mimicked the natural form of indolicidin) at concentrations of 32 μg/ml and above, consistent with the data of Ahmad et al. (2). However, the nonmethylated indolicidin and both CP-11 and CP-11C at concentrations of 128 μg/ml or below did not lyse erythrocytes at 4 h and thus were less toxic than indolicidin C.

Binding of peptides to P. aeruginosa H103 LPS. Antimicrobial cationic peptides, including indolicidin, have been demonstrated to cross the outer membrane of gram-negative bacteria via the self-promoted uptake pathway (9, 20, 22). The initial step in this process is the binding of the cationic peptide to surface LPS, causing the displacement of divalent cations that stabilize adjacent LPS molecules. Dansyl polymyxin was used as a probe for LPS binding. When positively charged compounds bind LPS and displace the bound dansyl polymyxin probe, this results in decreased fluorescence of the dansyl polymyxin. The maximum displacement of dansyl polymyxin was between 62 and 88% for all peptides and was 50% for CP-11C, whereas CP-11C demonstrated a PC50 of 0.6 μg/ml. Overall, the ability to permeabilize the outer membrane of E. coli was proportional to the relative MICs of the peptides for E. coli (r2 value of 0.98 by linear regression).

The ability of each compound to facilitate the uptake of the larger protein lysozyme (Mw, 14,000) was determined as previously described (13). As with the NPN assay, polymyxin B facilitated the uptake of lysozyme at concentrations of less than 1 μg/ml (13, 19). However, neither CP-11 nor CP-11C permeabilized E. coli to lysozyme even at peptide concentrations as high as 70 μg/ml. The relatively small size of indolicidin was maintained in CP-11, and although the latter was more positively charged, the ability of these peptides to cause substantial damage to the outer membrane, as indicated by the uptake of lysozyme, might well have been size dependent. It should be noted that neither indolicidin nor indolicidin-C in the presence or absence of lysozyme caused cell lysis even at concentrations in excess of threefold the MIC (9).

Inner membrane permeabilization. Indolicidin permeabilized the inner membrane of E. coli ML-35, as determined by unmasking of cytoplasmic β-galactosidase in this permease-negative mutant as described previously (9). At 16 μg/ml, indolicidin caused permeabilization of the inner membrane after a lag of less than 1 min, and this peptide exhibited dose-dependent activity. However, as shown in Fig. 4, at 4 μg/ml, CP-11 permeabilized the inner membrane of ML-35 six times

ability to permeabilize the outer membrane of wild-type E. coli to NPN (Mw, 200) and lysozyme (Mw, 14,000) was determined for each of the peptides. As reported previously (9), polymyxin B facilitated the uptake of NPN, with a concentration resulting in 50% permeabilization (PC50) of 0.15 μg/ml, and indolicidin and its methyl ester permeabilized E. coli to NPN, with PC50s of 5.5 and 3.0 μg/ml, respectively (Fig. 3). The corresponding value for CP-11 was 2.0 μg/ml, which was comparable to that for indolicidin-C, whereas CP-11C demonstrated a PC50 of 0.6 μg/ml. Overall, the ability to permeabilize the outer membrane of E. coli was proportional to the relative MICs of the peptides for E. coli (r2 value of 0.98 by linear regression).

FIG. 2. Killing of E. coli UB1005 (△), P. aeruginosa PAO1 H103 (●), S. aureus ATCC 25923 (+), and C. albicans (▲) by 32 μg of CP-11 per ml in 10 mM HEPES (pH 7.2).

FIG. 3. Peptide-mediated uptake of NPN in E. coli UB1005. E. coli cells were incubated with NPN in the presence of various concentrations of polymyxin B (●), CP-11 (○), and CP-11C (▲). Enhanced uptake of NPN was measured by an increase in fluorescence caused by partitioning of NPN into the hydrophobic outer membrane. Indolicidin (△) and indolicidin-C (▲) data were taken from the work of Falla et al. (9).

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more effectively than did indolicidin at the same concentration. The MICs of these peptides for ML-35 were 4 and 16 μg/ml, respectively.

**DISCUSSION**

Antimicrobial cationic peptides offer a new resource for the development of novel anti-infective agents. However, peptides from natural sources have only moderate MICs for most bacteria and tend to be toxic for non-host cells. Indeed, only a small number of compounds have reached clinical trials to date. Here we have demonstrated that modifications to increase the overall charge and amphipathic character of the extended-helix, proline- and tryptophan-rich peptide indolicidin. This has resulted in a molecule significantly more effective than did indolicidin at the same concentration. The MIC of CP-11C for *P. aeruginosa* and *C. albicans* of up to eightfold.

Here we have demonstrated that sequence modification based on the secondary structure and mechanism of action can be used to improve the activity of the unique tryptophan-rich peptide indolicidin. This has resulted in a molecule significantly less toxic than its parent peptide with activity against gram-negative and gram-positive bacteria and yeast at between 1 and 8 μg/ml. Further modifications based on these findings could produce a broad range of anti-infective agents suitable for clinical use.

**ACKNOWLEDGMENTS**

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**REFERENCES**


