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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 carboxymethylated 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 (ILPWKWPWWPWRR), 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 lyses 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-galactoside (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* UB1005 and its antibiotic-supersusceptible derivative DC2 (21), *Pseudomonas aeruginosa* PAO1 H103 (12), *P. aeruginosa* K799 and its antibiotic-supersusceptible derivative Z61 (6), *Salmonella typhimurium* 14028s and its defensin-supersusceptible derivative MS7953s (10), *Staphylococcus aureus* ATCC 25923, 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 lyoph-

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TABLE 1. MICs of indolicidins

Species and strain	Phenotype[a]	MIC (μg/ml)				
		CP-11	CP-11C	Indolicidin[b]	Indolicidin-C[b]	Polymyxin B
E. coli						
UB1005	WT	4	2	16	4	0.5
DC2	SS	2	2	4	4	0.1
P. aeruginosa						
H103	WT	16	8	64	64	0.5
K799	WT	16	8	64	64	0.5
Z61	SS	2	2	4	4	0.1
S. typhimurium						
14028s	WT	16	8	64	32	1.0
MS7953s	DSS	2	1	8	2	0.25
S. aureus ATCC 25923	WT	16	8	8	4	64
S. epidermidis	WT	2	1	4	1	64
C. albicans	WT	8	8	>64	>64	>64

[a] WT, wild-type strain; SS, antibiotic-supersusceptible strain; DSS, defensin-supersusceptible strain.
[b] Previously reported data (9).

ilized. The peptide was obtained in 90 to 95% yield relative to the starting amount of amino groups.

MIC assay. The MIC of each peptide for a range of microorganisms was determined by the broth microdilution method (4). Serial dilutions of each antibiotic were made in Luria-Bertani (1% [wt/vol] tryptone and 0.5% [wt/vol] yeast extract) (LB) medium in 96-well microtiter plates. Each well was inoculated with 10 μl of 10^4 to 10^5 CFU of the test organism per ml. The MIC was determined after overnight incubation of the plates at 37°C. The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited.

Bacterial killing assay. Bacteria or fungi were grown at 37°C in LB medium to the mid-exponential phase of growth and were then centrifuged and resuspended in 10 mM sodium HEPES buffer (pH 7.2) at approximately 10^7 cells/ml. Peptide was added at a final concentration of 32 μg/ml, and the killing assays were performed by incubating the suspension at 37°C. Samples were taken at various times after peptide addition and were diluted and plated onto LB medium to obtain viable colonies.

Erythrocyte lysis assay. Freshly collected human blood with heparin was centrifuged at 1,000 × *g* for 10 min to remove the buffy coat, and the erythrocytes obtained were washed three times in 0.85% saline and stored at 4°C. Serial dilutions of the peptides in saline were prepared in round-bottom microtiter plates by using 100-μl volumes. Erythrocytes were diluted with saline to a 1/25 packed volume of cells, and 50 μl was added to each well. The plates were incubated with rocking at 37°C, and the concentration required for lysis was determined after 4 h.

Dansyl polymyxin B displacement assay. The relative binding affinity of each peptide for LPS was determined by the dansyl polymyxin B displacement assay of Moore et al. (18). Briefly, dansyl polymyxin B (2.5 μM) and *P. aeruginosa* H103 LPS (3 μg/ml) were mixed in 1 ml of 5 mM HEPES (pH 7.2). This resulted in >90% maximum fluorescence, as measured with a fluorescence spectrophotometer. The decrease in fluorescence (excitation wavelength, 340 nm; emission wavelength, 485 nm) due to dansyl polymyxin B displacement was recorded upon the addition of samples of 5 μl of each of the peptides. The fraction of dansyl polymyxin B bound to LPS was plotted as a function of peptide concentration. By using this plot the concentration of each peptide required to displace 50% of bound dansyl polymyxin B (IC_{50}) was determined.

Outer membrane permeabilization assays. The membrane permeabilization activity of the peptides was determined by the NPN assay of Loh et al. (17) and the lysozyme lysis assay of Hancock et al. (13). In overview, an overnight culture of *E. coli* UB1005 was diluted in LB medium and was grown to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6. The cells were harvested, washed, and resuspended in the same volume of buffer (5 mM HEPES [pH 7.2], 5 mM KCN). For the NPN assay, 1 ml of cells and 10 μM NPN were mixed, and the fluorescence was measured with a fluorescence spectrophotometer (excitation wavelength, 350 nm; emission wavelength, 420 nm). The increase in fluorescence due to partitioning of NPN into the outer membrane was measured by the addition of various concentrations of peptide. All experiments were performed three times, and the trends that were observed were reproducible. For the lysozyme lysis 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 OD_{600}.

Inner membrane permeabilization assay. Inner membrane permeability was determined by the ability of the peptides to unmask cytoplasmic β-galactosidase activity in *E. coli* ML-35 by using ONPG as the substrate (16). Logarithmic-phase bacteria were washed in 10 mM sodium phosphate (pH 7.4) and were resuspended in 0.75 ml of the same buffer containing 1.5 mM ONPG. At time zero different amounts of indolicidin were added, and the production of *o*-nitrophenol was monitored at 420 nm.

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-like helix upon interaction with lipid membranes (9). 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 Trp^6, Trp^8, Trp^9, and Trp^{11}. However, the orientation of Trp^4 in this model did not position it in the hydrophobic plane. It has been shown that cationic peptides tend to adopt an amphipathic configuration in three-dimensional space, with distinct hydrophobic and hydrophilic, positively charged faces (14). The substitution of Pro^3 and Trp^4 with Lys^3 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 Lys^{13} increased 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 (Fig. 2). As with indolicidin, the defensin-supersusceptible *phoP/phoQ* mutant strain MS7953s of *S. typhimurium* was more susceptible than its parent strain to CP-11. Similarly, the outer membrane-altered supersusceptible strains *E. coli* DC2 and *P. aeruginosa* Z61 were more susceptible than their parent strains. Thus, passage across the outer membrane may still be the rate-limiting step in the actions of these peptides.

CP-11	IL**KK**WPWWPW**RRK**
CP-11C	IL**KK**WPWWPW**RRK**-C
Indolicidin	ILPW**K**WPWWPW**RR**
Indolicidin-C	ILPW**K**WPWWPW**RR**-C

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.

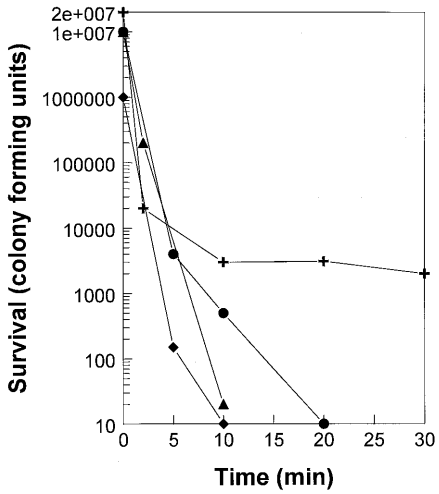

FIG. 2. Killing of *E. coli* UB1005 (▲), *P. aeruginosa* PAO1 H103 (●), *S. aureus* ATCC 25923 (+), and *C. albicans* (◆) by 32 μg of CP-11 per ml in 10 mM HEPES (pH 7.2).

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 group (18). The maximum displacement of dansyl polymyxin was between 62 and 88% for all peptides and was 50% for $MgCl_2$, suggesting similar numbers of binding sites on LPS for all peptides studied. The concentration of peptide resulting in 50% maximal displacement of dansyl polymyxin was expressed as an I_{50}, which served as an indicator of relative binding affinity. As previously described, the I_{50}s of indolicidin (8.5 μM) and indolicidin-C (1.2 μM) for LPS were comparable to that of polymyxin B (2.0 μM), while Mg^{2+}, the native divalent cation that is normally associated with LPS in cells, showed a substantially lower affinity (I_{50}, 620 μM). CP-11 (I_{50}, 4.3 μM) and CP-11C (3.1 μM) did not show a significant increase in LPS binding affinity compared to that of the parent compound or its methyl ester. Thus, the addition of two positively charged residues in CP-11 did not appear to increase its ability to bind to LPS.

Outer membrane permeabilization. The displacement of divalent cations from surface LPS destabilizes the gram-negative outer membrane and leads to the uptake of the destabilizing compound. This has been demonstrated for defensins (22), cecropin-melittin hybrids (19, 20), and indolicidin (9). The ability to permeabilize the outer membrane of wild-type *E. coli* to NPN (M_r, 200) and lysozyme (M_r, 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 (PC_{50}) of 0.15 μg/ml, and indolicidin and its methyl ester permeabilized *E. coli* to NPN, with PC_{50}s 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 PC_{50} 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* (r^2 value of 0.98 by linear regression).

The ability of each compound to facilitate the uptake of the larger protein lysozyme (M_r, 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

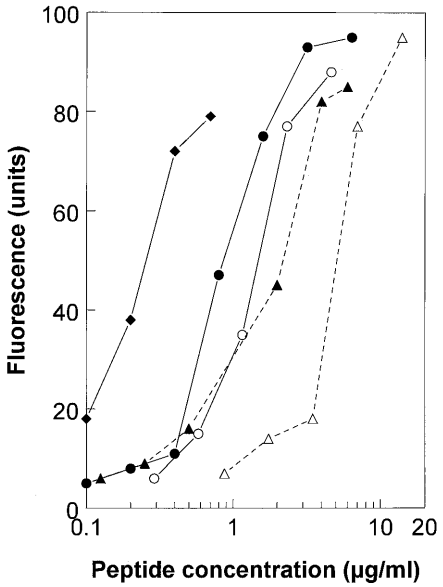

FIG. 3. Peptide-mediated uptake of NPN in *E. coli* UB1005. *E. coli* cells were incubated with NPN in the presence of various concentrations of polymyxin B (◆), CP-11 (○), and CP-11C (●). Enhanced uptake of NPN was measured by an increase in fluorescence caused by partitioning of NPN into the hydrophobic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

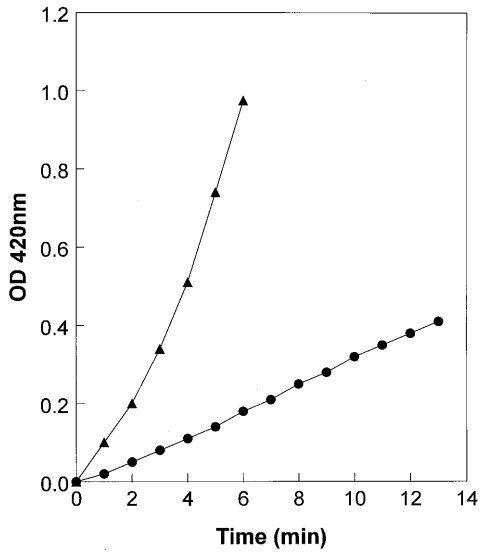

FIG. 4. Peptide-mediated inner membrane permeabilization of *E. coli* ML-35. Permeabilization was determined by following the unmasking of cytoplasmic β-galactosidase activity spectrophotometrically at 420 nm. *E. coli* (approximately 10^6 CFU/ml) was resuspended in 10 mM sodium phosphate buffer (pH 7.5) containing 1.5 mM substrate. The effects of CP-11 (▲) and indolicidin (●) at 4 μg/ml are presented.

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 resulted in a molecule, CP-11C, which exhibited reduced toxicity and broad-spectrum antimicrobial activity. Molecular modeling and preliminary circular dichroism spectral data indicated that CP-11C showed a structure rather similar to that of indolicidin, despite a reduction in proline content from 23 to 15%.

The mechanism of action of indolicidin against gram-negative bacteria has been established (9), and it has been shown here that CP-11C acts by the same mechanism. However, the ability of CP-11 to permeabilize both the outer and cytoplasmic membranes was significantly greater than that of indolicidin. While the inner membrane permeabilization assay reflects in part the movement of peptide across the outer membrane to access the inner membrane, we feel that overall the data favor an improved ability to interact with both membranes, since the modifications made to CP-11, compared to the structure of indolicidin, seemed to have a greater effect on the inner membrane permeabilization assay. The increased ability to interact with membranes combined with the increased positive nature of the peptide was also probably responsible for the increase in activity against gram-negative bacteria and *C. albicans*. The most significant improvement in activity was the reduction in 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.

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