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Biological Properties of Structurally Related α-Helical Cationic Antimicrobial Peptides

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A series of α-helical cationic antimicrobial peptide variants with small amino acid changes was designed. Alterations in the charge, hydrophobicity, or length of the variant peptides did not improve the antimicrobial activity, and there was no statistically significant correlation between any of these factors and the MIC for *Pseudomonas aeruginosa*, *Escherichia coli*, or *Salmonella typhimurium*. Individual peptides demonstrated synergy with conventional antibiotics against antibiotic-resistant strains of *P. aeruginosa*. The peptides varied considerably in the ability to bind *E. coli* O111:B4 lipopolysaccharide (LPS), and this correlated significantly with their antimicrobial activity and ability to block LPS-stimulated tumor necrosis factor and interleukin-6 production. In general, the peptides studied here demonstrated a broad range of activities, including antimicrobial, antiendotoxin, and enhancer activities.

Systemic disease associated with the presence of pathogenic microorganisms or their toxins in the blood often involves gram-negative bacteria and the release of an outer membrane component, endotoxin (23). The toxicity of endotoxin, also known as lipopolysaccharide (LPS), is contained within the lipid A portion of LPS. Antibiotics used to treat the bacterial infection can actually be harmful in that they can stimulate the release of endotoxin (5, 22). The physiological mechanism whereby endotoxin exerts its effect on humans involves the release of cytokines, of which tumor necrosis factor alpha (TNF-α) appears to be very important (13).

There is substantial interest in identifying novel strategies to overcome not only sepsis but also the underlying infection. Many new strategies, including neutralizing antibodies, soluble cytokine receptors, and various endotoxin-binding factors, have been tested with mixed results (4, 10, 20, 24). Recently, a new generation of LPS-binding antimicrobial agents, termed cationic antimicrobial peptides, has been discovered. In this study, we have investigated α-helical peptides derived from a hybrid of silk moth cecropin and bee melittin peptides (CEME [3], also called MBI-27 [9]) which contains the first 8 amino acids of cecropin followed by the first 18 amino acids of melittin. CEME and CEMA (MBI-28 [9]) were found to have strong antimicrobial activity against gram-negative bacteria, a high affinity for bacterial endotoxin (15), and endotoxin-neutralizing activity in murine macrophages and in mice (9). CEME and CEMA are proposed to cross the outer membrane by self-promoted uptake (15). In this process, the peptides interact with LPS divalent cation-binding sites on the outer membrane surface of gram-negative bacteria and competitively displace these cations (Mg^{2+} or Ca^{2+}). The bulky peptides then cause distortions of the outer membrane which allow probe molecules such as lysozyme and 1-*N*-phenylnaphthylamine to cross the membrane and which are proposed to permit the peptides themselves to move across the outer membrane. Although it is known that the peptides can subsequently cause a general collapse of membrane integrity with a resulting loss of the cytoplasmic permeability barrier, the exact nature of the mechanism of killing is not known (11).

Since the amphipathic nature of the peptides is considered important for their activity, amino acid changes were made to the peptides with the aid of a helical wheel to create a more amphipathic molecule. This resulted in peptides CP26 and CP29 (8). From these four peptides, a series of variants with small amino acid changes (Table 1) were designed and synthesized by Fmoc (9-fluorenylmethoxycarbonyl) chemistry in order to study structure-function relationships to gain insight into the peptide characteristics that are important for activity.

Antimicrobial activity of the peptides. Bacteria were grown on Mueller-Hinton medium supplemented with 1.5% (wt/vol) agar. The strains employed for peptide MIC determinations were *Pseudomonas aeruginosa* K799 (parent of Z61; 2), Z61 (antibiotic supersusceptible), H744 (*nalB* multidrug efflux mutant; 17), H374 (*nalA* DNA gyrase mutant, 19), and H547 (β-lactamase-depressed mutant from our laboratory stock collection); *Escherichia coli* UB1005 (18); *Salmonella typhimurium* 14028s (7); and *Burkholderia cepacia* ATCC 25416. The MIC of each peptide for a range of microorganisms was determined by the modified broth dilution method (25).

As shown in Table 2, all of the peptides were inactive against *B. cepacia*, which we have previously shown to be resistant to cationic antibiotics by virtue of its lack of a self-promoted uptake pathway across the outer membrane (14). Disruption of the outer membrane barrier (2) in *P. aeruginosa* Z61 had only about a twofold effect on the MIC (cf. its parent strain, K799), indicating that outer membrane passage was not limiting on activity (similar data was obtained with the outer membrane barrier mutant *E. coli* DC2). There was no obvious trend to resistance due to derepression of the *nalB*-regulated *mexA mexB oprM* efflux pump, in contrast to the situation recently described with certain peptides in *Neisseria* efflux mutants (21). Relatively minor changes to the peptides had major effects on MICs including the change of the W in position 2 of peptide CP207 to K in CP208 and the removal of KW in CM5. Peptide CP202 differed from peptide CP201 only by an S-to-K change at position 4. This change restored some of the gram-negative activity of peptide CP202.

Synergy of peptides with conventional antibiotics. The checkerboard assay was used to determine whether there was

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TABLE 1. Peptide amino acid sequences

Peptide	Amino acid sequence[a]	No. of amino acids	Charge	% Hydrophobic amino acids
CP26	-KWKSFIKK-LTSAAKKVVTTAKPLISS	26	+7	46
CEME	-KWKLF-KKIGIGAVLKVLTTGLPALIS	26	+5	69
CEMA	-KWKLF-KKIGIGAVLKVLTTGLPALKLTK	28	+7	64
CP29	-KWKSFIKK-LTTAVKKVLTTGLPALIS	26	+6	50
CPα1	-KWKSFIKK-LTSAAKKV-TTAAKPLTK	25	+8	44
CPα2	-KWKKFIKKIGIGAVLKVLTTGLPALKLTKK	30	+9	60
CPα3	KKWKKFIKKIGIGAVL---TTPGAKK	23	+8	57
CM1	-KWKSFIKK-LTSAAKKVVTTAKPLALIS	27	+7	56
CM2	-KWKSFIKK-LTKAAKKVVTTAKKPLIV	26	+9	54
CM3	-KWKKFIKS-LTKSAAKTVVKTAKKPLIV	26	+9	52
CM4	-KWKLF-KKIGIGAVLKVLTTGLPALKLTLK	29	+7	66
CM5	---KLF-KKIGIGAVLKVLTTGLPALKLTLK	26	+6	65
CM6	-KWK-F-KKIGIGAVLKVLTTGLPALKLTLK	27	+7	63
CM7	KLWKLF-KKIGIGAVLKVLTTGLPALKLTLK	29	+7	66
CP201	-KWKSFI-KNLTKGGSKILTTGLPALIS	26	+5	54
CP202	-KWKKFI-KNLTKGGSKILTTGLPALIS	26	+6	54
CP203	-KWKSFI-KKLTSAAKKVLTTGLPALIS	26	+6	54
CP204	KKWWKAQKAVNSGPNA-LQTLAQ	22	+4	50
CP205	KKWWKAKKFANSGPNA-LQTLAQ	22	+5	50
CP206	KKWWKFIKKAVNSGTTGLQTLAS	23	+5	48
CP207	-KWKSFI-KKLTSVLKKVVTTAKPLISS	26	+7	46
CP208	-KKKSFI-KLLTSAKVSVLTTAKPLISS	26	+6	46
CP209	--WKVFKSFIKKASSFAQSVLD	20	+4	50
CP210	KKWRK-SFFKQVGSFDNSV	18	+4	39

[a] Dashes were inserted to show alignment of amino acids.

antibiotic-peptide synergy (1). Synergy was defined as a fractional inhibitory concentration (FIC) index of less than 0.5. Many of the peptides were found to have an FIC index of around 0.5 or less, indicating synergy (Table 3). Some of the peptides that had very good antimicrobial activity (e.g., CM7 and CPα2) did not show strong synergy activity, whereas synergy was observed with peptides that were completely unable to kill bacteria. Although ciprofloxacin had an MIC of 0.25 μg/ml against the *P. aeruginosa nalB* mutant, many of the peptides at 1 to 4 μg/ml were able to reduce this value two- to fourfold. Carbenicillin had a very high MIC (64 μg/ml) against the β-lactamase-derepressed mutant (H547) of *P. aeruginosa*. With the addition of 1- to 4-μg/ml peptide, this value could also be decreased two- to fourfold, although only peptides CM5, CP202, and CP206 showed synergy in this situation. Nalidixic acid has an extremely high MIC (3,200 μg/ml) against both H744 (multidrug efflux mutant of *P. aeruginosa*) and H374 (DNA gyrase mutant). Peptide addition had a very pronounced effect on this MIC, reducing it by up to 64-fold.

Determination of LPS-binding affinity. *E. coli* O111:B4 (smooth) and J5 (rough mutant of O111:B4) LPSs were purchased from Sigma Chemical Co. (St. Louis, Mo.). The relative binding affinity of each peptide for LPS was determined by using the dansyl polymyxin B (PMB) displacement assay (14). Dansyl PMB and *E. coli* O111:B4 LPS (300 μg/ml) were mixed in 1 ml of 5 mM HEPES (pH 7.2), resulting in >90% of the maximum fluorescence. The decrease in fluorescence due to dansyl PMB displacement by the peptides was recorded. The relative affinities of the peptides for LPS were determined by calculating the 50% dansyl PMB displacement concentrations (I_{50}s) directly from the graph. The I_{50} represented the peptide concentration that resulted in 50% maximal displacement of dansyl PMB from the LPS (Table 4). The peptides showed a large range of LPS-binding affinities. CP29, CM2, CM3, and CP207, all of which had good antimicrobial activity against gram-negative bacteria, had the highest binding affinities (I_{50}s, 14, 16, 13, and 14 μg/ml, respectively). Peptides CP201, CP202,

TABLE 2. Activities of cationic antimicrobial peptides against gram-negative bacteria

Peptide	MIC (μg/ml)[a] for:							
	E. coli	*S. typhimurium*	*P. aeruginosa*					*B. cepacia*
			K799	Z61	H744	H374	H547	
CP26	1	3	4	3	2	2	4	>64
CEME	2	2	5	4	2	2	2	>64
CEMA	2	3	3	2	2	2	4	>64
CP29	2	2	6	3	2	2	2	>64
CPα1	2	43	64	24	8	8	16	>64
CPα2	2	2	4	4	2	4	2	>64
CPα3	4	4	64	32	4	8	16	>64
CM1	2	5	4	4	2	—[b]	16	>64
CM2	2	4	3	3	1	—	4	>64
CM3	1	3	4	3	2	2	2	>64
CM4	3	3	4	3	4	—	4	>64
CM5	5	29	32	19	4	—	32	>64
CM6	2	4	6	5	1	—	2	>64
CM7	2	3	4	3	16	—	32	>64
CP201	4	43	64	32	16	8	32	—
CP202	2	16	32	19	4	4	16	—
CP203	2	4	5	3	4	4	8	>64
CP204	>64	>64	>64	>64	>64	—	>64	—
CP205	>64	>64	>64	>64	>64	>64	>64	—
CP206	8	>64	>64	>64	16	8	>64	—
CP207	2	3	5	3	4	8	8	>64
CP208	32	>64	>64	>64	64	>64	>64	—
CP209	11	>64	>64	64	>64	>64	>64	—
CP210	>64	>64	>64	>64	>64	>64	>64	

[a] Strains were *E. coli* UB1005; *S. typhimurium* 14028s; *P. aeruginosa* K799 (wild type), Z61 (antibiotic supersusceptible), H744 (*nalB* multidrug efflux mutant), H374 (*nalA* DNA gyrase mutant), and H547 (β-lactamase depressed); and *B. cepacia* ATCC 25416 (the same data was achieved with strains H543 and ATCC 25609). MICs were determined on at least three separate occasions. The unusual values for some peptides were due to postassay corrections of peptide concentrations after amino acid quantitation.

[b] —, not done.

TABLE 3. Synergy of the peptides with ciprofloxacin, carbenicillin, and nalidixic acid against *P. aeruginosa* strains

Peptide	FIC index[a]			
	Ciprofloxacin vs H744 *nalB*	Carbenicillin vs H547	Nalidixic acid vs H374 *nalA*	Nalidixic acid vs H744 *nalB*
CPα1	0.5	0.75	0.38	0.38
CPα2	0.69	1	0.5	0.63
CPα3	0.63	0.75	0.5	0.5
CM1	B	B	—[b]	—
CM2	B	B	—	—
CM3	—	0.75	—	—
CM4	0.75	B	—	—
CM5	0.75	0.38	—	—
CM6	A	B	—	—
CM7	0.63	B	—	—
CP201	0.49	0.53	0.42	0.75
CP202	0.49	0.63	0.5	0.66
CP203	0.78	B	0.38	0.44
CP204	0.26	B	—	—
CP205	0.52	B	0.31	0.42
CP206	0.63	0.53	0.42	0.33
CP207	0.65	B	0.5	0.5
CP208	0.6	B	0.38	0.58
CP209	0.52	B	0.31	0.58
CP210	0.52	B	0.31	0.58

[a] To calculate the FIC index, the following formula was used: FIC index = [A]/(MIC A + [B])/MIC B, where [A] was the concentration of drug A in a well that represented the lowest inhibitory concentration in its row, MIC A was the MIC of drug A alone, [B] was the concentration of drug B in a well that represented the lowest inhibitory concentration in its row, and MIC B was the MIC of drug B alone. An FIC index of 0.5 or less is taken to imply synergy. An FIC index of 0.5 to 0.9 is marginal synergy. An FIC index of 1.0 implies that the two agents are additive. An FIC index of 2.0 implies antagonism. A and B imply that the MIC of agent A (the peptide) or B (the conventional antibiotic) did not change at any peptide or antibiotic concentration, respectively. The FIC indexes shown are averages of two or three determinations.

[b] —, not done.

TABLE 4. Binding of peptides to *E. coli* O111:B4 LPS and inhibition of the production of IL-6 and TNF by LPS-stimulated macrophages as tested by ELISA and L-cell assay[a]

Peptide	LPS binding (I_{50} [μg/ml])	% Inhibition of LPS-stimulated production		
		IL-6 (ELISA)	TNF (ELISA)	TNF (L-cell assay)
PMB	4	98	98	99
CP26	18	90	91	99
CEME	20	76	94	98
CEMA	10	82	90	97
CP29	14	96	98	96
CPα1	—[b]	82	51	65
CPα2	—	94	93	92
CPα3	—	18	15	63
CM1	18	81	95	83
CM2	16	91	93	86
CM3	13	88	94	85
CM4	14	88	85	94
CM5	30	44	42	47
CM6	17	74	47	66
CM7	9	95	99	87
CP201	40	19	10	66
CP202	32	50	43	71
CP203	26	90	98	99
CP204	32	5	0	16
CP205	31	18	9	19
CP206	29	23	18	62
CP207	14	93	97	97
CP208	22	7	0	73
CP209	27	16	0	82
CP210	30	16	0	70

[a] All values had <10% standard deviation from the mean.

[b] —, not done.

and CP210 were generally poorly active peptides and had weak binding affinities (I_{50}s, 40, 32, and 30 μg/ml, respectively), even though CP201 and CP202, but not CP210, had good antimicrobial activity against *E. coli*.

Blockage of TNF and IL-6 induction in RAW macrophage cells by smooth LPS. The murine cell line RAW 264.7 was obtained from the American Type Culture Collection, Manassas, Va., and maintained and passaged as described previously (12). TNF and IL-6 induction experiments with LPS were performed for 6 h as described by Kelly et al. (12), using LPS at a final concentration of 100 ng/ml. At the same time as LPS addition, cationic peptides were added to a final concentration of 20 μg/ml. Control assays were performed to demonstrate that peptides, at the highest concentrations utilized, did not induce TNF and were not cytotoxic as judged by trypan blue exclusion and continued adherence of RAW 264.7 cells.

TNF was measured in cell culture supernatants on the basis of cytotoxicity for L929 fibroblast cells (12). TNF activity was expressed in units as the reciprocal of the dilution of TNF that caused 50% cytotoxicity to L929 cells. One unit of TNF corresponded to 62.5-pg/ml recombinant murine TNF (R & D Systems, Minneapolis, Minn.). The concentration of TNF-α and IL-6 in the macrophage supernatants was also measured by enzyme-linked immunosorbent assay (ELISA; R & D Systems and Endogen [Hornby, Ontario, Canada]). The ELISA measured all of the TNF-α found in the supernatants tested, whereas the L929 cytotoxicity assay measured only bioactive TNF-α (TNF that was toxic to the TNF-sensitive L929 fibroblast cells). When 20 μg of peptide was incubated with the macrophage cells for 6 h, only 12 to 21 U of TNF per ml was produced (as assessed by the L929-cell assay), values that were not significantly higher than those obtained with medium alone (14 ± 4 U/ml), indicating that the peptides did not themselves stimulate cytokine production. Treatment with 100 ng of LPS led to the induction of 14,060 U of TNF per ml. The peptides varied greatly in the ability to inhibit the induction of TNF secretion by macrophage cells (Table 4; data is presented as mean percent inhibition of three independent assays done in duplicate). The ELISA results demonstrated that the inhibition of LPS-induced TNF production by the peptides was consistently lower than when measured by the L-cell assay (with the sole exception of CM7). This seems reasonable since the ELISA would measure total TNF-α, whether bioactive or not. Several of the peptide variants were equivalent to the previously studied α-helical peptides CEME and CEMA, with CP29, CPα2, CP207, CP203, CM4, and CM7 having similar or slightly better activities. The most active peptides were similar to PMB in the ability to reduce LPS-stimulated production of TNF. There was a large variance in TNF production as measured by ELISA and the L-cell assay for peptides CPα3, CP201, CP206, CP208, CP209, and CP210. Peptides CP204 and CP205 had a very minor inhibitory effect on TNF production (both about 0 to 20%). These peptides also had no antimicrobial activity and had a low binding affinity for *E. coli* O111:B4 LPS (displacing about 30% of dansyl PMB; Table 3). The active peptides have also been found to block *P. aeruginosa* PAO1 and *S. typhimurium* R595 LPS-stimulated production of TNF in RAW macrophage cells, demonstrating a broad range of activity (21a).

The effect of the peptides on production of IL-6 by *E. coli*

TABLE 5. Spearman rank correlation values for peptide data[a]

Parameter	MIC for *E. coli*	MIC for *P. aeruginosa*	MIC for *S. typhimurium*	IL-6 ELISA result	TNF ELISA result	TNF L-cell assay result	LPS binding
MIC for *E. coli*	1	0.80	0.83	0.82	0.82	0.72	0.66
MIC for *P. aeruginosa*	0.80	1	0.79	0.81	0.77	0.73	0.75
MIC for *S. typhimurium*	0.83	0.79	1	0.86	0.84	0.80	0.77
IL-6 ELISA result	0.82	0.81	0.86	1	0.93	0.77	0.77
TNF ELISA result	0.82	0.77	0.84	0.93	1	0.73	0.70
TNF L-cell assay result	0.72	0.73	0.80	0.77	0.73	1	0.66
LPS binding	0.66	0.75	0.77	0.77	0.70	0.66	1

[a] All Spearman rank values significantly correlate at a level of $P < 0.001$.

O111:B4 LPS-stimulated macrophages was examined by ELISA (Table 4). The peptides showed a wide range of abilities to inhibit the LPS-stimulated production of IL-6 by the macrophage cell line. CP29 and related peptides CP203 and CP207 very effectively antagonized LPS-stimulated IL-6 production, and CP26 and related peptides CM1, CM2, and CM3 were also quite effective (91 to 95% inhibition of IL-6 production). CM4, CM7, and CPα2 (88, 95, and 94% inhibition) were all better than their parent peptides, CEME (76% inhibition) and CEMA (82% inhibition). Peptides CPα3, CP201, CP204, CP205, CP206, CP208, CP209, and CP210 had little activity, in that the IL-6 production by the macrophages was not much different from that obtained with LPS alone. These results corresponded to the effect of the peptides on TNF production and indicated that small amino acid changes can have a large effect. For example, peptide CP207 was very active in inhibiting LPS-stimulated production of IL-6 by 97%, but peptide CP208 had lost all activity (0% inhibition), despite having a similar affinity for LPS. These peptides had similar charges, hydrophobicities and lengths and only seven sequence changes, of which the least conservative were W to K at position 2 and VLKK to AKVS in the center of the peptide.

Structure-activity correlations. Many of the peptides studied here exhibited antibacterial activity against a wide variety of bacteria. The peptides were most effective against *E. coli*, with the exception of CP204, CP205, and CP210, which had no activity against any of the bacteria tested (MICs of >64 μg/ml), but were completely ineffective against *B. cepacia*. No significant correlation was found between the length, charge, or hydrophobicity of the peptides and antimicrobial activity, as assessed by the Spearman rank correlation test. There was a trend for shorter peptides to be less active, but this would probably be sequence dependent, since peptides as short as 13 amino acids with activity against gram-negative and gram-positive bacteria have been demonstrated (6).

Many of the peptides with reduced LPS-binding affinity (i.e., high I_{50}s) also had decreased antimicrobial activity. There was significant ($P > 0.001$ by the Spearman rank correlation test; Table 5) correlation between the MICs of the peptides against *P. aeruginosa* and *E. coli* and the peptides' LPS-binding affinities. This implies that the interaction of the peptides with the outer membrane LPS as part of self-promoted uptake may be rate limiting for antibacterial activity.

Similar patterns of peptide inhibition of the production of TNF by LPS-stimulated macrophages, as measured by ELISA and the L-cell assay, and the LPS-stimulated production of IL-6 were observed ($P < 0.001$ by the Spearman rank correlation test; Table 5). This suggested the possibility of a similar mechanism of action. Interestingly, these data on inhibition of LPS-stimulated cytokine production also correlated significantly with LPS binding and MICs against *E. coli* and *P. aeruginosa* (Table 5). Both CEMA (9) and CP26 (21a) were able to inhibit LPS-stimulated TNF secretion by the macrophage cell line, even when added 30 or 60 min after LPS. Thus, binding to LPS probably cannot explain fully the inhibition of LPS-induced cytokine secretion by CP26 and CEMA, and these peptides may also have been affecting the macrophages themselves (e.g., the peptides may interfere at the cell membrane level).

There was a statistically significant correlation between the IL-6- and TNF-suppressing activities of peptides. The most active peptides had good antimicrobial and antiendotoxin activities, as well as higher LPS-binding affinity. However, there were also exceptional peptides; for example, CM6 had an LPS-binding affinity similar to that of CP26, but it only suppressed TNF by 66% and IL-6 by 47%, while CP26 inhibited TNF by 81% and IL-6 by 90%. It appears that there were other factors besides LPS binding that contributed to the peptides' ability to be a good antiendotoxin. This suggests that the peptides do more than interact with the LPS to prevent binding to macrophage cells. Several important factors are involved in the activity of the peptides that should be taken into account, including the three-dimensional structure of the peptide, the positioning of charges and hydrophobic residues, and also the peptide's ability to form α-helices.

Although the peptides discussed here may not be as potent as some of the recent β-lactams and quinolones, they do have certain potential advantages, including the enhancer (or synergistic) activity of cationic peptides (16; Table 3) and also the ability to block endotoxemia, in contrast to β-lactams and quinolones, which are known to promote endotoxin release (22). Thus, one can envision their use in combination with conventional antibiotics to increase killing and, at the same time, neutralize LPS released by these antibiotics.

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