ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1999, p. 1274–1276 Vol. 43, No. 5
0066-4804/99/$04.00+0

Improved Derivatives of Bactenecin, a Cyclic Dodecameric Antimicrobial Cationic Peptide

MANHONG WU AND ROBERT E. W. HANCOCK*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Received 8 October 1998/Returned for modification 14 January 1999/Accepted 11 February 1999

Both linear and cyclic derivatives of the cyclic 12-amino-acid antimicrobial peptide bactenecin were designed based on optimization of amphipathicity and charge location. In general, increasing the number of positive charges at the N and C termini and adding an extra tryptophan residue in the loop not only increased the activities against both gram-positive and gram-negative bacteria but also broadened the antimicrobial spectrum.

The rapid emergence of antibiotic resistance has been of great concern in recent years (7). Thus, there is great interest in the development of new classes of antimicrobial agents (2). Among the possible candidates, a group of antimicrobial cationic peptides has attracted increasing research and clinical interest due to their unique properties (3, 5). Cationic peptides have been found in a variety of sources, from prokaryotes to eukaryotes (4). In recent years, it has become clear that these endogenous peptide antibiotics constitute part of the first line of host defense; for more primitive life forms, like insects and plants, they constitute a host's primary defense system (1).

Bactenecin (also called bovine dodecapeptide) from bovine neutrophils (8) is the smallest natural cationic antimicrobial peptide, being only 12 amino acids long, including 4 arginine residues, 2 cysteine residues, and 6 other hydrophobic residues. The two cysteine residues form a disulfide bond to make bactenecin a loop molecule.

Bactenecin was previously shown to form a β-turn structure regardless of its environment (9). It tended to be weakly active only against gram-negative bacteria. Studies of its mechanism of action suggested that it was taken up across the outer membrane by the process of self-promoted uptake and that it failed to cause substantial depolarization of the cytoplasmic membrane, in contrast to most other peptides (9, 10). In contrast, when bactenecin was linearized either by reduction of the disulfide bridge or by alteration of the cysteines to serine residues, the peptide lost its activity against gram-negative bacteria, except for mutants that were generally supersusceptible to antibiotics due to an altered outer membrane barrier. The linearized peptides were dramatically altered in their interaction with cells. They interacted poorly with the outer membrane but were quite effective in permeabilizing (depolarizing) the cytoplasmic membrane. In addition, they adopted a different structure, being unstructured in free solution and adopting a β-turn structure upon interacting with membranes. Thus, its small size, unique mechanistic properties, and single disulfide bond make bactenecin an interesting candidate for research and drug development.

It is known that hydrophobicity, positive charge, disulfide bridging, and amphipathicity are important factors in the antimicrobial activities of cationic peptides (4). Analogues were designed to investigate the effects of modification of these factors on antimicrobial activities (Table 1). Peptides were designed by computer modeling with the program Insight II on a Silicon Graphics Indy computer. They were synthesized by *N*-9-fluorenylmethoxycarbonyl chemistry with an Applied Biosystems, Inc. (Foster City, Calif.), model 431 peptide synthesizer. The purchased bactenecin and its derivatives were in their fully reduced form. The disulfide bond was formed by air oxidation in 0.01 M Tris buffer, pH 7.6, at 23°C for 24 h, and then the oxidized form was purified as described previously (9). Concentrations of bactenecin and its derivatives were determined by amino acid analysis. Control experiments demonstrated that the disulfide bonds of linearized (reduced) bactenecin did not reform under the experimental conditions employed for MIC measurements.

All bacterial strains used in these studies are listed in Table 2, footnote *a*. MICs were examined by the broth dilution microtiter method, modified for use with cationic peptides (9). Bacterial strains for antimicrobial activity testing were grown in Luria broth (10 g of Bacto-tryptone per liter and 5 g of Bacto-yeast extract per liter [both from Difco Laboratories]), except for the *Streptococcus* strains, which were grown in Todd-Hewitt broth (500 g of beef heart infusion per liter, 20 g of Bacto-neopeptone per liter, 2 g of Bacto-dextrose per liter, 2 g of sodium chloride per liter, 0.4 g of disodium phosphate per liter, and 2.5 g of sodium carbonate per liter).

Linear peptides. In a previous study (9), two linear derivatives of bactenecin, Bac2S (here called Lin Bac2S, to be consistent with the other linear derivatives) and reduced bactenecin (Lin Bac), were described. They were found to have high selectivity for gram-positive bacteria and little activity against the wild-type gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Amidation of the C terminus partially restored activity against gram-negative organisms to Lin Bac2S-NH_2 (9). To further confirm this observation, two more linear derivatives of bactenecin were made (Table 1), Lin Bac2A-NH_2 (with two Cys-to-Ala replacements) and Lin BacS-NH_2 (with a single Cys-to-Ser replacement at position 3). The hydroxyl groups in serine residues and the sulfhydryl groups in cysteine residues are capable of hydrogen bonding to water and are thus hydrophilic, which would tend to make linear bactenecin more hydrophilic than cyclic bactenecin, since the SH groups in native bactenecin form a disulfide bridge. To ensure that the hydrophobicity of linear bactenecin was as similar as possible to that of native bactenecin, Lin Bac2A-NH_2 had alanine substitutions at both cysteine positions, since alanines are hydrophobic residues. Lin Bac2A-NH_2 was similar to Lin Bac2S-NH_2 in that both were more active

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, B.C. V6T 1Z3, Canada. Phone: (604) 822-2682. Fax: (604) 822-6041. E-mail: bob@cmdr.ubc.ca.

TABLE 1. Amino acid sequences of bactenecin and its derivatives

Peptide	Sequence	No. of amino acids	Net charge
Cyclic peptides			
Bactenecin	RLCRIVVIRVCR	12	+3
BacR	RRLCRIVVIRVCRR	14	+5
BacP3R	RRRCPIVVIRVCRR	14	+5
BacP3R-V	RRRLCPIVIRVCRR	14	+5
Bac2I-NH2	RICRIVVIRCIR-NH2	12	+4
BacP2R-NH2	RLCPRVRIRVCR-NH2	12	+5
BacP1	RLCRIVPVIRVCR	13	+3
BacW	RLCRIVWVIRVCR	13	+3
BacW2R	RRLCRIVWVIRVCRR	15	+5
Linear peptides			
Linear (reduced) bactenecin	RLCRIVVIRVCR	12	+3
Lin Bac 2S-NH2	RLSRIVVIRVSR-NH2	12	+4
Lin Bac 1S-NH2	RLSRIVVIRVCR-NH2	12	+4
LinBac 2A-NH2	RLARIVVIRVAR-NH2	12	+4
Lin BacP3R	RRRCPIVVIRVCRR	14	+5
Lin BacP3R-V	RRRLCPIVIRVCRR	14	+5
Lin BacP1	RLCRIVPVIRVCR	13	+3
Lin BacW	RLCRIVWVIRVCR	13	+3
Lin BacR	RRLCRIVVIRVCRR	14	+5
Lin BacW2R	RRLCRIVWVIRVCRR	15	+5

against both gram-negative and gram-positive bacteria than linear (reduced) bactenecin (Table 2) and were almost as active as cyclic bactenecin against the gram-negative bacteria *E. coli*, *P. aeruginosa*, and *S. typhimurium*. Overall, these peptides demonstrated good activity against gram-positive bacteria. In contrast, BacS-NH_2, with only a single alteration from Cys-3 to Ser-3, was two- to fourfold less active than Bac2S-NH_2.

We previously demonstrated that amidation of Lin Bac2S improved the activities against both gram-positive and gram-negative bacteria by a factor of 2 to 8 (9). Unfortunately, we could not make amidated bactenecin despite two attempts (it is apparently not amidated in nature [8]), and so the remaining peptides were constructed in the unamidated form. The other linear peptides studied were largely less active than bactenecin, although Lin BacP3R-V had slightly better MICs, except against *Streptococcus pneumoniae*. Lin BacP1, Lin BacW, and Lin BacW2R were much less active against all three gram-negative bacteria.

Cyclic peptides. Native bactenecin has a type I β-turn structure, with two arginine residues at positions 4 and 9 adjacent to the disulfide bond (8). Previous studies indicated that native cyclic bactenecin was selective entirely for gram-negative organisms and had little activity against the gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis* (9). When a more extensive group of gram-positive bacteria were examined (Table 2), it was found that bactenecin had reasonable MICs (1 to 2 μg/ml) for *Corynebacterium xerosis* and *Streptococcus mitis* and measurable MICs for *Streptococcus pyogenes* and *Listeria monocytogenes*. Increasing the positive charge from +3 to +5 in BacR (9) led to improved activity against most gram-negative and gram-positive bacteria, with the exception of *S. aureus*, *C. xerosis*, and *S. pneumoniae* (Table 2). In this study, a series of peptide variants were made to test the importance of ring size (numbers of amino acids between the cysteine residues), charge, and amphipathicity (Table 1). Peptides with the same charge as BacR, BacP3R, and BacP3R-V had similar activities, with BacR having about twofold-lower MICs. Interestingly, the peptide BacP3R-V had only six residues between the two cysteines but had better antimicrobial activity than bactenecin. It was the position of the positive charges rather than the number that was important, since Bac2I-NH_2 and BacP2R-NH_2 (with charges of +4 and +5, respectively) appeared to have no advantages over bactenecin (the latter also had three charged residues in the ring, destroying the hydrophobicity of this portion of the peptide).

TABLE 2. MICs and MHCs[b] of bactenecin and its derivatives

Peptide	MIC (μg/ml)[a]											MHC (μg/ml)
	E. coli K-12	*P. aeruginosa*	*S. typhimurium*	*S. aureus*	*S. epidermidis*	*E. faecalis*	*L. monocytogenes*	*C. xerosis*	*S. pyogenes*	*S. mitis*	*S. pneumoniae*	
Bactenecin	8	8	8	32–64	>64	>64	8	1	16	2	>64	64
BacR	2	4	4	64	8	32	<0.125	1	8	0.5	>64	64
BacP3R	2	8	8	>64	16	32	0.5	4	8	1	>64	64
BacP3R-V	2	8	8	>64	16	>64	1	4	4	1	>64	>64
Bac2I-NH_2	4	16	8	32	8	>32	ND[c]	ND	ND	ND		32
BacP2R-NH_2	4	16	32	>32	16	>32	ND	ND	ND	ND		>32
BacP1	32	>64	>64	64	64	>64	2	8	64	4	>64	64
BacW	8	4	4	4	2	8	0.5	1	2	1	16	64
BacW2R	2	2	2	2	1	2	0.25	0.25	1	0.25	8	32
Linear bactenecin	64	>64	>64	>64	8	8	1	1	16	1	4	4
Lin Bac2S-NH_2	2	16	32	4	1	4	0.25	0.25	2	0.125	16	>64
Lin BacS-NH_2	4	16	>64	16	2	16	0.5	0.5	8	0.5	8	32
Lin Bac2A-NH_2	4	8	32	4	1	2	0.25	0.25	2	0.25	16	>64
Lin BacP3R	8	>64	>64	>64	16	64	2	0.5	16	0.5	>64	16
Lin BacP3R-V	4	32	32	>64	8	32	1	0.5	8	1	>64	32
Lin BacP1	16	32	64	>64	16	32	4	2	32	2	>64	32
Lin BacW	>64	>64	64	>64	32	32	2	1	16	1	32	4
Lin BacW2R	>64	>64	>64	>64	32	>64	4	2	32	2	64	8

[a] Strains utilized were *E. coli* UB1005 (9); *P. aeruginosa* K799 (9); *S. typhimurium* 14028s; *S. aureus* ATCC 25923; *Staphylococcus epidermidis* C621, a clinical isolate obtained from A. Chow, University of British Columbia (UBC); *E. faecalis* ATCC 29212; *L. monocytogenes*, a clinical isolated obtained from B. Finlay, UBC; *C. xerosis*, a clinical isolate from the Department of Microbiology Collection, UBC; *Bacillus subtilis*, an environmental isolate from the Department of Microbiology Collection, UBC; *S. pneumoniae* ATCC 49619; *S. pyogenes* ATCC 19615; and *S. mitis* a clinical isolate from the Department of Microbiology Collection, UBC. The method described in reference 9 was used to test MICs. No effect from the residual concentration of acetic acid used to suspend the peptide was observed in control experiments.

[b] MHC, minimum hemagglutination concentration.

[c] ND, not done.

We also tested the impact of increasing ring size. Inserting a proline in the middle of the ring in BacP1 actually destroyed most of the antibacterial activity. However, insertion of a hydrophobic tryptophan at the same place in BacW led to substantial improvement in all activities. Since proline is also nonpolar, we assume that it was the structure-perturbing properties of proline that were responsible for this substantial difference in activities. Based on these results, we designed BacW2R, which combined the optimal features of the two best peptides, BacR and BacW. It was the best bactenecin derivative produced in this study and had optimal activities against both gram-negative and gram-positive bacteria, with MICs of 2 μg/ml or less for most of the bacteria listed in Table 2. It is worth noting that reduced Lin BacW2R and Lin BacW lost all activity against the three gram-negative bacteria and *S. aureus*, just like the linear (reduced) bactenecin (Table 2), which further confirmed the importance of the disulfide bond for the interaction with the outer membrane of gram-negative bacteria (9). Indeed, reduction of the disulfide bond in both Lin BacW and Lin BacW2R also led to loss of activity against gram-positive bacteria to almost the same level as native bactenecin.

Agglutination activities of bactenecin and its derivatives. Hemolysis and hemagglutination by these peptides were tested in a multiwell dilution assay for 8 h with fresh human erythrocytes with the buffy coat removed by centrifugation and suspended in 0.85% saline, as previously described (6). Bactenecin and its derivatives did not lyse human erythrocytes. However, some of the cyclic molecules did cause agglutination of these cells (Table 2) at 32 to 64 μg/ml. In general, the reduced forms of the cyclic bactenecin derivatives showed two- to eightfold-higher agglutination activities than their oxidized equivalents. For example, linear (reduced) bactenecin caused agglutination of erythrocytes at a concentration of 16 μg/ml, four times lower than the hemagglutination concentration of native bactenecin (64 μg/ml). Reduced Lin BacW2R and Lin BacW caused agglutination at lower concentrations than did their disulfide-bridged equivalents, at 8 μg/ml (fourfold) and 4 μg/ml (eightfold), respectively. It seemed that the formation of the disulfide bond inhibited the agglutination of human erythrocytes by bactenecin peptides. On the other hand, the linear derivatives Lin Bac2A-NH_2, Lin BacS-NH_2, and Lin Bac2S-NH_2 did not agglutinate erythrocytes. The low agglutinating activities of many bactenecin derivatives, especially those that had a broad spectrum of antimicrobial activity, make these peptides interesting and valuable candidates for drug development.

We acknowledge the financial assistance of the Canadian Bacterial Diseases Network for the majority of this work and a research contract from Micrologix Biotech Inc., which provided us with samples of the amidated peptides Lin Bac2S-NH_2, Lin Bac1S-NH_2, Lin Bac2A-NH_2, Bac2I-NH_2, and BacP2R-NH_2 and support for characterizing these. R.E.W.H. was the recipient of a Medical Research Council of Canada Distinguished Scientist Award. M.W. received a BC Science Council GREAT studentship award.

REFERENCES

1. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:**61–93.
2. **Cohen, M. L.** 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science **257:**1050–1055.
3. **Hancock, R. E. W.** 1997. Peptide antibiotics. Lancet **349:**418–422.
4. **Hancock, R. E. W., T. Falla, and M. H. Brown.** 1995. Cationic bactericidal peptides. Adv. Microb. Physiol. **37:**135–175.
5. **Hancock, R. E. W., and R. I. Lehrer.** 1998. Cationic peptides: a new source of antibiotics. Trends Biotechnol. **16:**82–87.
6. **Kondejewski, L. H., S. W. Farmer, D. S. Wishart, C. W. Kay, R. E. W. Hancock, and R. S. Hodges.** 1996. Effect of ring size of gramicidin S analogs on structure, antibacterial and hemolytic activity. J. Biol. Chem. **271:**25261–25268.
7. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064–1073.
8. **Romeo, D., B. Skerlavaj, M. Bolognesi, and R. Gennaro.** 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. J. Biol. Chem. **263:**9573–9757.
9. **Wu, M., and R. E. W. Hancock.** 1999. Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. J. Biol. Chem. **274:**29–35.
10. **Wu, M., E. Maier, R. Benz, and R. E. W. Hancock.** Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochemistry, in press.