Structure–function analyses involving palindromic analogs of tritrypticin suggest autonomy of anti-endotoxin and antibacterial activities

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Abstract

Neutralization of invading pathogens by gene-encoded peptide antibiotics has been suggested to manifest in a variety of different modes. Some of these modes require internalization of the peptide through a pathway that involves LPS-mediated uptake of the peptide antibiotics. Many proline/tryptophan-rich cationic peptides for which this mode has been invoked do, indeed, show LPS (endotoxin) binding. If the mechanism of antibiotic action involves the LPS-mediated pathway, a positive correlation ought to manifest between the binding to LPS, its neutralization, and the bacterial killing. No such correlation was evident based on our studies involving minimal active analogs of tritrypticin. The anti-endotoxin activities of these analogs appear not to relate directly to their antibiotic potential. The two palindromic analogs of tritrypticin, NT7 (RRFPWWW) and CT7 (WWWPFRR), showed comparable antibacterial activities. However, while NT7 exhibited anti-endotoxin activity, CT7 did not. The LPS binding of two tritrypticin analogs correlated with their corresponding structures, but the antibacterial activities did not. Further structure–function analysis indicated specific structural implications of the antibacterial activity at the molecular level. Studies involving designed analogs of NT7 incorporating either rigid or flexible linkers between the specifically distanced hydrophobic and cationic clusters modulate the LPS binding. On the other hand, not knowing the target receptor for antibacterial activity is a drawback since the precise epitope for antibacterial activity is not definable. It is apparent that the anti-endotoxin and antibacterial activities represent two independent functions of tritrypticin, consistent with the emerging multifunctionality in the nature of cathelicidins.

Keywords: structure/function studies; synthesis of peptides and proteins; antibacterial peptides; bacterial infection; endotoxin shock

In addition to the adaptive arm of the immune system, we have also inherited diverse pathogen resistance mechanisms that are collectively referred to as innate immunity (Gudmundsson and Agerberth 1999; Medzhitov and Janeway Jr. 2000; Zasloff 2002). An innate immune system is more readily observable in insects and plants since they seem to live in harmony with microbes without the presence of an adaptive immune system. Even in vertebrates, innate immunity has an immense survival value as an immediate defense reaction because mobilization of the adaptive immune system involves a long delay and high metabolic cost. A major component of the mammalian innate immunity constitutes expression of a large number of multifunctional proteinaceous effector molecules by neutrophils that work as antibiotics after they are post-translationally processed (Medzhitov and

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Abbreviations: k_{ass}, association rate constant (M^{-1} s^{-1}); K_D, dissociation equilibrium constant (M); k_{diss}, dissociation rate constant (s^{-1}); LPS, lipopolysaccharide.

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Janeway Jr. 2000). The innate immune system thus provides the first line of defense against any kind of microbial invasion. It is only when the innate immunity mechanisms are overwhelmed that they help activate the adaptive component to combat the infection. In other words, rapid and effective defense against the invading pathogens is the hallmark of the innate immune response. The gene-encoded peptides possessing antimicrobial activity are among the principal effector molecules of the innate immune system. They are generally localized at specific sites, which are exposed to microbial invasion. Although some of these peptides are suggested to work through membrane damage, in many cases antibiotic action may involve intervention at the level of cellular mechanisms.

The expression of antimicrobial peptides at specific sites exposed to microbial invasion serves as a first line of defense in vertebrates. We have explored structure–function analyses involving many different peptide antibiotics including the proline/tryptophan-rich peptide antibiotics indolicidin (Nagpal et al. 2002) and tritrypticin (Nagpal et al. 1999), which has also been referred to as tritrpticin (Schibli et al. 1999). We had earlier proposed that the specificity of tritrypticin/indolicidin binding to the membrane surface may be achieved by the appropriate juxtaposition of the clusters of hydrophobic and cationic residues so as to match a complementary site on the receptor. Tritrypticin is a palindromic molecule. Its symmetric analog (SYM11) also shows enhanced antibacterial activity (Nagpal et al. 1999). Detailed structure–function analysis of the tritrypticin analogs indicated that the N-terminal heptamer (NT7) and the C-terminal heptamer (CT7) are both active and may correspond to two independent minimal functional domains of tritrypticin. The solution structure of tritrypticin showing two consecutive β-turns in the presence of SDS has been subsequently determined (Schibli et al. 1999).

A large number of patients suffer from sepsis all over the world (Hancock and Scott 2000). Sepsis is usually caused by the release of a bacterial outer membrane component, lipopolysaccharide, which is also referred to as ''endotoxin.'' The toxicity of LPS is contained within its lipid A portion. In a way, the antibiotics used to treat the bacterial infection can actually be harmful as they can stimulate the release of LPS. Thus, there is a curious relationship between the antibiotics and the endotoxicity. It has been shown that many cationic antibacterial peptides of innate immune origin also exhibit endotoxin-neutralizing activity. In fact, the role of endotoxin in the uptake of antibacterial peptides for killing the Gram-negative bacteria has been considered preeminent (Piers et al. 1994; Hancock et al. 1995; Falla et al. 1996). For example, it has been proposed that the specificity of indolicidin binding to the LPS on the membrane surface during the early event of recognition achieved by the appropriate juxtaposition of hydrophobic and cationic residues is a critical step in expressing the activity of this peptide (Nagpal et al. 2002).

Our structure–function analyses involving indolicidin and tritrypticin provided the scope for exploring a possible model for the mechanism of antibacterial action through LPS-mediated internalization. We have been exploring the correlation of these two functions and the structures of this family of peptides. Analyses of the antibiotic analogs constituting minimal active domains suggested an interesting structure–function correlation. We demonstrate here that the LPS-binding and antibiotic activities of these peptides may actually be independent. While the amphipathic structural model can explain the anti-endotoxin activity, it does not completely explain the possible mode of antibacterial activity.

Results

The smallest active analog(s) of tritrypticin having similar antibacterial activities against different strains

The structure–function analysis of tritrypticin (VRRFP WWWPFLRR) was earlier reported (Nagpal et al. 1999). The smallest 7 mer deletion analog CT7 (WWWPFRR), having significantly high antibacterial activity against *Escherichia coli*, was identified. The corresponding inversely equivalent peptide NT7 (RRFPWWW) also exhibited nearly similar antibacterial activity. The antibacterial activities of CT7 and NT7 against other Gram-negative strains were determined by the radial diffusion method. The dose-dependent increase in the antibacterial activities of both the peptides against a large number of antibacterial strains was evident. The bacterial strains like *E. coli, Salmonella typhimurium, Agrobacterium tumefaciens*, and *Pseudomonas aeruginosa* responded similarly to the two peptides CT7 and NT7 in terms of their antibacterial activities (Fig. 1). It was observed that the activities were similar for NT7 and CT7 at their doses of up to 10 nmol, but at the higher peptide doses (>10 nmol), CT7 showed higher activity compared to that of NT7 in all the bacterial strains against which the peptides were tested.

Endotoxin-binding affinities of NT7 and CT7

The endotoxin-binding activities of tritrypticin, NT7, and CT7 were directly probed using an affinity biosensor (Table 1). The binding of varying concentrations of peptides to immobilized LPS at 25°C was carried out, the sensograms for which are shown in Figure 2. Tritrypticin binds to LPS with a K_D value of 18 μM, like other cationic antibacterial peptides of the cathelicidin family

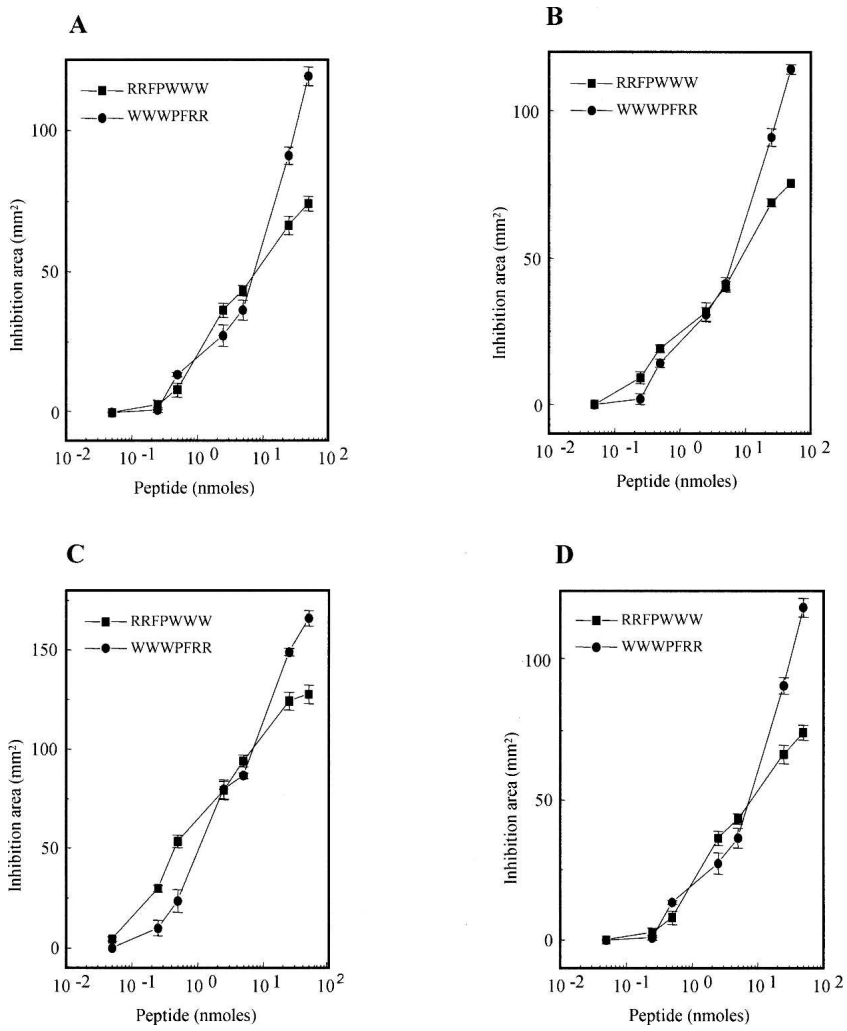

Figure 1. Comparison of dose-dependent antibacterial activity of NT7 and CT7 against (*A*) *S. typhimurium*, (*B*) *E. coli*, (*C*) *A. tumefaciens*, and (*D*) *P. aeruginosa* expressed in terms of inhibition zone area in the radial diffusion assay.

(Nagpal et al. 2002), whereas NT7 binds with a K_D value of 67 μM, and CT7 showed very poor affinity to endotoxin (K_D = 425 μM). The higher binding affinity of NT7 compared to that of CT7 could be entirely attributable to its higher association rate constant (k_{ass} = 3120 M^{-1} s^{-1}) and lower dissociation rate constant (k_{diss} = 0.21 s^{-1}). The corresponding parameters in the case of CT7 were k_{ass} = 920 M^{-1} s^{-1} and k_{diss} = 0.39 s^{-1}. The differences in k_{ass} and k_{diss} values indicate that the kinetics of binding to endotoxin is different in the two cases.

The effect of increasing temperature on the binding kinetics of NT7 to endotoxin was also determined (Table 2). In addition to the data at 25°C, the association and the dissociation rate constants of the NT7 binding to LPS were determined at 15°C, 20°C, 30°C, and 35°C (Table 2). Figure 2, D and E, represents the variation in k_{ass} and k_{diss} as a function of temperature. The kinetics of binding to endotoxin did not appear to be significantly influenced by variation in temperature during the association as well as the dissociation phase. Thus, the temperature changes did not affect the binding affinity of NT7 to LPS.

Effects of LPS binding on structures of NT7 and CT7

The differences in k_{ass} and k_{diss} values indicate that the kinetics of binding to endotoxin is different in the

Table 1. *Affinity parameters for the interaction of the three peptides with the endotoxin at 25°C*

Peptide	$k_{ass} \times 10^{-3}$ (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_D (μM)
VRRFPWWWPFLRR	6.64 ± 0.5	0.12 ± 0.03	18
RRFPWWW	3.12 ± 0.4	0.21 ± 0.04	67
WWWPFRR	0.92 ± 0.1	0.39 ± .0.04	426

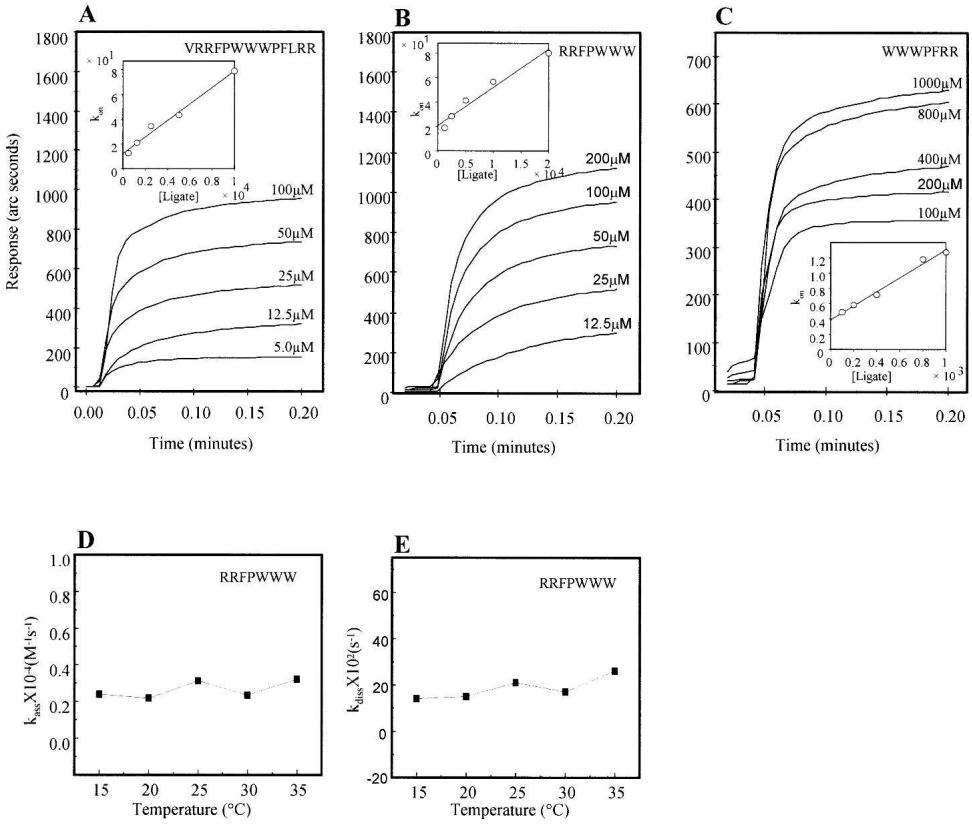

Figure 2. Surface plasmon resonance analysis of different peptides for binding to LPS and kinetic analysis of the binding of NT7 as a function of temperature. The sensograms representing the binding at 25°C to the immobilized endotoxin by (*A*) tritrypticin, (*B*) NT7, and (*C*) CT7 are shown as a function of peptide concentration. (*Inset*) Corresponding linear fit plots of the k_{on} (s^{-1}) as a function of peptide concentration. Analysis of plots of variation of (*D*) k_{ass} and (*E*) k_{diss} as a function of temperature for NT7.

two cases, probably because of the structural differences in these two peptides. We have earlier reported that CD profiles of CT7 and NT7 in aqueous medium showed very different structures (Nagpal et al. 1999). While CT7 showed characteristic of the PPII conformation, NT7 showed β-turn conformation. Circular dichroism spectra of NT7 and CT7 were analyzed in the presence of LPS and compared with the corresponding spectra in its absence (Fig. 3). Curiously, the presence of LPS affected the CD profile in both cases. The significant effect corresponds to the molar ellipticity at 228 nm, which is considered to be a signature of the conformational environment of the aromatic residues in the sequence (Woody 1985). While the negative molar ellipticity at 228 nm further decreased in the case of NT7, that in the case of CT7 marginally increased on the positive side.

Interference of NT7 and CT7 in polymyxin B–endotoxin binding

The direct affinity measurements of the LPS binding were consistent with the abilities of the peptide antibiotics for polymyxin B displacement, correlating with the possible endotoxin-neutralizing capabilities of these peptides. The displacement of dansyl-polymyxin B from its complex with endotoxin has been proven to be an excellent probe for identifying the anti-endotoxin activities of the peptide antibiotics. Dansyl-polymyxin B exhibits high fluorescence in the bound state, which decreases upon displacement by other ligands. It was observed that tritrypticin as well as its analog NT7 could displace polymyxin B bound to LPS with the comparable IC_{50} values of 60 μM and 45 μM, respectively (Fig. 4). On the other hand, the analog

Table 2. *Affinity parameters for the interaction of NT7 with the endotoxin at five different temperatures*

Temperature (°C)	$k_{ass} \times 10^{-3}$ (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_D (μM)
15	2.38 ± 0.4	0.14 ± 0.04	61
20	2.18 ± 0.3	0.15 ± 0.03	71
25	3.12 ± 0.4	0.21 ± 0.04	67
30	2.35 ± 0.2	0.17 ± 0.02	74
35	3.21 ± 0.3	0.26 ± 0.06	80

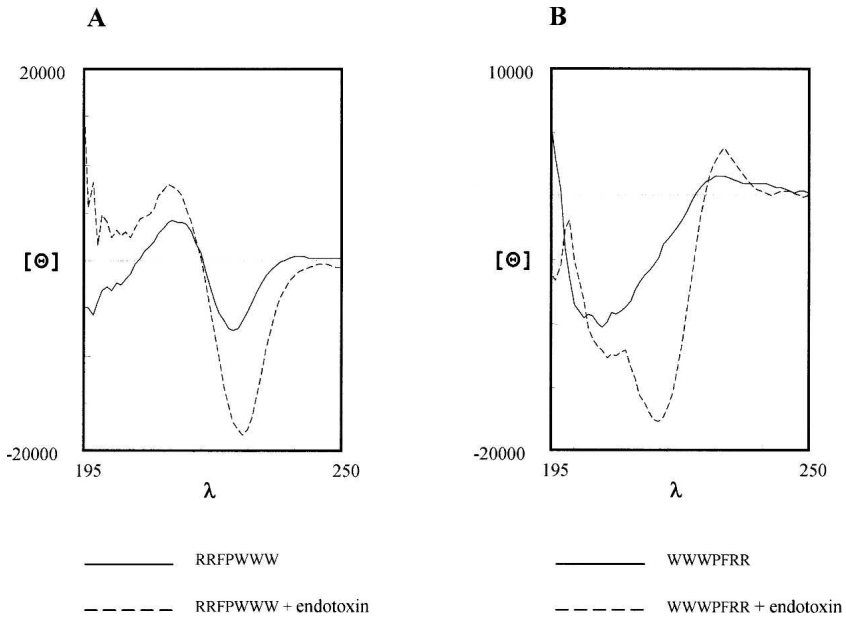

Figure 3. Comparison of circular dichroism profiles of (*A*) NT7 and (*B*) CT7, in free and LPS-bound forms.

CT7 could displace only ~28% of the bound polymyxin B from endotoxin even at 100 μM. Thus, NT7 has the ability to displace polymyxin B from LPS, whereas CT7 showed very low activity. In other words, NT7 has higher affinity for endotoxin in comparison to CT7. The ability of NT7 to displace polymyxin B from LPS was comparable to that of the native tritrypticin.

Inhibition of B-cell proliferation and NO release

The possibility of anti-endotoxin activities of tritrypticin and its small active analogs as indicated by polymyxin B displacement was confirmed by assaying for their interference in NO release and B-cell proliferation, the known markers associated with endotoxin activity.

Consistent with the binding studies, it was anticipated that tritrypticin and NT7 may interfere in the biological functions of LPS. Endotoxin is a known mitogenic stimulant of B-lymphocytes. Under physiological conditions, the interference or otherwise of the peptides—tritrypticin, NT7, and CT7—on endotoxin activity was measured by their effects on the LPS-mediated proliferation rate of B-cell lymphocytes. It was observed that the proliferating effect of endotoxin was inhibited in a dose-dependent manner in the presence of tritrypticin and NT7 but not by CT7 (Fig. 5A). Tritrypticin and NT7 completely neutralized the effect of endotoxin at 50 μM, whereas CT7 could not inhibit the cell proliferation completely even at the maximum concentration of 100 μM used.

It is known that macrophages exposed to such mitogens as endotoxin show high levels of nitric oxide (NO). The interference of these 7-mer peptide analogs as well as the native tritrypticin in the activation of macrophages by LPS was also assayed. As seen in Figure 5B, tritrypticin and NT7 led to 50% inhibition of the endotoxin-induced NO release at 50 μM and 75 μM, respectively. On the other hand, CT7 could affect not more than 30% inhibition even at a significantly higher dose of 200 μM. In other words, while NT7 binds to LPS with fairly high affinity, CT7 showed poor affinity. Thus, the comparative data of NT7 and CT7 with regard to the inhibition of B-cell proliferation and NO release correlate well with their binding affinities to endotoxin.

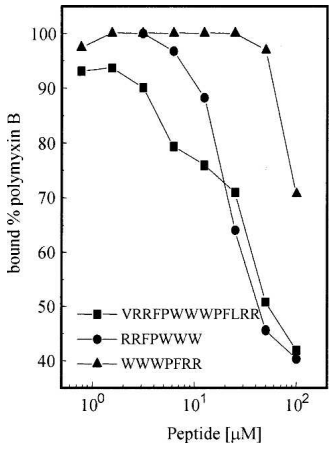

Figure 4. Comparison of NT7 and CT7 for LPS binding by competitive displacement of dansyl polymyxin B in a dose-dependent manner.

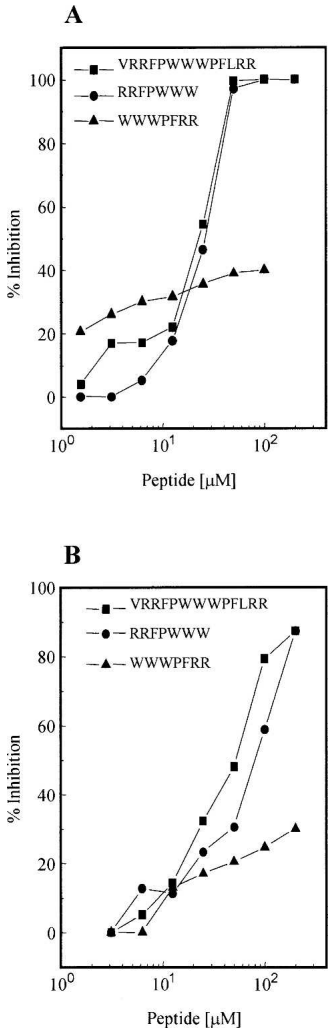

Figure 5. Functional comparison of endotoxin binding of NT7 and CT7. Dose-dependent inhibition of (*A*) B-cell proliferation and (*B*) NO release induced by endotoxin.

Analysis of designed analogs of NT7

It is evident that the tritrypticin analogs CT7 and NT7 exhibited similar antibacterial activities. However, the two peptides showed different anti-endotoxin activities. Interestingly, they exhibited distinct conformational characteristics. In order to explore the correlation of structure with the differential anti-endotoxin activities, we designed NT7 analogs with modification of the length and rigidity of the peptide. The NT7 analogs with increasing lengths using flexible and constrained linkers between two clusters were also analyzed to study their effect on the antibacterial activity. The antibacterial activity of native NT7 was compared with that of these designed analogs. Flexible linkers containing one (RRFGWWW), two (RRFGGWWW), or three (RRFGGGWWW) glycine residues between the aromatic and cationic clusters were introduced. All three NT7 analogs showed comparable antibacterial activity to that of native NT7 against *S. typhimurium* (Fig. 6A). An increase in the number of constrained proline residues—that is, two (RRFPPWWW) or three (RRFPPPWWW)—between the two clusters resulted in the loss of antibacterial activity of the analogs (Fig. 6B).

Comparison of the endotoxin binding of these analogs was carried out by an affinity sensor. Analogs incorporating glycine residues showed binding to LPS comparable to that of NT7 (Table 3). The k_{ass} and k_{diss} values were also comparable to those of NT7, indicating that the kinetics of binding was similar. Comparison of binding kinetics of NT7 analogs incorporating additional proline residues between the aromatic and cationic clusters showed a decrease in k_{ass} by sixfold to sevenfold, while the k_{diss} was similar to NT7, effectively resulting in about sixfold loss in the affinity to endotoxin of these analogs with respect to that of NT7 (Table 3).

Comparison of the anti-endotoxin activities of the analogs was carried out. It shows that with the successive increase in glycine residues in NT7 analogs, there was an incremental decrease in the inhibition of B-cell proliferation (Fig. 6C). On the other hand, the proline-extended analogs showed no inhibition at all (Fig. 6D).

Discussion

A large and diverse range of mechanisms by which a broad array of peptide antibiotics attack the bacterial cell have been proposed (Skerlavaj et al. 1990; Boman et al. 1993; Casteels and Tempst 1994; Shai 1995; Falla et al. 1996; Ludkte et al. 1996; Yang et al. 2006). It has been suggested that the initial event common to all the cationic peptide antibiotics is the binding of positively charged residues to the negatively charged LPS molecules in the case of Gram-negative bacteria and lipoteichoic acid (LTA) in the case of the Gram-positive bacteria exposed at the target cell surface, facilitating the uptake of the peptide antibiotics (Hancock 1997; Hancock and Scott 2000). The actual killing could take place subsequently, either by disrupting the membrane or permeabilizing and intervening at the level of the potential targets inside the cell.

The endotoxin-induced self-promoted uptake model for antibacterial activity of the peptide antibiotics (Hancock 1997) would necessitate positive correlation of the antibacterial activity, endotoxin binding, and its neutralization. This is because LPS would be required to mediate the uptake and subsequent internalization for transporting the peptide in the proximity of its target for killing. However, when the smallest active analogs of tritrypticin, NT7 and CT7, which exhibited similar antibacterial activities against different bacterial strains, were subjected to the anti-endotoxin assays, it was clear that they

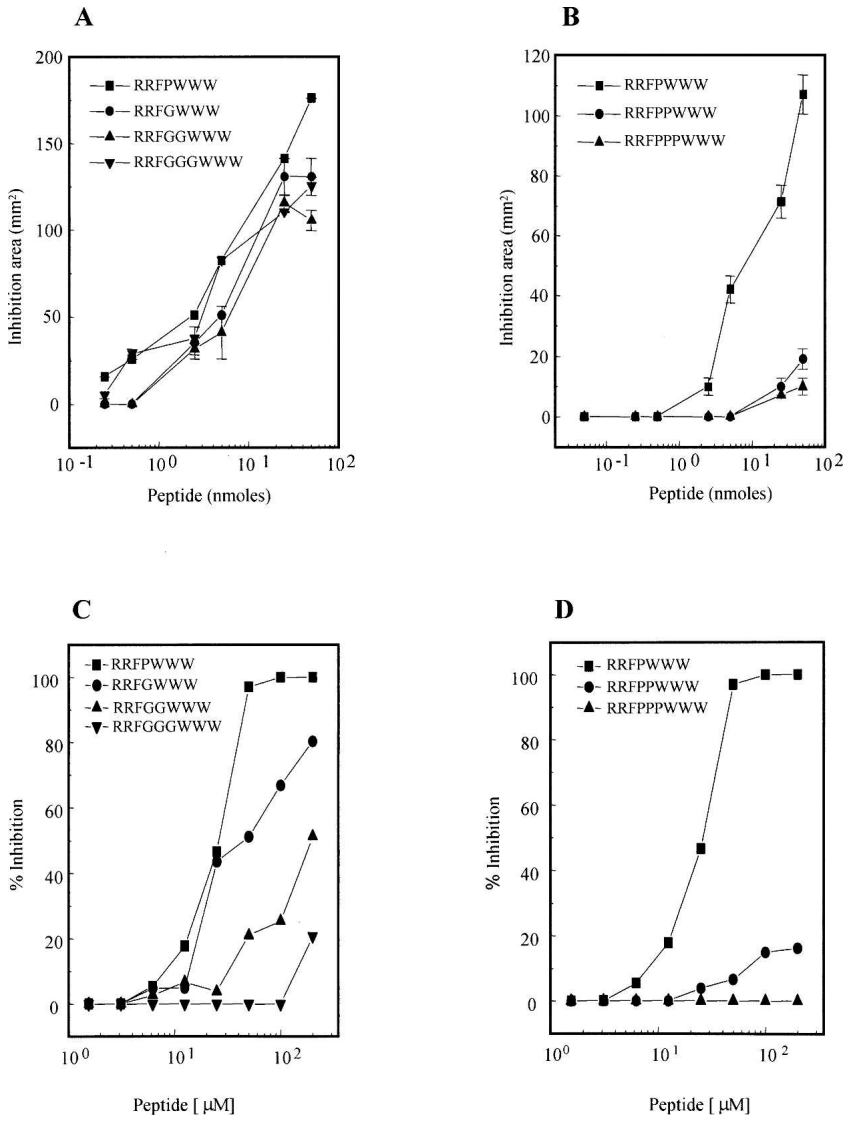

Figure 6. Functional comparison of various analogs containing different linker residues. Antibacterial activity against *S. typhimurium* of NT7 analogs containing different lengths of linker of (*A*) flexible Gly residues and (*B*) constrained Pro residues, in between cationic and aromatic clusters. Dose-dependent inhibition of B-cell proliferation of NT7 analogs containing different lengths of linker of (*C*) flexible Gly residues and (*D*) constrained Pro residues in between cationic and aromatic clusters.

were different from each other. NT7 showed binding to endotoxin with higher affinity in comparison to CT7. The differences were also evident in terms of polymyxin B displacement. The tritrypticin and NT7 bind to endotoxin at the same site where polymyxin B binds and neutralizes its activity. This indicated a possibility that the NT7 that binds to LPS with high affinity may also have endotoxin-neutralizing activity.

Binding of the peptide to endotoxin does not automatically imply that it possesses the potential to neutralize its activity. In fact, it may modulate the activity negatively as well as positively (Kirikae et al. 1998). Indeed, it is evident that NT7 neutralizes LPS-induced endotoxic effects as reflected by the inhibition of NO release or B-cell proliferation in a dose-dependent manner. CT7, which was unable to bind endotoxin, did not inhibit either B-cell proliferation or NO release. Thus, while NT7 and CT7 were found to be similar in terms of their antibiotic potential, they were distinct in terms of binding to endotoxin and its neutralization. In other words, a negative correlation between the antibacterial and anti-endotoxin activities of these two tritrypticin analogs was apparent.

Independence of the two activities—antibacterial and anti-endotoxin—was also indicated from other studies involving cathelicidins. Separate regions were suggested to be responsible for antibacterial and LPS-neutralizing

Table 3. *Kinetic parameters of binding of various NT7 analogs to endotoxin at 25°C*

Peptide	$k_{ass} \times 10^{-3}$ (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_D (μM)
RRFPWWW	3.12 ± 0.4	0.21 ± 0.04	67
RRFGWWW	2.17 ± 0.1	0.21 ± 0.01	96
RRFGGWWW	1.73 ± 0.1	0.17 ± 0.01	101
RRFGGGWW	4.47 ± 0.5	0.19 ± 0.05	43
RRFPPWWW	0.51 ± 0.1	0.22 ± 0.03	428
RRFPPPWWW	0.44 ± 0.1	0.17 ± 0.02	387

activities in CAP11 and CAP18, other members of cathelicidin family (Kirikae et al. 1998; Okuda et al. 2006). It has also been shown that the bactericidal activity of cathelicidins requires processing, whereas endotoxin neutralization is more potently expressed by the unprocessed protein (Zarember et al. 2002). Differential abilities for endotoxin neutralizations have been observed in many different peptide antibiotics that show comparable antibacterial activities (Rosenfeld et al. 2006). If a common mode of action is assumed in the case of these peptides, all these data were consistent with our results. Thus, the incongruity of the antibacterial and LPS detoxification activities is evident. It is also consistent with the suggestion that cathelicidins are capable of performing multiple functions (Gallo et al. 1994; Gao et al. 2000) besides showing antibacterial and anti-endotoxin functions. Indeed, neutrophil antibacterial peptides are multifunctional effector molecules constituting a primary bactericidal defense barrier, in addition to serving as a second wave of antibacterial defense effectors when recruited to sites of infection and inflammation (Gudmundsson and Agerberth 1999).

The two peptides, NT7 and CT7, showing different anti-endotoxin activities also exhibit distinct conformational characteristics. NT7 exhibits β-turn conformation, and CT7 shows PPII conformation (Nagpal et al. 1999). Both the conformations showed clear amphipathic nature with the segregation of cationic residues and aromatic residues. Analysis of the effect of LPS on the structures of the two peptides provided further insights. It was evident that the negative circular dichroism at 228 nm is correlated with the binding of LPS. In fact, the molar ellipticity at 228 nm has been suggested to represent the signature of the conformational environment of the aromatic residues in the sequence (Woody 1985). This has also been found in the case of indolicidin, where both native indolicidin as well as the retro-analog bind to LPS with comparable affinity (Nagpal et al. 2002). A structural model of indolicidin-LPS binding suggested that proper juxtaposition of the distinct clusters of the aromatic and cationic residues of indolicidin, analogous to a dumbbell shape, was critical while binding to LPS (Nagpal et al. 2002). Indeed, similar clustering of the aromatic and cationic residues was possible in the structures of both CT7 and NT7. However, these clusters would be spaced differently in the two peptides, the distance being more in the case of the PPII conformation compared with the β-turn conformation. Thus, the direct binding of the peptide to the LPS with appropriately juxtaposed hydrophobic and ionic clusters provides a mechanistic model for endotoxin neutralization.

While the functional difference between the two peptides in terms of LPS neutralization can be correlated well with respect to the dumbbell model for LPS binding, it certainly is contradicted in terms of antibacterial activity. Such a scenario would be consistent with a model for antibacterial activity involving a relatively nonspecific mechanism of bacterial killing. Analysis of antibacterial activity as well as LPS-neutralizing activities of the designed analogs, in which the distance between clusters of hydrophobic residues and positively charged residues in the dumbbell model was varied by introducing additional proline and glycine residues, indicated that this was not the case. While introduction of additional glycine residues in NT7 did not affect the antibacterial activity significantly but showed the differential ability to LPS neutralization, the sequential addition of prolines affected the antibiotic activity as well as the anti-endotoxic activities of the two peptides. Thus, the flexible extension involving glycine residues could maintain the distance required for functional juxtaposition of polar and hydrophobic residues, which was not possible with the addition of proline residues, suggesting that the antibacterial activity could not be considered entirely nonspecific.

Thus, while we were able to define structural determinants in the case of anti-endotoxin activity, such a description has not been possible in the case of antibacterial activity. Antibacterial activity implies bacterial killing, which may involve, at the molecular level, a variety of different mechanisms. These include membrane pore formation, lysing the membrane through the carpet effect, membrane permeabilization, and intervention in the DNA/RNA or protein synthesis (Boman et al. 1993; Shai 1995). The designed experiments involving flexible and rigid insertions indicated specificity of the antibacterial activity, although molecular determinations could not be delineated. On the other hand, endotoxin binding could be defined in terms of receptor peptide interactions providing a possible mechanism of endotoxin neutralization.

Materials and Methods

HMP (4-hydroxymethyl phenoxymethyl polystyrene) resin, solvents, and reagents used for synthesis were supplied by Applied Biosystems, Inc. Fmoc amino acid derivatives were procured from Bachem Feinchemikalein AG. Biotin cuvettes were obtained from IAsys Autoplus, Affinity Sensor. Lipopolysaccaride (LPS) of wild

S. typhimurium was obtained from Difco Laboratories. Other chemicals were purchased from Sigma.

The Gram-negative bacterial strains *S. typhimurium* 3261 PNP2 Gro A mutant, *E. coli* BL21(λD3), *P. aeruginosa*, and *A. tumefaciens* were used for radial diffusion assay. Agarose I (Biotechnology grade) was obtained from Amresco, and tryptic soy broth (TSB) was from Himedia Laboratories Pvt. Ltd.

Peptide synthesis, purification, and characterization

Peptides were synthesized by a solid-phase method using an automated peptide synthesizer Model 431A (Applied Biosystems, Inc.), employing standard Fmoc methodology. The peptides were cleaved from the resin by treatment with TFA/thioanisole/phenol/water/EDT in the ratio recommended by Applied Biosystems Inc. The crude peptides were purified using a C-18 column (Deltapak–100 Å, 15 μ, spherical, 19 × 300 mm; Waters), and peptide purity was verified using a C-18 analytical column (Deltapak-300 Å, 15 μ, spherical, 7.8 × 300 mm; Waters). Characterization was performed by molecular mass determination using single Quadruple mass analyzer (Fisons Instruments).

Antibacterial assay

The radial diffusion assay was performed using double-layered agarose as described previously (Nagpal et al. 1999). All the experiments were performed in triplicate, and data are presented as mean inhibition zone area ± SEM.

Dansyl polymyxin B displacement assay

Dansyl polymyxin B, a fluorescent derivative of polymyxin B, was prepared by condensing polymyxin B sulfate with dansyl chloride as described by Schindler and Teuber (1975). The comparative binding affinities of various antibiotic peptides for endotoxin were investigated by dansyl polymyxin B displacement assay (Moore et al. 1986). The data were expressed as the percent of dansyl polymyxin B bound to the endotoxin as a function of peptide concentration. The sample concentration resulting in 50% displacement (IC_{50}) of dansyl polymyxin B was thus determined from the graph.

Kinetic and thermodynamics of endotoxin binding

Binding kinetics were determined using an IAsys Autoplus, Affinity Sensor. Endotoxin was biotinylated with NHS-LC-biotin as described by de Haas et al. (1998). Biotinylated endotoxin pre-treated with EDTA and DOC as reported earlier (de Haas et al. 1998) was immobilized onto a streptavidin bound on biotin-coated surface at a concentration of 0.25 mg/mL in 10 mM PBS (pH 7.4). Approximately 600 arc seconds of endotoxin were immobilized, where 600 arc seconds corresponds to an immobilized protein concentration of 1 ng/mm^2. The unreacted sites were blocked with d-biotin. All measurements were carried in 10 mM HANK'S balanced salt solution at 25°C. The binding studies for NT7 were carried out at temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C. For the determination of association rate constants, various concentrations of antibiotic peptides in the same buffer were used. Dissociation rate constants were measured by replacing the sample by buffer. Following analyte binding, the surface was regenerated with 10 mM GlyHCl (pH 2.0).

Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensogram data using the FASTfit software package supplied with the IAsys instrument. The instrument response measured in arc seconds is proportional to the mass of bound ligate, resulting in

$$R_t = (R_{eq} - R_0)[1 - \exp(k_{on}\, t)] + R_0 \quad (1)$$

where R_t is the response at time t, R_0 is the initial response, R_{eq} is the maximal response, and k_{on} is the pseudo-first-order rate for the interaction. The response values are determined experimentally, and therefore k_{on} at a particular concentration of ligate can be derived. Multiple determinations of k_{on} are obtained by carrying out repeat associations at various concentrations of ligate. The value of k_{on} varies with ligate concentration [L] in a linear fashion:

$$k_{on} = k_{diss} + k_{ass}[L] \quad (2)$$

A plot of multiple k_{on} values, derived from interaction experiment, against the ligate concentration [L] at which they were carried out allows the determination of the association constant k_{ass} from the slope and dissociation rate constant k_{diss} from the intercept. The dissociation rate constant k_{diss} can also be measured by direct examination of dissociation data. The dissociation is observed as an exponential decay of the complex into its components, namely, immobilized ligand and free ligate with time as described by Equation 3:

$$R_t = R_0 \exp(-k_{diss}\, t) \quad (3)$$

The instrument response (R_t), measured in arc seconds at time t, is dependent on the initial response (R_0) and the dissociation rate constant (k_{diss}). The dissociation rate constant was determined from the dissociation data as well as from the intercept of the plot of k_{on} with respect to ligand concentration, in order to verify mutual concordance. The k_{diss} obtained from the dissociation data was used for subsequent analysis.

The dissociation equilibrium constant, $K_{D,}$ can be calculated from Equation 4:

$$K_D = k_{diss}/k_{ass} \quad (4)$$

Circular dichroism

The circular dichroism (CD) experiments were carried out on a JASCO 710 spectropolarimeter with a 1.0-nm bandwidth at 1.0-nm data pitch, and 1 sec response time using a 10-mm path length cell at 25°C. Typically, 25 scans with a speed of 200 nm/min in the range of 250–195 nm were accumulated and averaged. The spectra were recorded at the peptide concentration of 10 μM in the presence of LPS (10 μM) or absence of LPS in 10 mM phosphate buffer (pH 7.4). Results were expressed as mean residue molar ellipticity in deg cm^2/dmol.

B-cell proliferation assay

Splenic cells (0.5 × 10^6 / well) from unimmunized BALB/c mice were cultured (at 37°C and 5% CO_2) in RPMI, in 96-well flat-bottom tissue culture plates (Nunclon) for 48 h in the

presence of endotoxin (1 μg/mL). The RPMI was supplemented with 10% fetal calf serum (Biological Industries) and 40 μg/mL gentamicin. Various concentrations of the peptide were added to this culture in order to analyze dose-dependent effects of the peptide. At the end of 48 h, the cells were pulsed with methyl [^{3}H]thymidine (Amersham; 0.5 μCi/well) for 12 h and then harvested onto a filter-pad (Betaplate), and the radioactivity counts were recorded (LKB-Pharmacia). A control experiment, wherein only endotoxin (1 μg/mL) was added to the splenic cell culture, was also carried out. All experiments were performed in triplicate, and results are presented as the inhibition in proliferation as a function of peptide concentration. The inhibition levels of proliferation were calculated by comparison of the CPM of the wells with endotoxin and peptide and that of the control wells, which did not contain the peptide.

NO release assay

To prepare the macrophages, 1 mL of 8% thioglycolate solution was injected per mouse intraperitoneally. After 72 h, mice were sacrificed, and peritoneal exudates were received by flushing the abdomen cavity of mice with 10 mL of ice-cold RPMI using a 22-gauge needle. Macrophage were pooled together and centrifuged at 1500 rpm for 15 min at 4°C. The pellet was resuspended in CRPMI, and cells were counted. One hundred microliters of macrophage cell suspension (3×10^6 cells/mL CRPMI) was transferred to a 96-well microtiter plate and incubated for 3 h for cell adherence in a 5% CO_2 incubator at 37°C. Cells were washed with warm IRPMI in order to remove nonadherent cells. The macrophage were cultured with endotoxin (20 μg/mL) and in the absence and presence of increasing concentrations of peptides for 48 h in CO_2 in order to analyze the dose-dependent effect of peptides. After the incubation was complete, 100 μL of culture supernatant was taken in another microtiter plate and incubated with 100 μL of freshly prepared Griess reagent for 5 min at room temperature. Levels of NO production by the macrophage were estimated by measuring optical density (OD) of colored solution at 550 nm. All experiments were performed in triplicate, and results are presented as the inhibition in NO release as a function of peptide concentration. The levels of NO release inhibition were calculated by comparison of the OD from the wells obtained with endotoxin and peptide and that of the control wells, which did not contain the peptide.

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References

Boman, H.G., Agerberth, B., and Boman, A. 1993. Mechanism of action on *Escherichia coli* of Cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect. Immun.* **61:** 2978–2984.

Casteels, P. and Tempst, P. 1994. Apidaecin-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity. *Biochem. Biophys. Res. Commun.* **199:** 339–345.

de Haas, C.J., Haas, P.J., van Kessel, K.P., and van Strijp, J.A. 1998. Affinities of different proteins and peptides for lipopolysaccharide as determined by biosensor technology. *Biochem. Biophys. Res. Commun.* **252:** 492–496.

Falla, T.J., Karunaratne, D.N., and Hancock, R.E.W. 1996. Mode of action of the antimicrobial peptide Indolicidin. *J. Biol. Chem.* **271:** 19298–19303.

Gallo, R.L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. 1994. Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci.* **91:** 11035–11039.

Gao, Y., Lecker, S., Post, M.J., Hietaranta, A.J., Li, J., Volk, R., Li, M., Sato, K., Saluja, A.K., Steer, M.L., et al. 2000. Inhibition of ubiquitin–proteasome pathway-mediated IκBα degradation by a naturally occurring antibacterial peptide. *J. Clin. Invest.* **106:** 439–448.

Gudmundsson, G.H. and Agerberth, B. 1999. Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. *J. Immunol. Methods* **232:** 45–54.

Hancock, R.E.W. 1997. Peptide antibiotics. *Lancet* **349:** 418–422.

Hancock, R.E.W. and Scott, M.G. 2000. The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci.* **97:** 8856–8861.

Hancock, R.E.W., Falla, T., and Brown, M.M. 1995. Cationic bactericidal peptides. *Adv. Microb. Physiol.* **37:** 135–175.

Kirikae, T., Hirata, M., Yamasu, H., Kirikae, F., Tamura, H., Kayama, F., Nakatsuka, K., Yokochi, T., and Nakano, M. 1998. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)- derived peptide against murine endotoxemia. *Infect. Immun.* **66:** 1861–1868.

Ludkte, S.J., He, K., Heller, W.T., Harroun, T.A., Yang, L., and Huang, H.W. 1996. Membrane pores induced by Magainin. *Biochemistry* **35:** 13723–13728.

Medzhitov, R. and Janeway Jr., C. 2000. Innate immunity. *N. Engl. J. Med.* **3:** 338–344.

Moore, R.A., Bates, N.C., and Hancock, R.E.W. 1986. Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob. Agents Chemother.* **29:** 496–500.

Nagpal, S., Gupta, V., Kaur, K.J., and Salunke, D.M. 1999. Structure–function analysis of tritrypticin, an antibacterial peptide of innate immune origin. *J. Biol. Chem.* **274:** 23296–23304.

Nagpal, S., Kaur, K.J., Jain, D., and Salunke, D.M. 2002. Plasticity in structure and interactions is critical for the action of indolicidin, an antibacterial peptide of innate immune origin. *Protein Sci.* **11:** 2158–2167.

Okuda, D., Yomogida, S., Tamura, H., and Nagaoka, I. 2006. Determination of the antibacterial and lipopolysaccharide-neutralizing regions of guinea pig neutrophil cathelicidin peptide CAP11. *Antimicrob. Agents Chemother.* **50:** 2602–2607.

Piers, K.L., Brown, M.H., and Hancock, R.E.W. 1994. Improvement of outer membrane-permeabilization and lipopolysaccharide-binding activities of an antimicrobial cationic peptide by C-terminal modification. *Antimicrob. Agents Chemother.* **38:** 2311–2316.

Rosenfeld, Y., Papo, N., and Shai, Y. 2006. Endotoxin (LPS) neutralization by innate immunity host-defense peptides: Peptides' properties and plausible modes of action. *J. Biol. Chem.* **281:** 1636–1643.

Schibli, D.J., Hwang, P.M., and Vogel, H.J. 1999. Structure of the antimicrobial peptide tritrpticin bound to micelles: A distinct membrane-bound peptide fold. *Biochemistry* **38:** 16749–16755.

Schindler, P.R.G. and Teuber, M. 1975. Action of polymyxin B on bacterial membranes: Morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob. Agents Chemother.* **8:** 94–104.

Shai, Y. 1995. Molecular recognition between membrane-spanning polypeptides. *Trends Biochem. Sci.* **20:** 460–464.

Skerlavaj, B., Romeo, D., and Gennaro, R. 1990. Rapid membrane permeabilization and inhibition of vital functions of Gram-negative bacteria by Bactenecins. *Infect. Immun.* **58:** 3724–3730.

Woody, R.W. 1985. Circular dichroism of peptides. In *The peptides: Analysis, synthesis, biology* (eds. S. Udenfriend et al.), Vol. 7, pp. 15–114. Academic Press, London.

Yang, S.T., Jeon, J.H., Kim, Y., Shin, S.Y., Hahm, K.S., and Kim II, J. 2006. Possible role of a PXXP central hinge in the antibacterial activity and membrane interaction of PMAP-23, a member of cathelicidin family. *Biochemistry* **45:** 1775–1784.

Zarember, K.A., Katz, S.S., Tack, B.F., Doukhan, L., Weiss, J., and Elsbach, P. 2002. Host defense functions of proteolytically processed and parent (unprocessed) cathelicidins of rabbit granulocytes. *Infect. Immun.* **70:** 569–576.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415:** 389–395.