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Membrane permeability and antimicrobial kinetics of cecropin P1 against *Escherichia coli*‡

Steven Arcidiacono,[a]* Jason W. Soares,[a] Alexa M. Meehan,[a] Patrick Marek[b] and Romy Kirby[a]

The interaction of cecropin P1 (CP1) with *Escherichia coli* was investigated to gain insight into the time-dependent antimicrobial action. Biophysical characterizations of CP1 with whole bacterial cells were performed using both fluorescent and colorimetric assays to investigate the role of membrane permeability and lipopolysaccharide (LPS) binding in lytic behavior. The kinetics of CP1 growth inhibition assays indicated a minimal inhibitory concentration (MIC) of 3 μM. Bactericidal kinetics at the MIC indicated rapid killing of *E. coli* (<30 min). Membrane permeability studies illustrated permeation as a time-dependent event. Maximum permeability at the MIC occurred within 30 min, which correlates to the bactericidal action. Further investigation showed that the immediate permeabilizing action of CP1 is concentration-dependent, which correlates to the concentration-dependent nature of the inhibition assays. At the MIC and above, the immediate permeability was significant enough that the cells could not recover and exhibit growth. Below the MIC, immediate permeability was evident, but the level was insufficient to inhibit growth. Dansyl polymyxin B displacement studies showed LPS binding is essentially the same at all concentrations investigated. However, it does appear that only the immediate interaction is important, because binding continued to increase over time beyond cell viability. Our studies correlated CP1 bactericidal kinetics to membrane permeability suggesting CP1 concentration-dependent killing is driven by the extent of the immediate permeabilizing action of the peptide. Published in 2009 by John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; cecropin P1; antimicrobial activity; lipopolysaccharide; membrane permeabilization; *E. coli*

Introduction

Antimicrobial peptides (AMPs) are part of the innate defense system found in all organisms to protect them from microbial infection [1]. AMPs are generally 20–40 amino acids in length, cationic, and have a physical mode of action, exhibiting a broad range of antimicrobial activity against both Gram-negative and Gram-positive bacteria. More specifically, AMPs that target Gram-negative bacteria bind to the negatively charged lipopolysaccharide (LPS) predominantly via electrostatic and van der Waals interactions [2–4].

Cecropin P1 (CP1) is a 3339 Da, 31 amino acid peptide found in nematodes from the stomachs of pigs [5]. It exhibits antimicrobial activity predominantly against Gram-negative bacteria (with reduced activity against Gram-positive bacteria) and kills in a dose-dependent manner [6]. Understanding the CP1 mode of action is of interest because CP1 and other AMPs are potential therapeutic agents. In light of the emergence of multidrug resistant strains of bacteria, AMPs have been considered as possible alternatives to conventional antibiotics. CP1 has been found to be active against a number of clinically relevant bacteria, including *Pseudomonas aeruginosa* [7] and *Acinetobacter baumannii* [8]. Because of the physical mode of action of CP1, resistance would not be expected as with antibiotics that work by disrupting biochemical pathways. A limitation of the use of peptides for therapeutics is that many possess cytotoxic or hemolytic properties. However, unlike many peptides, CP1 does not exhibit hemolysis [9]. Other potential uses of CP1 include incorporation as pathogen-selective capture molecules [10] and secondary labels [11] in biosensor systems.

CP1 is part of the class of linear, cationic peptides that form amphipathic α-helical structures upon binding to the cell. CP1 is generally believed to function by the carpet mechanism in which it binds to the acidic bacterial membrane surface and disrupts the lipid bilayer, leading to membrane disintegration [12,13]. CP1 was found to be oriented parallel to the lipid membranes but did not enter the hydrocarbon membrane core [14]. Binding studies with *E. coli* have also led to an understanding of LPS as the initial binding site [4]. Whole cell studies have investigated CP1 membrane permeability by studying killing kinetics using *E. coli* strains with different membrane structure but could not distinguish between interactions with the inner and outer membranes [6]. More recently, investigations of membrane permeability using *o*-nitrophenyl-β-galactopyranoside (ONPG) have been performed to study the mode of action of many AMPs [15–18]. Results show that permeability is dose and time dependent, and that permeability occurs even at sublethal peptide concentrations. Many studies have related permeability with peptide minimal inhibitory concentration (MIC) and some have also examined killing kinetics. However, correlation between permeability and

* *Correspondence to: Steven Arcidiacono, Biological Science and Technology Team, U.S. Army Natick Soldier Research, Development, & Engineering Center, Natick, MA 01760-5020, USA. E-mail: steven.arcidiacono@us.army.mil*

a *Biological Science and Technology Team, Warfighter Science Technology and Applied Research Directorate, U.S. Army Natick Soldier Research, Development, & Engineering Center (NSRDEC), Natick, MA 01760-5020, USA*

b *Performance Enhancement and Food Safety Team, Combat Feeding Directorate, U.S. Army Natick Soldier Research, Development, & Engineering Center (NSRDEC), Natick, MA 01760-5020, USA*

bactericidal kinetics has not been possible due to limitations of the ONPG assay [18].

The studies presented here use direct measurement techniques to further examine the cell interactions with CP1. Specifically, CP1 membrane permeability was investigated through SYTOX kinetic assays and binding to LPS was measured through dansyl polymyxin B (dansyl PMB) displacement assays. Here, binding and permeability was correlated with CP1 time-dependent antimicrobial action.

Materials and Methods

CP1, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and ONPG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CP1 was solubilized in water and quantitated by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). *E. coli* ML35 (ATCC 43 827), a lactose, permease-deficient strain that constitutively expresses cytoplasmic β-galactosidase, was obtained from American Type Cell Culture (ATCC, Manassas, VA, USA). The cells were grown to mid-log phase at 37 °C to $OD_{600} = 1.0$ (approximately 10^8 CFU/ml) as a working stock for each assay. Mueller Hinton II broth (MHII), Luria broth (LB), Nutrient broth (NB) and agar were from Becton Dickenson and Co. (Franklin Lakes, NJ, USA). SYTOX Green nucleic acid stain and dansyl PMB were from Invitrogen Corp. (Carlsbad, CA, USA). PopCulture reagent was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Phosphate-buffered saline (PBS) pH 7.2 consisted of 137 mM NaCl, 2.7 mM KCl, 4.4 mM Na_2HPO_4, and 1.4 mM KH_2PO_4.

Antimicrobial Activity

The MIC of CP1 was determined using a microplate broth dilution assay [19]. Cells were grown as stated above and diluted to a 10^5 CFU/ml working stock in MHII medium. Twofold dilutions of CP1 from a 6 μM stock solution were made in PBS. Peptide was tested in triplicate in a microplate against 100 μl aliquots of the working stock, or 10^4 CFU/well. Control wells were inoculated with cells without peptide present. The microplates were incubated overnight at 37 °C and OD_{595} was determined every 30 min on a GENios fluorescent microplate reader (TECAN Austria GmbH, Grödig, Austria).

Bactericidal kinetics was determined using a modified version of the assay described by Lehrer *et al*. [20]. CP1 at the MIC concentration of 3 μM was incubated with 10^5 CFU/ml for 30 min. Aliquots at 10-min intervals were serially diluted 10-fold in PBS, plated on LB agar and incubated overnight at 37 °C to determine cell viability. Peptide-free controls were also run to determine control cell concentrations.

Membrane Permeability

Permeabilization of the outer/inner membrane was investigated using ONPG hydrolysis and SYTOX Green uptake assays. In the ONPG hydrolysis method [18], cells were grown to mid-log in LB and washed 2× with an equal volume of PBS. Cells were diluted in PBS to 10^7 CFU/ml ($OD_{600} \sim 0.5–0.7$), and 15 μl ($\sim 10^5$ CFU) was added to 135 μl of PBS supplemented with 1.5 mM ONPG and CP1 (0–2× MIC). Maximum permeability was determined by evaluating cells pretreated with PopCulture detergent for 10 min. The rate of permeability was evaluated through ONPG hydrolysis by measuring absorbance at 405 nm on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

The SYTOX Green uptake studies [21] used cells grown to mid-log in NB and washed 2× with an equal volume of 5 mM HEPES pH 7.0. Cells ($\sim 10^7$ CFU/well) were added in white microplates (Nalge NUNC International, Rochester, NY, USA) to 150 μl HEPES supplemented with 2.5 μM SYTOX and CP1 (0–2× MIC). Fluorescence was measured on a GENios fluorescent microplate reader with an excitation of 485 nm and an emission of 535 nm. To determine maximum permeability, cells were pretreated with 70% isopropanol for 1.5 h at ambient temperature to permeate all cells.

Fluorescent Microscopy

Images of fluorescent *E. coli* ML35 cells were captured on an Olympus BX-60 epi-fluorescence microscope coupled to a Qimaging Retiga EXi SVGA high-speed cooled digital camera M-12-C system run by IPLab v4.0 (BD Biosciences Bioimaging, Rockville, MD, USA). Fluorescence from the SYTOX Green infiltrated cells were captured using the excitation and emission filter cube 41 001 from Chroma Technologies Corp. (Rockingham, VT, USA) with a 100 ms exposure time under a 100× oil immersion objective.

LPS Binding

CP1 binding to LPS was evaluated using the dansyl PMB displacement assay [22]. Cells were grown to mid-log in LB, washed 2× with an equal volume of 5 mM HEPES pH 7.0 and diluted in HEPES to 10^7 CFU/ml. In white microplates, 15 μl of diluted cells (final cells/well $\sim 10^5$ CFU) was added to 150 μl HEPES supplemented with dansyl PMB (final concentration of 5 μM) and CP1 (0–2× MIC). Samples were read on a GENios fluorescent microplate reader at an excitation wavelength of 340 nm and an emission wavelength of 485 nm. Maximum fluorescence was determined by evaluating dansyl PMB and cells without peptide and subtracting background due to free dansyl in solution. Displacement is measured by the reduction in maximum fluorescence due to addition of CP1.

Results and Discussion

The time-dependent nature of peptide killing kinetics, membrane interaction, and membrane permeation and how they relate is not completely understood. Here, we attempted to expand the understanding of the role of membrane permeability and LPS binding in the CP1 bactericidal action through kinetic-based assays.

CP1 activity against *E. coli* ML35 was assayed through a modified microdilution plate method in which the kinetics of growth inhibition at 1.5, 3, and 6 μM were investigated (Figure 1). The MIC was determined to be 3 μM, the concentration in which *E. coli* ML35 growth was completely inhibited. Below the MIC (1.5 μM), the growth lag was greater than the zero peptide control. This indicates some initial inhibitory effect at 1.5 μM, but the interaction was not sufficient to inhibit cell growth. After 24 h of incubation, growth was still not evident at or above the MIC (data not shown). To further explore the antimicrobial action at the CP1 MIC (3 μM), bactericidal kinetic assays were conducted to determine the rate of lysis. A 1-log reduction was seen after 10 min, and complete lysis was evident by a 5-log reduction after 20 min (Figure 2). The no-peptide control did not exhibit any reduction.

To investigate the role of membrane permeability, a colorimetric assay was performed by measuring β-galactosidase activity [18].

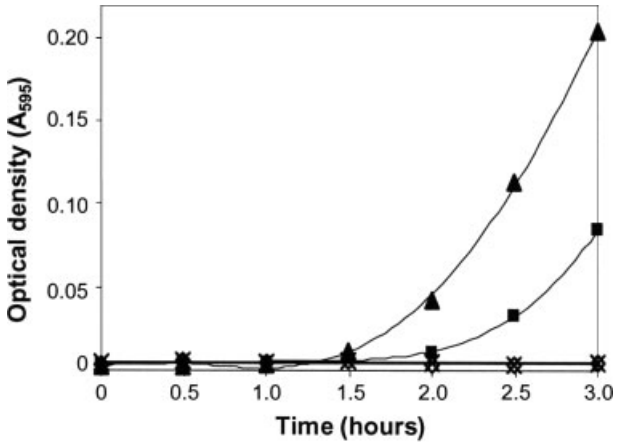

Figure 1. MIC of CP1 against *E. coli* ML35. Time-dependent cell growth in the presence of no peptide (▲), 1.5 (■), 3 (◊), and 6 μM (×) CP1. At 1.5 μM, an increased lag is evident because the onset of growth is ∼2 h as compared with the positive control (∼1.5 h). At >1.5 μM, growth is not evident, indicating cell growth has been inhibited. The MIC = 3 μM. (SD <10%, $n = 3$).

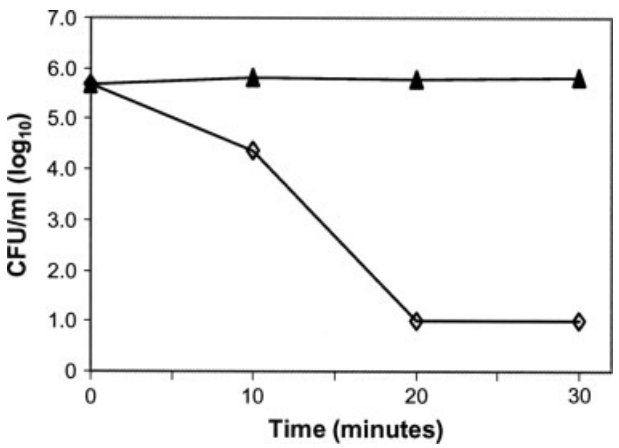

Figure 2. Bactericidal kinetics of CP1 *versus E. coli* ML35. A 5-log reduction occurs after 30 min at the MIC (3 μM). Untreated cells (▲), 3 μM (◊) (SD ≤10%, $n = 4$).

The enzyme is constitutively expressed within the cytoplasm of *E. coli* ML35 and released upon membrane permeation. The substrate ONPG is hydrolyzed by β-galactosidase to *o*-nitrophenol, which is detected by absorbance at 405 nm. At the MIC of 3 μM, permeation with CP1 reached a maximum at ∼2 h, after which the signal began to decrease due to substrate depletion (Figure 3). Above the MIC, the rate or extent of permeability did not change. For 1.5 μM CP1 (0.5× MIC), the time to reach maximum signal was greater, occurring at ∼3 h. Minimal background signal was observed in cells when no peptide was present. Researchers have reported permeability at sub-MIC for temporin L [16], cecropin A [18], and SMAP29 [23]. For SMAP29, the rate to maximum permeability was dose-dependent up to the MIC (the greatest concentration tested), whereas for cecropin A and temporin L the rate was dose-dependent for concentrations greater than or equal to the MIC. This is in contrast to our studies with CP1, where dose dependence was seen until the MIC was reached, at which point permeability remained unchanged.

Bactericidal kinetics showed complete killing of *E. coli* in <30 min; however, at the MIC, the ONPG studies show maximum

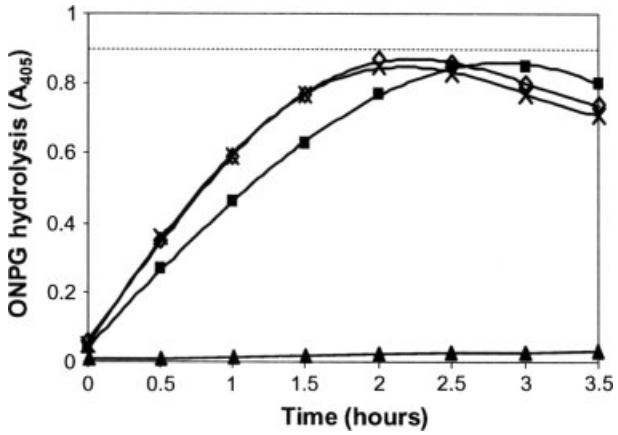

Figure 3. Membrane permeability against *E. coli* ML35 assayed by ONPG hydrolysis with no peptide (▲), 1.5 (■), 3 (◊), and 6 μM (×) CP1. Cells without peptide were not permeabilized and generated minimal signal. Maximum permeation occurs at ∼2 h at or above the MIC of 3 μM. Sub-MIC (1.5 μM) also reaches maximum permeability but does so at ∼3 h. Dotted line is maximum fluorescence from detergent lysed cells (SD ≤10%, $n = 3$).

permeability occurs after 2 h. The disconnect between the kinetics of activity and the membrane permeation determined through ONPG hydrolysis has been observed for other peptides including insect cecropin A [18] and defensins [17]. Mangoni *et al*. [16] suggested a connection, but did not present the time-dependent nature of the permeability. For ONPG, the rate of substrate hydrolysis may account for the time differential between bactericidal activity and membrane permeation. ONPG hydrolysis in cells lysed with detergent (assay positive control) also took about ∼2 h to reach maximum signal (data not shown). This data suggests that the reaction, not permeation, is the cause of the increased time needed for signal development.

To explore a more definitive correlation of bactericidal action and membrane permeability, investigations using SYTOX Green uptake assays were undertaken. SYTOX Green is a fluorescent nucleic acid–binding stain that is fully excluded from intact bacterial cells [21]. The fluorophore has weak fluorescence when bound to the outer membrane. When the membrane is permeabilized, the stain enters the cell and binds readily to nucleic acids, producing an enhanced fluorescent signal throughout the cell. Fluorescent images were taken to visually evaluate cell permeability due to CP1 treatment at 3 μM after 2 h (Figure 4A). Fluorescent microscopy indicated that the cells are permeabilized by CP1. To evaluate the kinetics of membrane permeability, microplate-based SYTOX uptake assays were performed at 1.5, 3, and 6 μM CP1 (Figure 4B). Cells exhibited minimal background fluorescence in the absence of peptide. Membrane permeability was observed to be a concentration- and time-dependent process. At the MIC and above, maximum permeability is approached after ∼30 min, with about 90% of maximum ultimately achieved. The permeabilizing action of CP1 at the MIC directly correlates to the bactericidal kinetics in Figure 2, which could not be achieved with the ONPG studies. Interestingly, below the MIC, maximum permeability equivalent to that seen with 3 μM is still achieved, but it takes ∼1.5 h. This suggests that complete permeation alone is not sufficient for lysis. The rate over time for 3 and 6 μM is similar, whereas 1.5 μM is slightly faster. Therefore, the ability of the cells to remain viable is not dependent on the peptide permeabilizing rate over time, but rather the time it takes to reach maximum permeability. The time to reach

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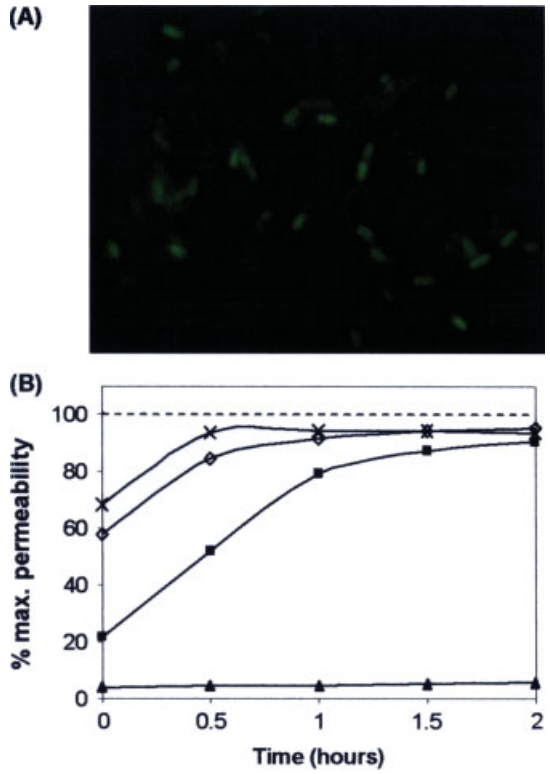

Figure 4. (A) Fluorescence microscopy showing uptake of SYTOX dye into cells after treatment with 3 μM CP1 for 2 h. (B) Kinetics of CP1 membrane permeability against *E. coli* ML35 through SYTOX Green uptake assay. Peptide concentrations plotted over 2 h for no peptide (▲), 1.5 (■), 3 (◊), and 6 μM (×) CP1 and compared with maximum permeability (dotted line) from isopropanol-treated cells. Immediate permeability is concentration-dependent, whereas reaching maximum permeability is time-dependent. At the MIC and above, ~90% of maximum permeability is evident after 30 min. Below the MIC, similar permeability is achieved after 1.5 h. The extent of background signal was minimal as evidenced by weak fluorescent signal due to non-peptide–mediated permeability (SD <20%, $n \geq 3$).

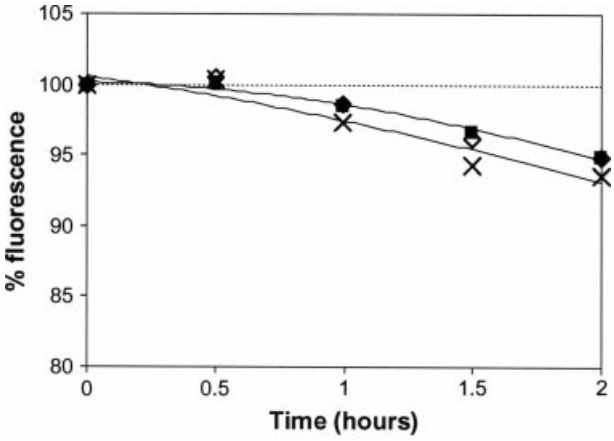

Figure 5. Dansyl PMB displacement assay to investigate CP1 binding to *E. coli* ML35 LPS. Displacement is plotted *versus* time for 1.5 (■), 3 (◊), and 6 μM CP1 (×), represented as % fluorescence remaining after peptide addition. Dotted line is maximum fluorescence from cells before peptide exposure. All concentrations of CP1 evaluated exhibited equivalent minimal displacement (~5%) over 2 h (SD ≤10%, $n = 3$).

maximum was a function of the initial CP1–cell interaction. As concentration increased, the permeabilizing action of the peptide upon immediate interaction with the cells increased (Figure 4B). The ~20% immediate permeation of 1.5 μM is not sufficient to inhibit growth is evident by the increased lag time and subsequent growth seen in Figure 1. CP1 at sub-MIC appears capable of causing disruptions to the membrane, resulting in damaged cells that subsequently recover. This has also been seen in sub-MIC studies with temporin L where significant alteration of membrane integrity was evident; however, cell death did not accompany the permeabilizing effect [16]. At and above the MIC, the immediate permeabilization caused damage too significant for the cell to recover and resume growth.

It is evident from the SYTOX Green studies that immediate permeability is essential for the CP1 antimicrobial action. However, the extent of membrane interaction required to initiate the permeability is not well understood. It is generally believed that LPS is the initial peptide-binding site, from which the peptide subsequently permeabilizes the cell membrane. To gain an understanding of the role of LPS binding in the bactericidal action of CP1, kinetic dansyl PMB displacement assays were performed. This method probes the electrostatic interaction of cationic peptides with LPS and has been used to investigate LPS binding of a number of peptides including defensins [17], cecropin A-melittin hybrids [2,24], and polymyxin B [25]. Dansyl PMB fluoresces intensely when bound to LPS and only weakly in solution. Thus, displacement by the addition of peptide causes a reduction in fluorescent signal, directly measuring peptide binding to the anionic membrane. Displacement was evaluated over time to determine the rate and extent of 1.5, 3, and 6 μM CP1 binding to LPS (Figure 5). All concentrations of CP1 evaluated exhibited equivalent minimal displacement (~5%) over 2 h. After 6 h, 1.5, 3, and 6 μM CP1 exhibited ~19%, ~20%, and ~23% displacement, respectively (data not shown). The lack of concentration-dependent LPS binding has previously been observed for CP1. Lad *et al*. [26] reported that CP1 accumulated on the anionic lipid layer but did not exhibit increased interaction at concentrations above 0.7 μM.

Although it is unclear what role electrostatic binding plays during the lytic action of CP1, it appears that only the immediate interaction is important, which is presumed to dictate the level of initial permeability. Dathe *et al*. [27] have shown that cationic peptides interact with cell membranes both electrostatically and hydrophobically. It is possible the initial interaction of CP1 is more hydrophobic in nature, which may not be detectable by the dansyl PMB displacement assay. Dansyl PMB electrostatically bridges the LPS molecules resulting in displacement of divalent cations [28]. The cationic peptide (or any positively charged molecule) can disrupt the weak association, displace the dansyl molecules, and bind in their place. It may be that primarily hydrophobic interactions drive the permeability before the electrostatic binding behavior is exhibited.

An interesting aspect to the LPS-binding studies is that binding continues to increase over time, which at the MIC occurs after bactericidal action is complete. It is generally accepted that upon transient hole formation, peptide molecules line the hole, gaining access to the membrane causing bilayer disruption, micelle formation, and ultimately cell death [14,29]. Recently, investigations with phospholipid vesicles showed that only a minimal amount of peptide translocated into the pore while the majority remained on the surface [30]. The surface molecules were able to disassociate and interact with other vesicles in solution. Peptide disassociation and association with cell debris and membrane components after cell death may explain why

LPS binding continues to occur in our assays, beyond the time of bactericidal action of the peptide.

In correlating CP1 kinetics of killing, membrane permeability, and LPS binding, it is evident that CP1 bactericidal action is driven by the immediate cell permeability. However, it is difficult to envision how permeability can be concentration-dependent because the binding does not appear so. It is well accepted that CP1 accumulates parallel to the bilayer surface [12,14,29]. At a critical concentration, CP1 undergoes a conformational change to an α-helical structure with defined hydrophobic and polar regions on opposing sides of the helix. Chen *et al*. [31] have shown that peptides can self-associate in solution through their hydrophobic regions in the conformed α-helical state. It is possible that greater than or equal to the MIC, CP1 self-associates on the membrane during initial interaction with LPS, thus preventing further displacement in the LPS-binding assays. With increasing peptide concentration, self-association may cause peptide stacking, creating additional stress on the cell membrane and thus inducing greater immediate permeability. Below the MIC, insufficient peptide molecules may be present to self-associate and create the stress required for sufficient immediate permeability to prevent cell growth and induce lysis, as seen in our studies.

Here, we directly correlated CP1 bactericidal behavior with membrane permeability and LPS binding. The role binding plays is unclear; however it does appear that only the immediate interaction is important, because binding continued to increase over time beyond cell viability. Furthermore, it was determined that membrane permeability alone is not sufficient for lysis. The time to maximum permeability, as dictated by the immediate permeabilizing action, is the mediator of the bactericidal action and critical in preventing cell recovery and subsequent outgrowth.

Conclusions

The interaction of CP1 with *E. coli* was investigated to correlate membrane permeation and LPS binding to the kinetics of lysis. At the MIC (3 μM), maximum permeability correlated well with bactericidal kinetics, with both occurring at $<$30 min. Maximum permeability was also achieved below the MIC, suggesting that simply reaching the maximum does not assure bactericidal action. The extent of immediate permeability, rather than the rate to reach maximum permeation, is the key mediator for bactericidal action. The quantity of LPS binding required for membrane permeation is not known, and it is inconclusive as to the role binding plays. However, this research indicates that only the initial interaction is important, which is presumed to dictate the level of immediate permeability. Additional studies are needed regarding hydrophobic interactions and their role in the onset of the permeabilizing behavior. This research furthers the understanding of the time-dependent antimicrobial action of CP1.

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