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Different modes in antibiotic action of tritrpticin analogs, cathelicidin-derived Trp-rich and Pro/Arg-rich peptides

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Abstract

The cathelicidin-derived antimicrobial tritrpticin could be classified as either Trp-rich or Pro/Arg-rich peptide. We recently found that the sequence modification of tritrpticin focused on Trp and Pro residues led to considerable change in structure and antimicrobial potency and selectivity, but their mechanisms of microbial killing action were still unclear. Here, to better understand the bactericidal mechanisms of tritrpticin and its two analogs, TPA and TWF, we studied their effect on the viability of Gram-positive *S. aureus* and Gram-negative *E. coli* in relation to their membrane depolarization. Although TWF more effectively inhibited growth of *S. aureus* and *E. coli* than TPA, only a 30 min exposure to TPA was sufficient to kill both bacteria and TWF required a lag period of about 3–6 h for bactericidal activity. Their different bactericidal kinetics was associated with membrane permeabilization, i.e., TWF showed negligible ability to depolarize the cytoplasmic membrane potential of target cell membrane, whereas we observed significant membrane depolarization for TPA. In addition, while TPA caused rapid and large dye leakage from negatively charged model vesicles, TWF showed very little membrane-disrupting activity. Interestingly, we have looked for a synergism among the three peptides against *E. coli*, supporting that they are working with different modes of action. Collectively, our results suggest that TPA disrupts the ion gradients across the membrane, causing depolarization and a loss of microbial viability. By contrast, TWF more likely translocates across the cytoplasmic membrane without depolarization and then acts against one or more intracellular targets. Tritrpticin exhibits intermediate properties and appears to act via membrane depolarization coupled to secondary intracellular targeting.

Keywords: Cathelicidin; Tritrpticin; Membrane depolarization; Intracellular target; Synergy

1. Introduction

In mammals, endogenous antimicrobial peptides are small molecules that have the capacity to kill and/or inhibit a broad range of microorganisms, including Gram-positive and Gram-negative bacteria and fungi [1–4]. The primary and secondary granules of neutrophils contain a variety of these peptides, which represent important components of the innate host defense [5,6]. One major class of these peptides, the cathelicidins, appears to be expressed in response to cytokines produced early during infection [7–9]. Endogenous cathelicidins share strongly conserved N-terminal pre-proregions and highly varied C-terminal domains, which correspond to their antimicrobial properties. Two hypotheses have been proposed to explain the mechanism of antimicrobial action of cathelicidin-derived peptides: some appear to have the ability to permeabilize the microbial cytoplasmic membranes, while others appear to cross the cell membrane and interact with intracellular targets [10–12]. For example, BMAP-27 [13] and PMAP-23 [14], amphipathic α-helical peptides, are membrane-active and kill microorganisms through membrane permeabilization. By contrast, PR-39 [15] and Bac7 [16], Pro-rich peptides are not linked to membrane disruption and both inhibit bacterial DNA synthesis or cause a rapid reduction in RNA and protein synthesis.

The cathelicidin-derived antimicrobial peptide tritrpticin, which was first identified through screening a porcine bone marrow

Abbreviations: PP-II, polyproline type II; CD, circular dichroism; MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration; CFU, colony forming units; $diSC_3(5)$, 3,3′-dipropylthiacarbocyanine; LPS, lipopolysaccharide; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; LUVs, large unilamellar vesicles; SDS, sodium dodecyl sulfate

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cDNA library [17], assumes a unique amphipathic turn structure when bound to SDS micelles [18]. The primary structure of tritrpticin is remarkable by virtue of its high content of Arg, Trp and Pro residues, which are crucial for its interaction with phospholipids. Several studies aimed at clarifying the antimicrobial action of tritrpticin have been carried out, but their results reveal no consistent mechanism. In one study Nagpal et al. [19] proposed that tritrpticin adopts a β-turn structure in aqueous buffer and undergoes functional activation through a conformational transition as an initial event in bacterial killing. By contrast, Schibli et al. [18] reported that while tritrpticin shows a disordered structure in Tris–HCl buffer, the peptide adopts an amphipathic turn structure in SDS micelles, so that its antibacterial action might involve nonspecific interactions with the cell membrane. In addition, the results of a recent electrophysiological study by Salay et al. [20] suggested that tritrpticin has channel-like activity in azolectin planar lipid bilayers. Since there is much ambiguity concerning the mode of action of tritrpticin, it is of interest to clarify the structure-dependent membrane interaction and mode of antimicrobial action. In a recent study, we reported that apparent differences in their CD patterns induced by modifying the sequence of tritrpticin are closely related to pronounced changes in the molecule's activity and selectivity [21]. For example, the analog TPA, substituting the two Pro residues with Ala, adopted an α-helical structure, but not amphipathic turn structure, and exerted a slightly reduced antibacterial potency. In contrast, the analog TWF, in which Trp was replaced with Phe, showed a 2- to 4-fold increase in antimicrobial activity against Gram-negative bacteria and was as active as tritrpticin against Gram-positive bacteria. On the other hand, it showed significantly reduced activity against human erythrocytes, suggesting it is largely that Trp in some way mediates erythrocyte damage by these peptides. Still, the mechanisms of antimicrobial action of tritrpticin and its two analogs (TWF and TPA) remain unclear.

Here, we carried out a systematic analysis of the effects tritrpticin, TWF, and TPA on the viability of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, with the aim of better understanding their diverse mechanisms of action and providing a structural basis for the design of efficient low-molecular-weight peptide antibiotics.

2. Experimental procedures

2.1. Bacterial killing assays

Gram-positive *Staphylococcus aureus* (KCTC 1621) and Gram-negative *Escherichia coli* (KCTC 1682) were grown to mid-log phase at 37 °C in Luria Bertani medium. The cells were washed twice with 10 mM phosphate buffer (pH 7.4) and resuspended in the same buffer to give approximately 2×10^6 colony forming units (CFU)/ml. Peptides were then added to give a 64 μg/ml peptide concentration and 10^6 CFU/ml. This mixture was incubated at 37 °C, and the viabilities of the cells were estimated at timed intervals. After 200-fold dilution with 10 mM phosphate buffer, 100 μl aliquots were spread on heart infusion agar plates and incubated for 20 h to allow full colony development. The resultant colonies were counted and the killing rate was determined by plotting the log CFU/ml against time. The results of three independent experiments were averaged.

2.2. Membrane depolarization assay

Measurements of cytoplasmic membrane depolarization were made using a membrane potential-sensitive probe, 3,3′-dipropylthiacarbocyanine [diSC$_3$(5)] as previously reported [22]. *Staphylococcus aureus* and *E. coli* cells were grown at 37 °C to mid-log phase, centrifuged (3500 rpm, 7 min) and washed with 5 mM HEPES buffer (pH 7.2) containing 20 mM glucose and resuspended in buffer (5 mM HEPES buffer, 20 mM glucose, 100 mM KCl, pH 7.2) to an OD_{600} of 0.05. Changes in fluorescence due to the collapse of the cytoplasmic membrane potential were continuously monitored at 20 °C using a Shimadzu RF-5301 spectrofluorometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. When the dye uptake was maximal, as indicated by a stable reduction in fluorescence due to quenching of the accumulated dye in the membrane interior, peptides were added to the cells. Complete collapse of the membrane potential for *S. aureus* and *E. coli* was obtained using gramicidin D (0.22 nM), which forms ion channels in the cytoplasmic membrane of both bacteria, thereby dissipating the membrane potential. Measurements were repeated two times under each condition to ensure reproducibility.

2.3. Peptide-induced dye leakage from large unilamellar vesicles

Large unilamellar vesicles (LUVs) within which dye was entrapped were prepared by hydrating a POPC/POPG (2:1) mixture with dye solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) as previously described [23]. The mixed solution was put through five freeze–thaw cycles and then extruded 20 times through polycarbonate filters (Liposo-Fast; pore diameter, 100 nm). Untrapped dye was then removed by gel filtration chromatography on a Sephadex G-50 column. The fluorescence intensity of the calcein released from the vesicles was monitored at 520 nm (excited at 490 nm) on a Shimadzu RF-5301 spectrofluorometer. To obtain 100% leakage, 10% w/v Triton X-100 (20 μl) was added to dissolve the vesicles. Measurements were repeated three times under each condition.

2.4. Synergistic activity

To examine the synergistic actions of tritrpticin and its analogs, the peptides and bacteria (10^6 CFU/ml) were incubated in 10 mM phosphate buffer (pH 7.4) for 15 min at 37 °C and then spread on agar plates and incubated for 20 h. The resultant colonies were counted, and synergic effects of the peptides on antibacterial activity was expressed as killing (%) using the following formula: killing (%) = [1 − (number of colonies in the presence of peptides/number of colonies in the absence of peptides)] × 100. The recorded killing (%) was the average from three independent assays. In addition, the fractional inhibitory concentration (FIC) was determined using checkerboard titrations [24], in which one peptide was diluted (2-fold serial dilution in 1% peptone) along the rows of 96-well microtiter plates and the other was diluted along the