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The importance of cyclic structure for Labaditin on its antimicrobial activity against *Staphylococcus aureus*

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ABSTRACT

Antimicrobial resistance has reached alarming levels in many countries, thus leading to a search for new classes of antibiotics, such as antimicrobial peptides whose activity is exerted by interacting specifically with the microorganism membrane. In this study, we investigate the molecular-level mechanism of action for Labaditin (Lo), a 10-amino acid residue cyclic peptide from *Jatropha multifida* with known bactericidal activity against *Streptococcus mutans*. We show that Lo is also effective against *Staphylococcus aureus* (*S. aureus*) but this does not apply to its linear analogue (L_1). Using polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS), we observed with that the secondary structure of Lo was preserved upon interacting with Langmuir monolayers from a phospholipid mixture mimicking *S. aureus* membrane, in contrast to L_1. This structure preservation for the rigid, cyclic Lo is key for the self-assembly of peptide nanotubes that induce pore formation in large unilamellar vesicles (LUVs), according to permeability assays and dynamic light scattering measurements. In summary, the comparison between Labaditin (Lo) and its linear analogue L_1 allowed us to infer that the bactericidal activity of Lo is more related to its interaction with the membrane. It does not require specific metabolic targets, which makes cyclic peptides promising for antibiotics without bacteria resistance.

1. Introduction

Staphylococcus aureus is a Gram-positive bacteria that can cause several human illnesses such as skin infection, pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS) and sepsis [1]. *S. aureus* is also the major cause of deadly bacteremia, much more aggressive than caused by other pathogens [2]. The treatment of these diseases is fraught with problems owing to the development of bacterial resistance. A classical case is the methicillin-resistant Staphylococcus aureus (MRSA), a strain of *Staphylococcus* bacteria that became resistant to antibiotics used for treating ordinary infections. This calls for the discovery of new antibiotics whose mechanism of action differs from the conventional ones, normally related to specific metabolic targets [3].

Antimicrobial peptides (AMPs) are promising as the next generation of antibiotics [4,5], for they act on bacterial membranes and other generalized targets, thus making bacterial resistance unlikely [6]. AMPs develop an immunological role in a variety of plant and animal species, protecting these organisms from pathogen invasion [7–9]. They are able to differentiate cell membranes from eukaryotic to prokaryotic organisms [10], mostly due to their being usually positively charged and binding preferentially to anionic membranes, such as bacterial surfaces [11,12]. The mechanism of action of linear AMPs is well documented, including several models of membrane permeabilization/disintegration. On the other hand, not much is known about cyclic peptides [13], whose antibiotic activity depends on their conformational restrictions [14] and higher proteolytic stability compared to their linear analogues [15].

Labaditin (Lo) is a small head-to-tail cyclic AMP (VWTVWGTIAG) from *Jatropha multifida* with high hydrophobic content. In contrast to most of AMPs, it has no charged residues [16,17]. It has been proven effective against *Streptococcus mutans*, while its linear analogue (L_1) is not [17]. Also, no hemolytic effect was observed for

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concentrations up to 100 μM of Labaditin, indicating high selectivity of this peptide to microorganisms cells [17]. In this study, we designed a series of experiments to explain why Lo – but not L_1 - is active, for which we used *S. aureus* as the bacteria of reference. In addition to performing bactericidal activity tests, we investigated the interaction of Lo and L_1 with cell membrane models represented by Langmuir monolayers. Motivation for membrane studies arose from the hypothesis that bactericidal activity is directly correlated with interaction with the cell membrane. For elucidating the mechanism of action of Lo we also resorted to leakage assays and dynamic light scattering performed in large unilamellar vesicles (LUVs) to probe membrane permeabilization.

2. Materials and methods

2.1. MIC determination

S. aureus cells (ATCC 25923) were cultured in LB (Luria-Bertani) broth without shaking, and at a late exponential phase (OD_{600} = 1.0) the culture was diluted to OD_{600} of 0.1 and used as inoculum. Microdilution was performed using a series of 200 μL of LB broth and containing two fold serial dilution of one of the synthetic peptides (Labaditin Lo or its linear analogue L_1), which were prepared in the 96-well microtiter plates. Approximately 10^4 cells from the inoculum as described above were inoculated. The plates were incubated for 18 h at 37 °C, and the tests were performed in triplicate.

2.2. Langmuir monolayers

The peptides Lo and L_1 were obtained from Aminotech Research (>95% purity), while 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) and 14:0 Cardiolipin (CL) were purchased from Avanti Polar Lipids. All the solutions were prepared using Millipore Direct-Q ultra pure apyrogenic water (resistivity of 18.2 MΩ cm at 25 °C), and the reagents were of the highest commercially available purity grade. For producing Langmuir monolayers, we simulated the phospholipid composition of *S. aureus* with 55% DOPG and 45% CL, according to the literature [18–20], named from now on *S. aureus* PL. It is worth mentioning that we do not neglet the importance of lipid composition, with the presence of branched lipids for example, for the bacterial susceptibility against antimicrobial peptides [33]. However, we decided to focus our efforts on determining the role of peptideís cyclic structure on its interaction with the membrane, taking into account that many Gram-positive bacteria contain mostly anionic lipids [34]. The stock solution was prepared in chloroform:methanol (4:1 v/v). Surface pressure isotherms were measured in a mini-KSV Langmuir trough (KSV Instruments Ltd, Helsinki, Finland) equipped with a Wilhelmy plate made of filter paper, at 21 °C. The isotherms were obtained by spreading 50 μL of the lipid from a stock solution at 627 μM on the air/water interface. Prior to the π-A isotherms, adsorption kinetics of the peptide at different concentrations were obtained on the lipid monolayer (at null surface pressure). The surface pressure was monitored with time to ensure adsorption had reached equilibrium before compressing the monolayer. Compression was carried out using two movable barriers at 10 cm^2 min^{-1}. Surface pressure isotherms were performed in triplicate, and the maximum error found was 3 $Å^2$/molecule.

Polarization-Modulated Infrared Reflection-Absorption Spectroscopy (PM-IRRAS) measurements were performed using a KSV PMI 550 instrument (KSV Instruments Ltd, Helsinki, Finland) in a mini KSV Langmuir trough. The light beam reached the monolayer at a fixed incidence angle of 81°, for which the upward-oriented bands indicate a transition moment preferentially parallel the surface plane, whereas downward bands indicate orientation perpendicular to the surface. All the experiments were carried out in a clean room at 21.0 ± 0.1 °C. The experimental setup was the same used above in Langmuir monolayers. In these PM-IRRAS experiments, we used the highest concentration of each peptide to amplify the band signal. Spectra were collected every 5 mN/m of surface pressure.

2.3. Carboxyfluorescein (CF) release from LUVs

For the leakage assays, LUVs were prepared with *S. aureus* PL at a concentration of 15 mM. The mixture of lipids was dried under a N_2 stream and left in vacuum for 6 h to form a lipid film. First, multilamellar vesicles were obtained by mechanical stirring with a 30 mM HEPES buffer solution, pH 7.4, with 50 mM CF and 86 mM glucose, added to adjust the solution osmolarity. The suspension was then extruded to get vesicles with uniform size, using a polycarbonate porous membrane to render 100 nm unilamellar vesicles. This solution was eluted by size-exclusion chromatography through a Sephadex G-50 column to remove the free CF outside the vesicles, using 30 mM HEPES buffer, pH 7.4, with 100 mM NaCl. The CF-LUVs were collected in tubes, diluted and the phospholipid concentration was determined by phosphorous analysis according to the methodology by Rouser et al. [35]. The fluorescence emission of CF was monitored at λ = 517 nm, with excitation at λ = 492 nm (slit widths 5 nm), using a spectrofluorometer (Cary Eclipse, Varian). Different concentrations of peptide (Lo and L_1) were added to the CF-LUVs suspension. At the end of each experiment Triton X-100 (1% v:v) was added for the release of all CF. The percentage of CF leakage was calculated with the equation: 100(Ft−Fo)/(Fmax−Fo), where Ft is the fluorescence at a given time, Fo is the initial fluorescence (before addition of peptide), and Fmax is the maximum fluorescence after addition of Triton X-100 [36–38]. All experiments were performed in triplicate.

Dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer Nano S equipped with a 633 nm laser and a detector at 173° backscattering angle operating at 25 °C. Carboxyfluorescein-entrapped LUVs (100 nm, 1 mL, 15 mM) prepared with the phospholipid composition of the *S. aureus* was incubated with Lo and L_1 (0.071 μM) peptides for 10 min. Particle size distributions were analysed using the intensity distribution. All experiments were performed in triplicate.

3. Results and discussion

3.1. Bactericidal activity

Lo and L1 bactericidal activity were evaluated against *S. aureus* ATCC 25923. For the cyclic peptide, no bacterial growth was observed, even for concentrations as low as 0.23 μM, indicating high activity against *S. aureus*. For the linear analogue, L1, the ability to kill *S. aureus* was observed for concentrations above 28.6 μM. These data show that Lo is a potent antibiotic against *S. aureus*, with considerably higher activity than its linear analogue L1. In order to understand the importance of the cyclic structure on the bactericidal activity at the molecular level, Langmuir monolayers were used to mimic the bacterial membrane.

3.2. Surface pressure-area isotherms

The peptides Lo and L_1 adsorbed on a bare air/water interface to form Gibbs films, whose isotherms are shown in Fig. S1 of the Supporting information for an injected concentration of 0.071 μM and after waiting 6 h for the adsorption. The linear nature of L_1 leads to a more condensed film, with higher collapse pressure (25 mN/m)