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The enigmatic mode of action of the lantibiotic epilancin 15X

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ABSTRACT

Epilancin 15X is a lantibiotic that has an antimicrobial activity in the nanomolar concentration range towards *Staphylococcus simulans*. Such low MICs usually imply that these peptides employ a mechanism of action (MoA) involving high affinity targets. Here we studied this MoA by using epilancin 15X's ability to dissipate the membrane potential of intact *S. simulans* cells. These membrane depolarization assays showed that treatment of the bacteria by antibiotics known to affect the bacterial cell wall synthesis pathway decreased the membrane depolarization effects of epilancin 15X. Disruption of the Lipid II cycle in intact bacteria using several methods led to a decrease in the activity of epilancin 15X. Antagonism-based experiments on 96-well plate and agar diffusion plate pointed towards a possible interaction between epilancin 15X and Lipid II and this was confirmed by Circular Dichroism (CD) based experiments. However, this interaction did not lead to a detectable effect on either carboxyfluorescein (CF) leakage or proton permeability. All experiments point to the involvement of a phosphodiester-containing target within a polyisoprene-based biosynthesis pathway, yet the exact identity of the target remains obscure so far.

1. Introduction

Bacterial resistance to traditional antibiotics is a growing concern. Therefore, there is an urgent demand for substitutes. Peptides from the family of lantibiotics exhibit high antibacterial activity against a broad spectrum of gram-positive bacteria including several antibiotic-resistant pathogens [1]. Lantibiotics are a type of peptide antibiotics with an extremely low minimal inhibitory concentration (MIC). They belong to bacteriocin Class I and are ribosomally synthesized peptides produced by Gram-positive bacteria [2,3]. These peptides contain the dehydrated amino acids 2, 3-didehydroalanine (Dha) and 2, 3-didehydrobutyrine (Dhb) as well as lanthionine (Lans) and/or methyllanthionine (MeLans) residues that form the typical thio-ether linked rings (Fig. 1). These rings and unnatural amino acids are formed by post-translation modification [1]. The low MIC of the lantibiotics implies that they use targets with high affinity on the bacterial cells for their efficient killing. The most well-known lantibiotic nisin, uses the essential bacterial cell wall precursor, Lipid II, to efficiently kill bacteria by forming pores in the cytoplasmic membrane [4,5]. There are several lantibiotics with unknown MoAs [6]. One example of such a lantibiotic is epilancin 15X, which has an antibacterial activity in the nanomolar concentration range towards *S. simulans*. Elucidating the mechanism of action of epilancin 15X may provide novel insight in tackling the problematic multiresistant staphylococci.

Epilancin 15X is produced by *Staphylococcus epidermidis* 15X154 and was isolated and structurally characterized in 2005 (Fig. 1A) [7]. It contains 31 amino acid residues including 10 modified residues and 3 lanthionine rings with a molecular weight of 3172.75 Da [7]. Epilancin 15X is a highly positive charged lantibiotic that has 8 positively charged amino acids, and those amino acids probably play an important role in supporting electrostatic interactions between the cationic peptides and components of the bacterial envelope, such as the negatively charged membrane lipids. Moreover, epilancin 15X acts against several antibiotic-resistant pathogens including vancomycin-resistant *Enterococci* (VRE) and methicillin-resistant *Staphylococcus. aureus* (MRSA) displaying low MICs in the range of 0.125–0.5 μg/mL [8,9], which implies that epilancin 15X targets a special binding site different from vancomycin and methicillin.

Epilancin K7 highly resembles epilancin 15X. It has 31 amino acid residues, including 10 modified residues and 3 lanthionine rings similar to epilancin 15X, and a molecular weight of 3033 Da (Fig. 1B) [10]. Interestingly, the C-terminal half of the structures of epilancin 15X and

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K7 starting from residue 17 are very similar to the C-terminal half of nisin (starting from residue 20) (Fig. 1C). This C terminal part of nisin including the hinge region and rings D/E has been suggested to be important for pore-formation [11]. Additionally, the rings D and E of nisin have been implicated in recognition by NisFEG, proteins that are important for the self-protection of nisin-producing strains [12]. Hence, the B/C rings of the epilancins might have a similar function. Notably, the epilancins do not have the A/B-ring system of nisin that is responsible for binding to Lipid II. Indeed, for epilancin K7, earlier results showed that it could not induce leakage from model membranes containing Lipid II, which led the authors to conclude that it does not target Lipid II [13].

Lipid II is an important precursor within the bacterial peptidoglycan biosynthesis pathway, which harbors many potential targets of antibiotics especially in Gram-positive bacteria. The peptidoglycan (PG) layer is a main constituent of the Gram-positive cell wall together with the wall teichoic acids (wTA) that are embedded within this PG-layer [14,15]. The PG synthesis steps start with the synthesis of Lipid I on the cytosolic side of the plasma membrane from UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and undecaprenyl phosphate (C55-P). This step is catalyzed by the integral membrane protein MraY [16]. Then, an *N*-acetyl-D-glucosamine (GlcNAc) moiety of UDP-GlcNAc is transferred to Lipid I by MurG to produce Lipid II [5]. Lipid II is flipped to the external leaflet of the plasma membrane and polymerized and linked to the existing PG-layer by penicillin binding proteins (PBPs) [17–20]. The C55-PP that is resulting from the Lipid II polymerization step is dephosphorylated to C55-P by BacA and the C55-P is recycled to the cytosolic side of the plasma membrane to complete the cycle [21]. The wTA synthesis pathway is connected with PG synthesis via the shared use of the precursor C55-P. There are two main types of wTA known, the poly(glycerol-phosphate) based wTA and the poly(ribotol-phosphate) based that have very similar biosynthesis pathways. What follows is a description of the poly(glycerol-phosphate) based wTA synthesis pathway of *Bacillus subtilis*. The first teichoic acid synthesis step on membrane starts with the synthesis of lipid α from UDP-GlcNAc and C55-P, a step catalyzed by the integral membrane enzyme TagO, a paralogue of MraY [22]. Then a *N*-acetylmannosamine (ManNAc) moiety from UDP-ManNAc is transferred to lipid α to produce a lipid β by TagA [23]. TagB adds an sn-glycerol-3-phosphate unit from CDP-glycerol to lipid β to form lipid φ.1 and more glycerol-phosphate units (n) are polymerized onto lipid φ.1 by TagF to form lipid φ.n [24,25]. Lipid φ.n is modified with glucose units by TagE before it is transferred to the external leaflet of the plasma membrane, presumably by TagGH [26,27]. Finally, the wTA polymer is D-alanylated and transferred from its polyisoprenoid anchor to the PG layer [28]. The remaining, C55-PP is dephosphorylated and recycled as described above.

In this research, we used *S. simulans*, *M. flavus* and *B. subtilis* to study effects of antibiotics that target the PG and wTA biosynthesis pathways on the activity of epilancin 15X. Precursors, enzymes, and products of the above described biosynthesis pathways are popular targets for many antibiotics. For instance, nisin binds to peptidoglycan precursor Lipid II; Bacitracin binds to C55-PP; penicillin binds to the transpeptidation domain and moenomycin binds to the transglycosylation domain of PBPs. Targocil targets wTA translocase TarGH and tunicamycin inhibits MraY as well as TagO of both pathways, albeit with different efficacies [29–37].

This research explores potential targets of epilancin 15X by tackling precursors and enzymes that are involved in the pathways involved in the bacterial cell wall synthesis. The results point to a target that possesses a phosphodiester and functions within a polyisoprenoid-based biosynthesis pathway.

2. Materials and methods

2.1. Materials

Nisin A and Lipid II (DAP/Lys) was prepared as previously described [11,38]. Nisin(AB)-C14 was obtained from Nathaniel I. Martin (Leiden University). The TagO mutant of *B. subtilis* was obtained from Richard Daniel (The Centre for Bacterial Cell Biology, Newcastle University).

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-(cytidine diphosphate) (CDP-DAG) were purchased from Avanti Polar Lipids. Carboxyfluorescein (CF) was obtained from Molecular Probes. $DiSC_2(5)$, HPTS were purchased from Sigma-Aldrich. G-25 Sephadex and G-50 Sephadex were from Amersham Pharmacia Biotech AB. Tween-20 was purchased from Merck. Triton X-100 was purchased from Sigma-Aldrich. All other chemicals used were of analytical or reagent grade.

2.2. General procedures

The concentration of peptides was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher). The tubes used were siliconized by SurfaSil Siliconizing Fluid (Thermo Scientific™) using Siliconizing Fluids. All fluorescence related experiments were measured by a Cary Eclipse fluorescence spectrophotometer.

2.3. Bacterial strains and culture conditions

Epilancin 15X producer strain *Staphylococcus epidermidis* 15X154 [39] was grown on Muller-Hinton broth at 37 °C. Indicator strain *S. simulans* 22 was grown on Tryptic Soy Broth (TSB) at 37 °C [11].

2.4. Epilancin 15X purification

The strain *Staphylococcus epidermidis* 15X154 was inoculated in 60 mL Muller-Hinton broth at 37 °C and 200 rpm overnight. The pre-

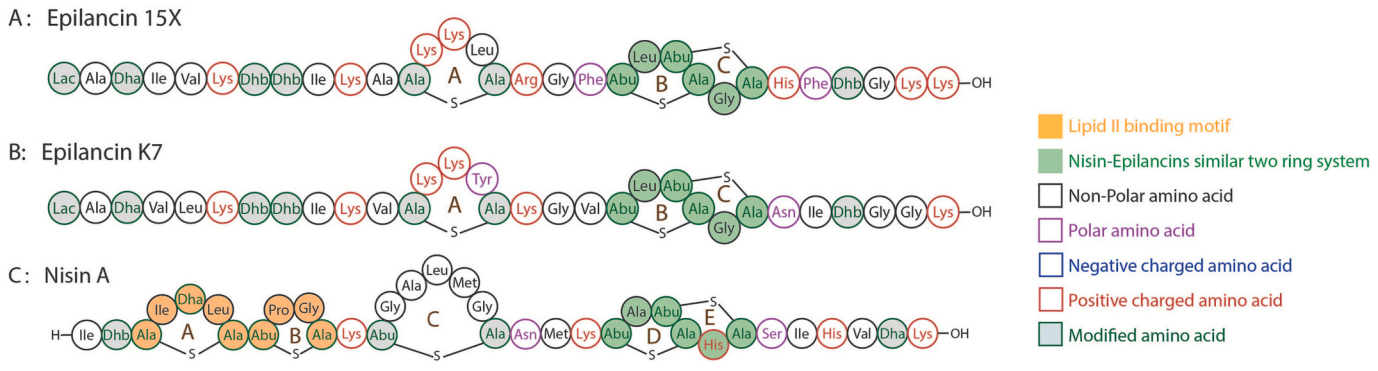

Fig. 1. Structures of the lantibiotics epilancin 15X, epilancin K7, nisin A. The lipid II binding motif is highlighted in orange. The similar two C-terminal ring systems are highlighted in green.

culture was transferred into sterile 6 × 1 L TSB in 2 L Erlenmeyer flasks and grown for 18 h at 37 °C aerobically (1 % inoculum). The cells were removed by centrifugation at 8000 ×*g* for 20 min at 4 °C, and the supernatant was sterilized using a 200 nm filter. The supernatant was applied to a cation exchange column (SP Sepharose Fast Flow) which was equilibrated with 20 mM sodium acetate, pH 5. Epilancin 15X was eluted using a linear gradient from 100 mM to 1 M NaCl in 20 mM sodium acetate pH 5. Fractions containing antibacterial activity were collected and applied to a RP-18 column (LiChrosprep, 40–63 μm of 23.5 mm × 50 mm, d x h) and epilancin 15X was eluted by a step gradient of 10 % methanol, 50 % methanol, 90 % methanol, and 95 % acetonitrile in water. Finally, epilancin was purified from the fractions containing antimicrobial activity using a C18 RP-HPLC column (LiChrospher, 250 mm × 4.6 mm, 5 μm) equilibrated in eluent A (5 % acetonitrile, 95 % water, 0.05 % TFA) and using a linear gradient from 0 % to 100 % eluent B (95 % acetonitrile, 5 % water, 0.05 % TFA) in 30 min (flow rate at 0.8 mL/min, UV detection at 214 nm). Epilancin 15X appeared as a broad peak at ~90 % B (Fig. S1).

To reach maximal activity of epilancin 15X it was necessary to remove the peptide-bound TFA. For this, 13.5 μL of 1 M HCl was added for every 100 mL epilancin 15X solution containing 0.01 % TFA. Hereafter, the sample was evaporated at 40 °C to dryness and the peptide redissolved in 20 mL 50 % Methanol 50 % water and again evaporated. This last step was done a total of three times. Finally, epilancin 15X was dissolved in water and freeze-dried for 24h. The molecular weight was determined by Mass Spectroscopy, and epilancin appeared in 4 main peaks of 636.2 (5 H^+), 794.8 (4 H^+), 1059.5 (3 H^+) and 1588.4 (2 H^+) (Thermo Finnigan) (Fig. S2).

2.5. Minimal inhibitory concentration (MIC) determination

The minimal concentration of epilancin 15X that did not allow *S. simulans* growth after 24 h was defined as the MIC. This was determined using 1 mL cultures of *S. simulans* at a start OD_{600} of 0.05 in TSB broth containing a serial dilution of epilancin 15X in sterilized and siliconized glass tubes. The tubes were shaken at 37 °C, 200 rpm and the OD_{600} was read after incubation for 24 h. Experiments were repeated three times.

2.6. Membrane potential depolarization assay

The fluorescent dye 3,3′-diethylthiadicarbocyanine iodide $DiSC_2(5)$ (excitation at 650 nm and emission at 670 nm) was used to inspect the ability of epilancin 15X to dissipate the membrane potential of *S. simulans* [40]. Precultures of *S. simulans* were grown at 37 °C, 200 rpm overnight, and then diluted to an OD_{600} of 0.05 with fresh TSB medium. The culture was further grown for 4 h and spun down at 4000 ×*g* for 20 min at 4 °C. The cells were washed twice with 250 mM glucose, 5 mM $MgSO_4$, 100 mM KCl, 10 mM potassium-phosphate buffer at pH 7 (buffer A). The cells were then resuspended to an OD_{600} of 5. From this cell suspension 10 μL was added into a cuvette containing 1 mL buffer A, followed by the addition of 2 μL of a stock solution of 0.1 mM $DiSC_2(5)$ dissolved in DMSO. Antibiotics were added after a stable curve was obtained and at the end of the experiment Triton X-100 was added to a final concentration of 0.2 % to fully dissipate the membrane potential. Experiments were repeated three times.

2.7. Preincubation of S. simulans with vancomycin

Precultures of *S. simulans* were grown at 37 °C, 200 rpm overnight, and then diluted to an OD_{600} of 0.05 with fresh TSB medium. The culture was further grown for 4 h and diluted to OD_{600} of 0.05 again with fresh TSB medium. The diluted *S. simulans* were divided into 3 tubes containing 30 mL of bacteria. Then 10 μM (final concentration) of vancomycin was added to each tube. Cells in the first tube were immediately centrifuged and washed twice with buffer A. The pellet was put on ice. The second and the third tubes were incubated at 37 °C, 200 rpm for 5 and 10 min respectively, and were then centrifuged and washed as for the first sample and stored on ice. Cells from the three tubes were then resuspended to an OD_{600} of 5. From this cell suspension 10 μL was added into a cuvette containing 1 mL buffer A for the membrane potential assay. Experiments were repeated three times.

2.8. Agar diffusion assay

A preculture of *S. simulans* was grown at 37 °C, 200 rpm overnight. 100 μL of pre-cultured *S. simulans* was added into 10 mL 0.6 % agar TSB medium at around 50 °C. After the plate became solid, 10 μL of samples were spotted on the agar. The plate was incubated at 37 °C overnight.

The ability of Lipid II to antagonize antibiotic activity was also tested using an agar diffusion assay, normally used to determine antibiotic activity. 20 μL of epilancin 15X and nisin stock solution (20 μM) were added on 1 % pre-cultured *S. simulans* containing agar plate, and it was incubated at 30 °C overnight. Then the diameters of inhibition haloes were measured. Subsequently, Lipid II-Lys, lipid II–DAP (0.02 nmol, 0.1 nmol and 0.2 nmol dissolved in 0.1 % Tween-20) and DOPG (0.04 nmol, 0.2 nmol, 0.4 nmol dissolved in 0.1 % Tween-20) were spotted on a new 1 % pre-cultured *S. simulans* containing agar plate at a distance from the planned antibiotic spot that corresponds to the previously measured radius of the inhibition halo. The lipid spots were allowed to dry, which was then followed by spotting 20 μL of an epilancin 15X and nisin stock solution (20 μM). The plate was incubated at 30 °C overnight. Experiments were repeated three times.

2.9. Assay for antagonism by Lipid II in liquid medium

Overnight pre-cultured *S. simulans* was diluted by TSB medium containing 0.01 % Tween-20 to an OD_{600} of 0.05. The molecular ratios of lantibiotics (epilancin 15X and nisin) to Lipid II-Lys, Lipid II-DAP were varied from 1:0 to 1:10. DOPG was used as a control for electrostatic effect of concentration two times that of Lipid II. Lipids, lantibiotics and *S. simulans* were added into 96 well plate (Ultra-Low Attachment Surface, 3474 Corning) with the final volume of 200 μL each well. The 96 well plates were incubated at 37 °C and 200 rpm overnight. Experiments were repeated three times.

2.10. Model membrane vesicle preparation

Large unilamellar vesicles (LUVs) containing phospholipids (DOPC, DOPG) with/without Lipid II were prepared for CF leakage, proton permeability assay and direct binding assay. Lipids were dried by N_2 and were hydrated by adding 25 mM Tris-HCl, 150 mM NaCl, pH 7.5 (buffer B) also containing 50 mM CF for carboxyfluorescein leakage, or by adding buffer 0.2 M pH 7 potassium-phosphate containing 2 mM HPTS for proton permeability assay, or by adding buffer B for direct binding assay.

The LUVs were prepared in 10 freeze-thaw cycles in liquid nitrogen and extruded over a 200 nm filter for 10 times. The vesicles for CF leakage were passed through a 2.5 mL spin column filled with sephadex G50 which was equilibrated with buffer B. The vesicles for proton permeability assay were passed through a 2.5 mL spin column filled with sephadex G25 column which was equilibrated with 0.2 M pH 7 potassium-phosphate.

Small unilamellar vesicles (SUVs) containing phospholipids (DOPC, DOPG) with/without Lipid II were prepared for Circular Dichroism (CD) Measurements. Lipids were dried by N_2 and were hydrated by adding 20 mM HEPES, 40 mM Na_2SO_4, pH 7.4. The SUVs were prepared by sonication using a Branson 250 tip sonicator for about 5 min with 10-s time intervals and an input power of 40 W until the dispersions were clear. Residual particles of vesicles for direct binding assay were removed by centrifugation at 14000 rpm for 20 min at 4 °C.

The concentrations of phospholipids and vesicle preparations were

determined as described [41]. The phospholipids dispersed in phosphate buffer were extracted using the method described by Bligh and Dyer [42].

2.11. CF leakage assay using model membrane vesicles

LUVs with the fluorescent dye CF (excitation at 492 nm and emission at 515 nm) enclosed were used to measure the pore-forming ability of epilancin 15X [43]. LUVs were diluted with buffer B to a concentration of 5 μM in a 1 mL cuvette, followed by the addition of the lantibiotics after 30s. Maximum fluorescence was reached by adding Triton X-100 to a final concentration of 0.2 % to destroy all LUVs. Experiments were repeated three times.

2.12. Proton permeability assay using model membrane vesicles

Proton permeability assay was measured in LUVs, using the fluorescent signal of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) (excitation at 450 nm and emission at 508 nm) which is a type of pH-dependent fluorescent probe [44]. The LUVs were diluted in buffer 0.2 M pH 7 potassium-phosphate to a final concentration of 35 μM in 1 mL cuvette, and antibiotics were added after 1 min. Maximum fluorescence was reached by adding Triton X-100 to a final concentration of 0.2 %. Experiments were repeated three times.

2.13. CD measurements

CD spectra were measured with a Jasco-810 spectropolarimeter in a quartz cuvette with a 2 mm path length. The temperature was kept at 20 °C by a Jasco Peltier CDF 426S. The ellipticity was recorded between 190 nm and 260 nm at a 0.2-nm step size with 1-s response time. The spectra were averaged over 5 recordings with a scanning speed of 50 nm/min. Samples contained 50 μM epilancin 15X and buffer 20 mM HEPES, 40 mM Na_2SO_4, pH 7.4 was used for preparation and dilution. The lipid II containing SUVs were added to a final lipid II concentration of 50 μM. The DOPG containing SUVs were added to a final DOPG concentration of 100 μM. Experiments were repeated three times.

3. Results and discussion

3.1. Epilancin 15X has a low MIC indicating involvement of high affinity targets

In the course of our studies on the MoA of epilancin 15X, there were two major aspects that negatively affected the determination of the MIC value, glass adhesion and an inhibitory effect of TFA. Of note is the property of epilancin 15X to adhere to glass surfaces, hence it was essential that the glass tubes used during MIC determination were siliconized beforehand. Siliconization resulted in a reduction of the measured MIC towards *S. simulans* from 750 nM to 500 nM. The second, more severe effect was caused by the presence of TFA (as it is used in the HPLC-purification step) in the epilancin 15X solution, which significantly decreased the peptide's activity. Removal of the TFA from the peptide resulted in a 5-fold reduction of the measured MIC towards *S. simulans* from 500 nM to 100 nM. This large effect of TFA on the activity of epilancin 15X may be a general problem with HPLC-purified antimicrobial peptides that are highly positively charged.

These low MICs suggest that epilancin 15X uses high affinity targets in its mechanism of action. Previous studies showed that epilancin 15X clearly dissipated membrane potential of *S. simulans* at concentrations equal to its MIC, while it did not cause severe membrane disruption [45]. In view of this correlation, the effect on the membrane potential is likely to be related to its MoA and we therefore used this assay in our attempts to identify the target of epilancin 15X.

3.2. Epilancin 15X likely interacts with the cell wall synthesis precursor Lipid II

First, we performed the membrane depolarization assay with nisin and epilancin 15X at 200 nM and 500 nM, respectively, and a rapid dissipation of the membrane potential was observed (Fig. 2A), similar as was found before [45]. Nisin uses the essential cell wall precursor, Lipid II, as a target with which it efficiently forms pores [4] and we have shown before that vancomycin, an antibiotic that targets the D-Ala-D-Ala terminus of the pentapeptide of Lipid II, can inhibit the pore-forming activity of nisin [46]. Indeed, addition of vancomycin to the *S. simulans* cells shortly before the addition of nisin caused a significant inhibition of the ability of nisin to dissipate the membrane potential of these cells (Fig. 2B). As epilancin 15X does not have the archetypical A/B ring system of Lipid II targeting lantibiotics, we did not expect an effect of vancomycin on epilancin 15X. Indeed, addition of vancomycin shortly before the addition of epilancin 15X could not inhibit the membrane permeability of epilancin 15X, even at a two-fold higher concentration (Fig. 2C). The membrane depolarization effect of epilancin 15X was even slightly enhanced.

This picture completely changed when we used a truncated form of nisin which is composed of rings A and B fused to a lipid chain containing 14 carbon atoms (Nisin(AB)-C14) that, like nisin, binds to the pyrophosphate part of Lipid II but cannot form pores [47]. It was shown before that this truncated and acylated form of nisin can inhibit the Lipid II-dependent pore-formation of wild-type nisin in model membranes [47]. Fig. 2D shows that this truncated form of nisin could also inhibit the pore-formation of wild-type nisin in intact bacterial cells as a clear effect on the dissipation of the membrane potential by nisin could be observed (blue and green traces). Much to our surprise, we also observed an effect of nisin(AB)-C14 on the ability of epilancin 15X to dissipate the membrane potential of intact cells (Fig. 2D, orange and yellow traces). Nisin(AB)-C14 caused a clear inhibition of the activity of epilancin 15X, albeit that nisin was more sensitive to the acylated fragment as a higher inhibition could be observed at a two-fold lower concentration of the fragment. These results suggest that Lipid II may also be targeted by epilancin 15X despite that it lacks the archetypical A/B-ring system of Lipid II-targeting lantibiotics.

3.3. Epilancin interacts with Lipid II

To check whether epilancin 15X and Lipid II indeed interact, we first tested if Lipid II is able to antagonize the antibacterial action of epilancin 15X towards *S. simulans* in two different ways. In one of the approaches, we grew *S. simulans* cells in 96 wells plates in the presence of 5 times the MIC of nisin or epilancin 15X and increasing concentrations of Lipid II. In the case of nisin, bacterial growth could be observed at Lipid II concentrations above 225 nM. Hardly any difference could be observed between the lysine form of Lipid II or the diaminopimelic acid (DAP) form (Fig. 3A). Lipid II could also antagonize the antibacterial activity of epilancin 15X, albeit that the concentration of Lipid II needed for this antagonization effect was about 10-fold higher irrespective of the Lipid II variant used. This suggests that epilancin 15X and Lipid II do interact. DOPG, a control to test for electrostatic effects, also displayed antagonistic affects towards epilancin 15X at concentrations above 5 μM while DOPG did not affect the antibiotic activity of nisin.

As an alternative and independent approach, we tested the antagonization effect of Lipid II on epilancin 15X in an agar diffusion assay. For this we spotted different amounts of Lipid II at positions around the spot where the lantibiotic was applied. These positions were located at the predicted rim of the halo formed by the antibacterial action of the lantibiotic in the absence of any antagonist, which was determined using identical circumstances (e.g., amount of lantibiotic spotted, thickness of agar plate) (Fig. 3B) [6]. In case of nisin, clear deformations of the halo could be observed for amounts of Lipid II as low as 20 pmol. There did not appear to be a difference between the variants of Lipid II tested and

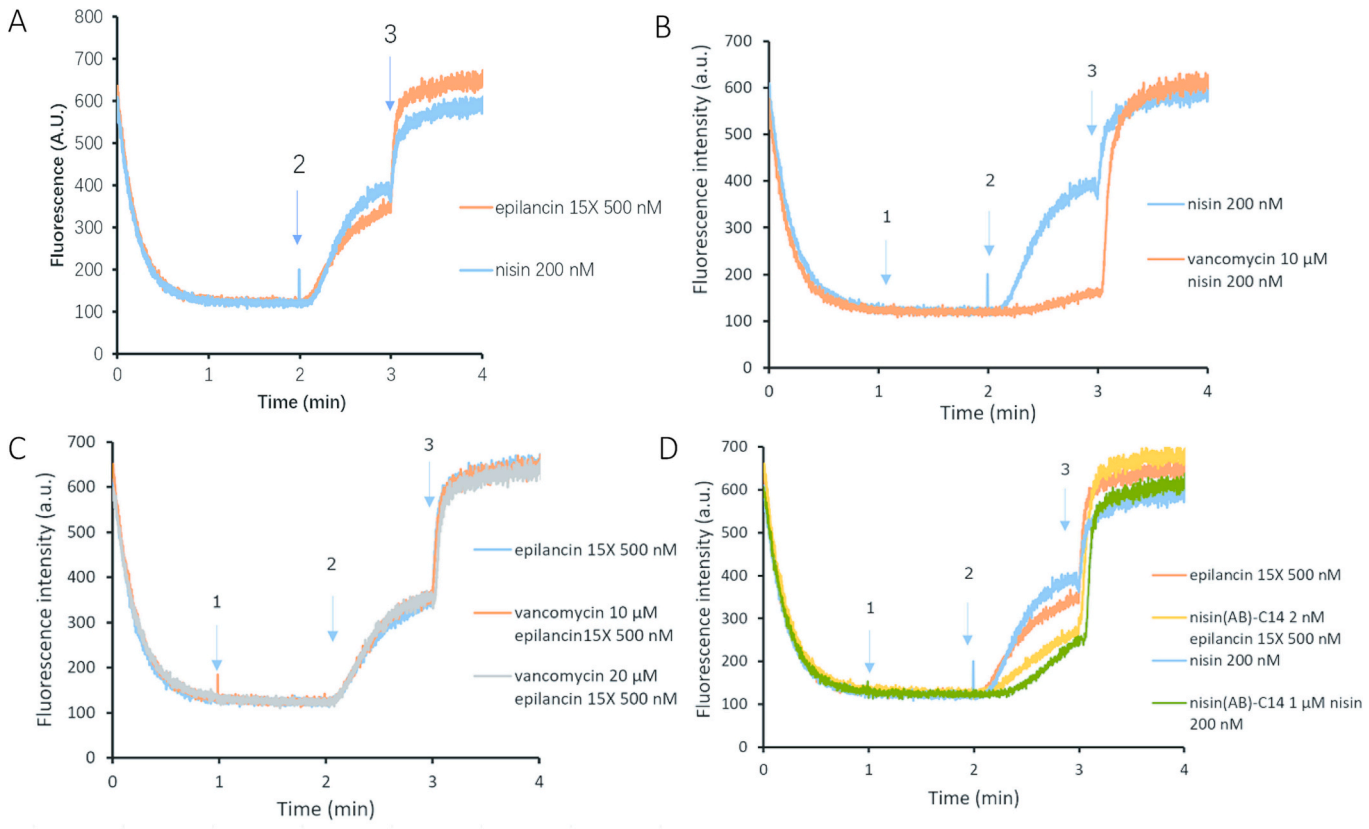

Fig. 2. Effect of addition of vancomycin and nisin(AB)-C14 on the ability of epilancin 15X and nisin to disrupt the membrane potential of *S. simulans* cells. (A) 200 nM nisin (blue) and 500 nM epilancin 15X (orange) were added at 2 min. (B) 200 nM nisin was added at 2 min (blue); 10 μM vancomycin was added at 1 min and 200 nM nisin added at 2 min (orange). (C) 500 nM epilancin 15X was added at 2 min (blue); 10 nM (orange) and 20 μM (gray) vancomycin was added at 1 min and 500 nM epilancin 15X added at 2 min. (D) 500 nM epilancin 15X was added at 2 min (orange), 2 μM nisin(AB)-C14 was added at 1 min and 500 nM epilancin 15X was added at 2 min (yellow); 200 nM nisin 15X added at 2 min (blue), 1 μM nisin(AB)-C14 was added at 1 min and 200 nM nisin was added at 2 min (green). Arrows mark the addition of antagonists (1), lantibiotics (2), and Triton X-100 (0.2 % final concentration) (3), which was added to fully dissipate the membrane potential.

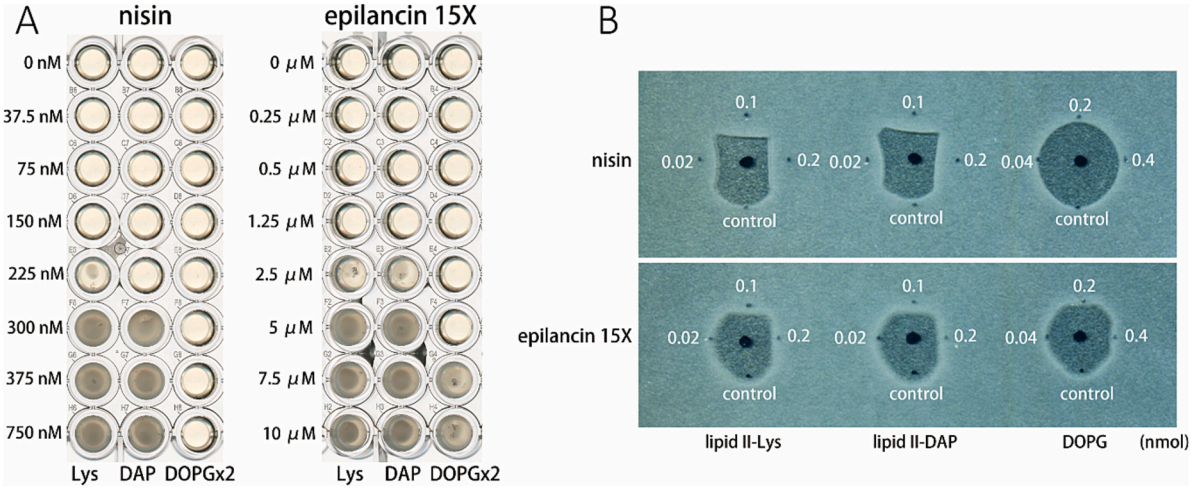

Fig. 3. Antagonistic effect of externally added Lipid II on the antimicrobial activities of epilancin 15X and nisin. (A) The concentrations of nisin and epilancin 15X were 375 nM and 250 nM respectively. The numbers displayed to the left of the wells are the concentrations of externally added Lipid II. Lys and DAP refer to the lysine or diaminopimelic acid variant of Lipid II, the concentration of externally added DOPG was twice that of Lipid II. (B) Different amount of Lipid II-Lys and Lipid II-DAP equal to 0.02 nmol, 0.1 nmol, 0.2 nmol were added at the edge of the predicted inhibition halo where the lowest concentration of lantibiotic that still inhibits *S. simulans* growth is present. Twice the amount of DOPG was added as compared to Lipid II.
(B was taken from [6].)

DOPG had no effect on the nisin activity. The halos of epilancin 15X also showed deformation when Lipid II was added and, like above, higher concentrations of Lipid II were needed to observe an effect on the halo. Again, DOPG had an effect on the activity of epilancin 15X as well.

These results clearly show that epilancin 15X interacts with Lipid II. However, the apparent affinity of epilancin 15X towards Lipid II seems

to be lower as compared to that of nisin, as significantly higher concentrations of Lipid II were needed to observe an antagonistic effect. This difference in affinity may point to a difference in binding mode between the two lantibiotics, as can be expected from the large differences in primary structure. Alternatively, this may point to a different target for epilancin 15X. We could detect no significant difference between Lipid II-Lys and Lipid II-DAP, suggesting that the penta-peptide part of Lipid II is not an important part of the interaction interface. In both test systems, DOPG affected the activity of epilancin 15X. This may suggest that the interaction of epilancin with Lipid II is mainly caused by (unspecific) electrostatic interactions, which may be stronger in the case of Lipid II as it contains more negative charges. Yet, since DAP-Lipid II has one negative charge more than the lysine form and we could not detect any difference between the two, this suggests that electrostatics aren't the main driving force for this interaction.

Next, we checked if the lantibiotic changes its conformation upon binding to Lipid II by circular dichroism (CD). We observed a clear change in the CD-spectrum of epilancin 15X in the presence of small unilamellar vesicles (SUVs) containing Lipid II (Fig. 4A). This points to a Lipid II-induced change in the secondary structure of epilancin 15X. It remains unclear what kind of secondary structure is induced upon binding to Lipid II. SUVs containing double the amount of DOPG as compared to Lipid II, to correct for the charge, did not induce any structural change in the peptide (Fig. 4B). Thus, the binding of epilancin to Lipid II is likely to be specific, and not only governed by electrostatics.[1]

3.4. Epilancin 15X-Lipid II binding does not lead to pore-formation

Next, we checked whether the interaction between epilancin 15 X and Lipid II leads to membrane permeabilization of model membrane vesicles loaded with either carboxy fluorescein (CF) or 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS). CF is loaded in self-quenching concentrations into the vesicles. Thus, leakage is monitored by the release of the probe, which relieves the self-quenching (molar mass 376 Da). Nisin could efficiently induce CF leakage as expected from a pore-former where about 50 % leakage could be observed from Lipid II containing LUVs at a nisin concentration of only 5 nM (Fig. S3A, red trace). No leakage could be observed from vesicles devoid of Lipid II (blue trace). Epilancin 15X did not cause leakage either in the absence or presence of Lipid II at ten times higher concentrations of 50 nM (Fig. S3B). Similar results were obtained when vesicles were used that also contained negatively charged phospholipids with the composition DOPC/DOPG (1:1) with/without 0.1 % Lipid II (Fig. S4).

We have shown before that epilancin 15X causes proton leakage across the membranes of *S. simulans*, *M. flavus* and *B. megaterium* [45]. Thus, it may be possible that epilancin 15X and Lipid II together might form small pores that allow only protons instead of bigger molecular such as CF (1 nm of molecular radius) to pass though. To check whether epilancin 15X causes proton permeabilization in the presence of Lipid II in model vesicles, we tested for proton leakage using HPTS containing LUVs [44,48]. HPTS is a pH sensitive fluorescent probe, and its fluorescent signal will decrease when protons from the more acidic exterior can enter the vesicles and lower the pH. As expected, nisin caused a rapid decrease in fluorescence when Lipid II was present (Fig. S3C). However, epilancin 15X failed to have any effect on the pH gradient even in the presence of Lipid II (Fig. S3D). Thus, even though epilancin 15X showed specific binding to Lipid II in vitro, this interaction did not lead to a detectable effect on the permeability of model membranes.

[1] In Fig. 4, a buffer composed of 20 mM HEPES, 40 mM $Na2SO_4$, pH 7.4 was used and the concentration of epilancin 15X was 50 μM. In Fig. S5A, a buffer composed of 0.1 % Tween-20, 50 mM phosphate, pH 5 was used and the concentration of epilancin 15X was 80 μM. The CD spectra of epilancin 15X changed likely due to the presence of Tween 20.

3.5. Interfering with the cell wall biosynthesis pathways affects epilancin 15X activity

The observation that nisin(AB)-C14 could inhibit epilancin while vancomycin could not, can be explained by the differences in substrate specificity of the two compounds. Vancomycin targets the D-Ala-D-Ala terminus of the pentapeptide of Lipid II and nascent peptidoglycan and thus prevents both further transglycosilation and transpeptidation. Nisin (AB)-C14 targets not only Lipid II, but also other undecaprenyl-pyrophosphoryl-linked precursors including C55-PP, lipid α, lipid β and lipid φ.1 (and n). Thus, epilancin may target another undecaprenyl-pyrophosphoryl-linked precursor rather than Lipid II. This would explain the failure of epilancin 15X to cause permeabilization in model membranes containing Lipid II.

To check whether C55-PP is the target of epilancin 15X, we made use of bacitracin in the membrane depolarization assays. Bacitracin binds to C55-PP and thus blocks the lipid II cycle at that position resulting in PG synthesis inhibition. The results showed that the presence of bracitracin inhibited the activity of both epilancin 15X and nisin (Fig. 5A). A two-fold higher concentration of bacitracin was needed to inhibit epilancin 15X to the same extent as that of nisin. These results suggest that C55-PP may play a role in the MoA of epilancin, so we tested it for possible antagonization activity in an agar diffusion assay. For this, we used farnesyl-pyrophosphate (C15-PP) that is water soluble and more stable as compared to C55-PP. The only difference between C55-PP and C15-PP is the length of lipid chain which insert in cell membrane. No effect of a large excess (7.5 μmol) of C15-PP could be observed on the shape of the halo formed by epilancin 15X's antibacterial activity (Fig. S5B). We also checked the effect of C15-PP on the secondary structure of epilancin 15X in the presence of 0.1 % Tween. In line with the agar diffusion assay, C15-PP did not induce any structural change in the peptide (Fig. S5A). Both these results suggest that it is unlikely that C55-PP is the direct target of epilancin 15X. Possibly, the effect of bacitracin on the epilancin activity can be explained by an indirect effect of bacitracin on the pool of other undecaprenyl-pyrophosphoryl-linked precursors. Bacitracin addition to the cells will lead to an accumulation of C55-PP in the external leaflet of the plasma membrane, which in turn will cause a depletion of undecaprenyl-phosphate and other members of the Lipid II cycle. This will also affect other biosynthesis pathways that rely on this compound, such as the wTA pathway.

Considering this possible indirect effect of bacitracin, it is hard to explain why vancomycin did not inhibit the membrane depolarization activity of epilancin 15X. An explanation for this could be that vancomycin needs more time to completely block the lipid II cycle before it can have an effect on epilancin's activity. To test this, we prolonged the preincubation time of *S. simulans* with vancomycin to 5 and 10 min. Indeed, the depolarization activity of epilancin 15X was greatly suppressed already after 5 min preincubation with vancomycin and a complete inhibition could be observed after 10 min (Fig. 5B). Notably, at time point 0 min, the activity of epilancin was already affected a bit (compare epilancin traces in Fig. 5A and B), which can be explained by an increased dead-time of the experiment due to a washing step. Thus, vancomycin induced accumulation of Lipid II and concomitant disruption of the Lipid II cycle also leads to inhibition of epilancin's effect on the membrane potential. Moreover, these results also indicate that Lipid II is probably not the target of epilancin 15X, as addition of vancomycin inhibited nisin but not epilancin 15X.

Our previous study showed that one should not rely on only a single bacterial strain for mode of action studies [45]. We therefore tested whether the above effects could also be observed for *M. flavus*. Indeed, similar inhibitory effects of bacitracin, nisin(AB)-C14 and vancomycin to nisin and epilancin 15X were observed using *M. flavus* (Fig. S6). With these bacteria, a prolonged incubation time with vancomycin was not needed to inhibit the membrane depolarization activity of epilancin 15X. From the results above a picture emerges in which the inhibitory activity of epilancin 15X is dependent on the correct functioning of the

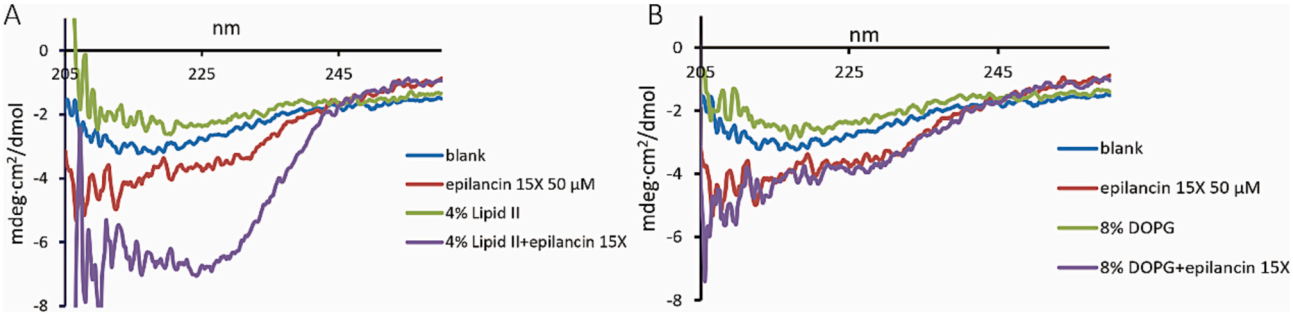

Fig. 4. Lipid II-induced changes in the secondary structure of epilancin 15X. CD spectra recorded for 50 μM epilancin 15X (tracing Red) and the control with buffer only (tracing blue). (A) Tracing purple was recorded for 50 μM epilancin 15X in the presence of 50 μM Lipid II in DOPC SUVs (4 mol%) and green was recorded for 50 μM Lipid II in DOPC SUVs (4 mol%) only. (B) Tracing purple and green were recorded for 50 μM epilancin 15X in the presence of 100 μM DOPG in DOPC SUVs (8 mol %) and 100 μM DOPC SUVs containing 8 mol% DOPG control.

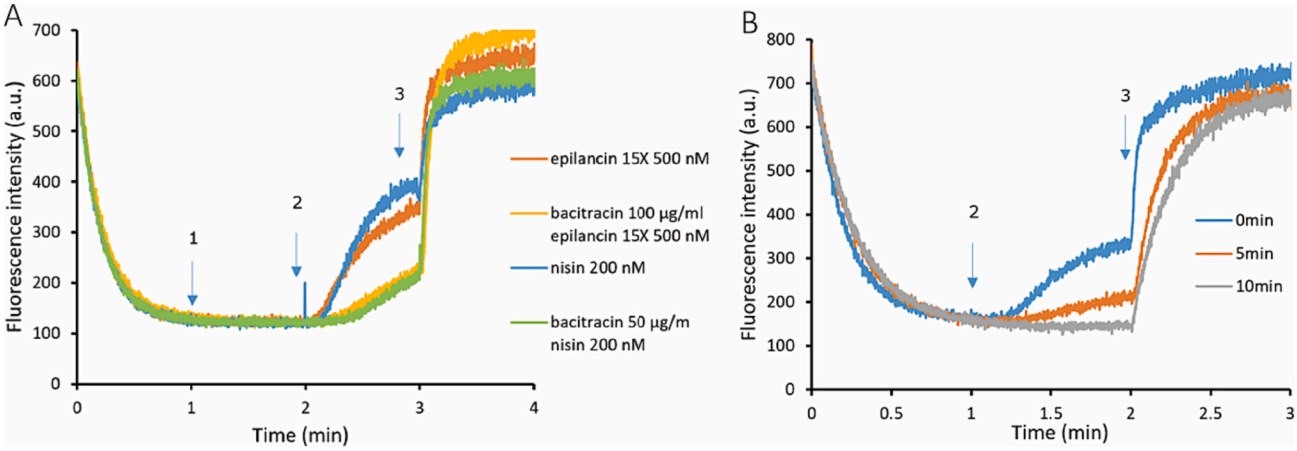

Fig. 5. Effects of bacitracin and prolonged incubation with vancomycin on epilancin 15X's ability to disrupt the membrane potential of *S. simulans* cells. (A) 500 nM epilancin 15X was added at 2 min (orange), 100 μg/mL bacitracin was added at 1 min and 500 nM epilancin 15X was added at 2 min (yellow); 200 nM nisin 15X added at 2 min (blue), 50 μg/mL bacitracin was added at 1 min and 200 nM nisin was added at 2 min (green). (B) Intact cells were treated with 10 μM vancomycin for 0 min (blue), 5 min (orange), 10 min (gray). 200 nM epilancin 15X was added at 1 min. Arrows match the addition of antagonists (1), lantibiotics (2), and Triton X-100 (0.2 % final concentration) (3), which was added to dissipate 100 % membrane potential.

Lipid II cycle. Possibly, inhibition of this cycle has an effect on the availability of epilancin's target. While Lipid II itself and C55-PP are ruled out as possible targets within the PG biosynthesis pathway, another (biosynthesis) pathway that is affected by a blockage of the Lipid II cycle likely harbors the target of epilancin 15X.

3.6. PG-synthesis inhibition at the level of PBPs and their effects on the activity of epilancin 15X

All the antibiotics that we used to test for possible targets of epilancin, i.e. vancomycin, bacitracin and nisin(AB)-C14 act at the level of

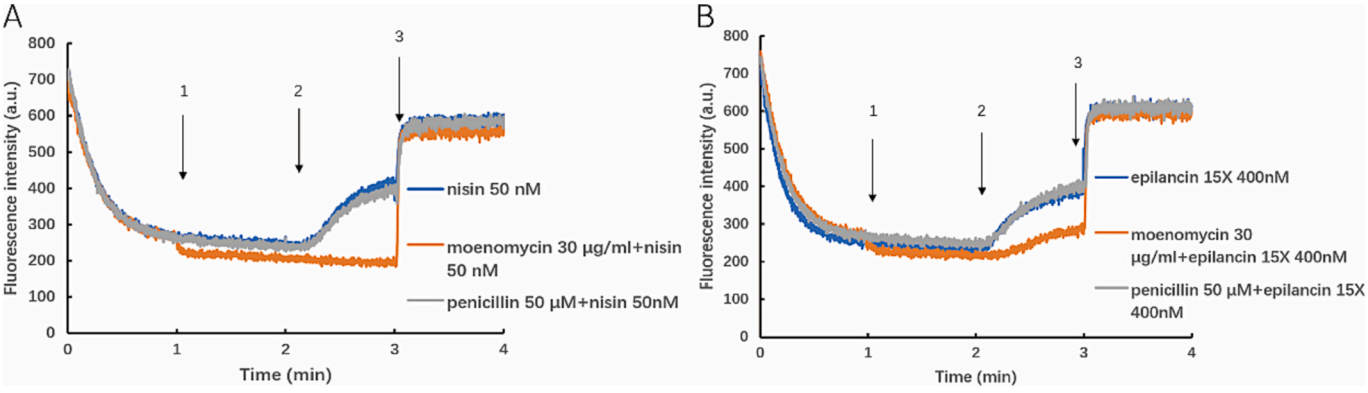

Fig. 6. Effects of penicillin and moenomycin on the membrane depolarization activity of nisin and epilancin 15X in *S. simulans* cells. (A) 50 nM nisin was added at 2 min (blue); 30 μg/mL moenomycin was added at 1 min and 50 nM nisin added at 2 min (orange), 50 μM penicillin was added at 1 min and 50 nM nisin was added at 2 min (gray). (B) 400 nM epilancin 15X was added at 2 min (blue); 30 μg/mL moenomycin was added at 1 min and 400 nM epilancin 15X was added at 2 min (orange), 50 μM penicillin was added at 1 min and 400 nM epilancin 15X was added at 2 min (gray). Arrows mark the addition of antagonists (1), lantibiotics (2), and Triton X-100 (0.2 % final concentration) (3), which was added to fully dissipate the membrane potential.

the Lipid II cycle of the PG-biosynthesis pathway. Next, we disrupted the PG pathway at the level of the PBPs to test effects of epilancin 15X. For this we used two PBP-targeting antibiotics penicillin G and moenomycin. Penicillin targets the transpeptidation activity of both monofunctional and bifunctional PBPs, which inhibits the crosslinking of polymerized peptidoglycan strains [31]. Moenomycin targets the transglycosylase domain of PBP which blocks the Lipid II polymerization step [32]. The addition of penicillin did not affect the membrane depolarization activity of nisin and epilancin 15X as can be expected since blocking transpeptidation/crosslinking does not have an effect on the Lipid II cycle (Fig. 6). Addition of moenomycin completely blocked the depolarization activity of nisin and also inhibits that of epilancin 15X, albeit to a lesser extent. The complete block of nisin (and also the partly block of epilancin) activity came as a surprise as it's known that moenomycin accumulates Lipid II in the bacterial membrane, which also holds true for *S. simulans* (unpublished observations). These observations can only be explained if the accumulated Lipid II is located at the cytosolic side of the plasma membrane opposed to the general belief that it accumulates at the outer leaflet. This points to a (strict) coupling of transport of Lipid II to its use in the polymerization step of the PG synthesis pathway in intact cells. Hence, moenomycin causes a block of the transport of Lipid II that is possibly indirect. Eventually, this accumulation will lead to a depletion of the undecaprenyl-phosphate pool available to the other biosynthesis pathways that rely on this pool. Thus, only the antibiotics that block the Lipid II cycle of the PG biosynthesis pathway inhibit the activity of nisin and epilancin 15X. Again, similar results were obtained with *M. flavus* as the test bacterium (Fig. S7), albeit that the effect of moenomycin on epilancin was less severe.

Interestingly, the addition of moenomycin caused a small decrease of the fluorescence, which points to a possible moenomycin induced increase in the membrane potential of the bacteria. In this, the effect resembles the effect of adding nigericin, a H^+/K^+ antiporter that increases the membrane potential at the expense of the ΔpH [49]. The origin of this effect of moenomycin remains unclear, but may be related to the block in Lipid II transport.

3.7. Wall teichoic acid (wTA) precursors are not the target of epilancin 15X

Direct inhibition of the Lipid II cycle by means of the compounds used so far all affected the activity of epilancin 15X, but this can also be attributed to their indirect effect on other pathways that make use of undecaprenyl-phosphate as a carrier. The other main pathway in Gram-positive bacteria that uses this precursor is the wTA pathway. To check whether epilancin 15X targets the wTA synthesis pathway, we first tried to use tunicamycin and targocil (a wTA flippase inhibitor) as antagonists in the membrane depolarization activity of nisin or epilancin 15X towards *S. simulans*. Unfortunately, both compounds had an effect on the membrane potential on their own account, where tunicamycin decreased the membrane potential of cells at 10 μg/mL and the addition of targocil in combination with nisin decreased the fluorescent signal actually pointing to an increase in membrane potential (Fig. S8). Thus, interaction of targosyl with the probe cannot be ruled out here. These observations make it impossible to use these compounds in our assay. Thus, we took an alternative approach that used a *B. subtilis* TagO deletion mutant to check whether wTA precursors are involved in the mode of action of epilancin 15X. TagO is a phosphosugar transferases enzyme that forms GlcNAc-pp-undecaprenyl, which is a reversible step [50]. After deleting the *tagO* gene, cells will not be able to form wTA, but remain viable. We performed an agar diffusion assay with a 2-fold serial dilution of epilancin 15X on the *B. subtilis* ΔTagO strain (Fig. S9A) and the corresponding wildtype (Fig. S9B). The halo sizes indicate that the ΔTagO strain was much more sensitive to epilancin 15X than the wildtype strain. A concentration of 0.16 μM epilancin 15X could still inhibit the bacteria of the ΔTagO strain as shown by the presence of a halo, this contrasts with epilancin's activity towards wild type bacteria where a similar sized halo could be observed at a 16-fold higher concentration of 2.5 μM epilancin 15X. So, wTA precursors are not the target of epilancin 15X. Instead, the negative charged wTA may even play a protective role in bacteria by trapping the positive charged epilancin 15X, thereby preventing the peptide to reach the membrane, similar to the proposed protective role of Lysyl-PG as suggested before [45].

3.8. Recognition of lipid-linked pyrophospates by epilancin

The effects of the inhibition of the Lipid II cycle on the activity of epilancin 15X and the interaction of epilancin 15X with Lipid II as shown by CD points to a target that resides in a polyprenol-based biosynthesis pathway. Yet, Lipid II is not likely to be that target in view of epilancin 15X's low affinity for Lipid II as well as the lack of any effect on the membrane permeability in model systems containing Lipid II. Similarly, wTA precursors are also not likely to be the target in view of the results with the TagO deletion mutant. This makes it difficult to draw conclusions on what constitutes the target of epilancin 15X. In all likelihood this target concerns a pyrophosphate containing molecule, which made us wonder if epilancin 15X would be able to recognize CDP-diacylglycerol (CDG-DAG). This lipid, an important precursor in the phospholipid biosynthesis routes, contains a diacylglycerol-linked pyrophosphate to which a cytidyl group is attached (Fig. S10A). So, some resemblance to the polyprenyl-pyrophosphate-linked sugars is apparent. We tested its possible interaction with epilancin 15X by CD, and could observe a significant change in the secondary structure of the peptide in the presence of CDP-DAG (Fig. S10B). This shows that epilancin 15X can recognize pyrophosphoryl groups that are not polyisoprenoid-linked and a hexose sugar following the pyrophosphoryl group isn't necessary either. As epilancin 15X did not interact with farnesyl-pyrophosphate, this indicates that epilancin 15X recognizes pyrophosphate groups that are derivatized on both sides, i.e. molecules containing phosphodiesters.

A very recent study of epilancin 15X also found that it can induce leakage from model membrane vesicles that contain negatively charged lipids in a Lipid II independent way [51]. Interestingly, the two bacteria tested in this study, *Staphylococcus carnosus* and *B. subtilis*, seemed to behave differently towards the addition of epilancin 15X, corroborating our advice to always include more than one bacterium in testing modes of action of antimicrobial peptides [45]. The finding that epilancin 15X induced expression of *vraR* and *vraS* in *S. carnosus* [51], matches with our results that epilancin likely targets a polyprenol-based cell wall biosynthesis precursor.

4. Conclusions

This study focused on exploring potential targets of epilancin 15X within the bacterial membrane and involved in the PG and wTA biosynthesis cycles. Several results suggested that the target of epilancin 15X is located in polyprenol-based biosynthesis pathways. Although a clear interaction between epilancin 15X and Lipid II was observed and Lipid II induced conformation change of epilancin 15X, this interaction failed to result in permeabilization of model membranes, which indicated that Lipid II is likely not the actual target. Similarly, C55-PP and wTA precursors are also unlikely to be the target of epilancin 15X. Interestingly, we could show an interaction between epilancin 15X and CDP-DAG, which implied that epilancin 15X does target phosphodiester containing molecules. However, the actual target of epilancin 15X was not identified yet, but it cannot be ruled out that epilancin 15X uses multiple targets within a bacterium.

CRediT authorship contribution statement

Xiaoqi Wang: Data curation, Formal analysis, Methodology, Writing – original draft. **Yang Xu:** Methodology, Writing – review & editing. **Nathaniel I. Martin:** Methodology, Writing – review & editing. **Eefjan**

Breukink: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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