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Non-proteinogenic Amino Acids in Lacticin 481 Analogues Result in More Potent Inhibition of Peptidoglycan Transglycosylation

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ABSTRACT: Lantibiotics are ribosomally synthesized and post-translationally modified peptide natural products that contain the thioether structures lanthionine and methyllanthionine and exert potent antimicrobial activity against Gram-positive bacteria. At present, detailed modes-of-action are only known for a small subset of family members. Lacticin 481, a tricyclic lantibiotic, contains a lipid II binding motif present in related compounds such as mersacidin and nukacin ISK-1. Here, we show that lacticin 481 inhibits PBP1b-catalyzed peptidoglycan formation. Furthermore, we show that changes in potency of analogues of lacticin 481 containing non-proteinogenic amino acids correlate positively with the potency of inhibition of the transglycosylase activity of PBP1b. Thus, lipid II is the likely target of lacticin 481, and use of non-proteinogenic amino acids resulted in stronger inhibition of the target. Additionally, we demonstrate that lacticin 481 does not form pores in the membranes of susceptible bacteria, a common mode-of-action of other lantibiotics.

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481 likely exerts its biological activity via interaction with lipid II.

We have recently reported the generation of a series of mutants of lacticin 481 with altered antimicrobial potencies using an in vitro biosynthetic platform.17,21 The analogues had both improved and attenuated activities, but the underlying mechanism for the changes in bioactivities is unknown and could involve differences in uptake, metabolism, or interaction with the target. In this study, we used the in vitro methodology17 to produce sufficient quantities of four analogues for further mode-of-action studies. These compounds contained the following mutations: N15R/F21H, N15R/F21Pal, N15R/F21H/W23Nal, and N15R/F21Pal/W23Nal, where Pal = 3-(4′-pyridyl)alanine and Nal = 3-(2-naphthyl)alanine. Previous evaluation of these analogues against L. lactis HP demonstrated that the N15R/F21Pal (IC_{50} = 213 ± 9 nM) and N15R/F21H (IC_{50} = 428 ± 21 nM) analogues displayed more potent growth inhibitory activity compared to authentic lacticin 481 (IC_{50} = 785 ± 19 nM). The triply substituted analogues N15R/F21H/W23Nal (IC_{50} = 1370 ± 48 nM) and N15R/F21Pal/W23Nal (IC_{50} = 2420 ± 60 nM) were less active than the natural product, thus yielding an IC_{50} value range from 200 to 2500 nM with authentic lacticin 481 as the median.17

We examined in this work whether changes in inhibitory activity could be correlated with a biochemical mode-of-action. Given the recently reported binding of nukacin ISK-1 to lipid II,20 we specifically examined if lacticin 481 inhibits the transglycosylation reaction involved in peptidoglycan biosynthesis. We used a previously reported in vitro assay that monitors the catalytic activity of a major transglycosylase, PBP1b, from Escherichia coli using a radiolabeled lipid II variant as substrate.22 The two lacticin 481 analogues with improved antibacterial activity compared to that of the wild type compound also displayed an enhanced inhibitory effect on the transglycosylation reaction (Figure 2), with IC_{50} values of 5.4 ± 1.2 and 7.0 ± 2.9 μM for N15R/F21Pal and N15R/F21H, respectively, compared to an IC_{50} of 12 ± 2.3 μM for wild-type lacticin 481. Likewise, the two less active analogues gave weaker inhibition with IC_{50} values of 27 ± 5.6 and 105 ± 34 μM for N15R/F21H/W23Nal and N15R/F21Pal/W23Nal, respectively. These IC_{50} values are higher than those for antimicrobial activity because these lantibiotics bind to the substrate, not the biosynthetic enzyme, and because of the relatively high concentrations of lipid II required for these assays compared to lipid II present in cell membranes. Importantly, the potency of transglycosylase inhibition observed for lacticin 481 is similar to that of the known lipid II binders haloduracin23 and ramoplanin24 using the same assay, suggesting that like these compounds, the binding affinity is in the mid-nanomolar range. Furthermore, the positive correlation between antimicrobial activity and transglycosylation inhibition in the series of analogues strongly suggests that lacticin 481 exerts its biological activity through inhibition of cell wall biosynthesis.

The mersacidin-like lipid II binding motif (TXS/TXD/EC, where X is any residue) is found in ring A of lacticin 481 (Figure 1) as well as other lantibiotics known to interact with lipid II,25 including the α-peptides of two-component systems such as lacticin 31426 and haloduracin.18,23 The importance of...
the conserved acidic residue in this motif for antimicrobial potency has been demonstrated in several instances, where mutation to a nonacidic residue abolishes or severely attenuates activity.\textsuperscript{23,27\textendash}30 It was therefore surprising that a previous study suggested that the conserved glutamate (Glu13) in the A-ring of lacticin 481 was not required for antimicrobial activity, because a weak zone of growth inhibition was seen for the E13A mutant that also lacked Lys1.\textsuperscript{31} Using our recently developed \textit{in vitro} methodology,\textsuperscript{17} we prepared wild type lacticin 481 and an E13A mutant containing Lys1 and tested their activity against \textit{L. lactis} HP. Whereas the zone of growth inhibition observed for the wild type compound prepared \textit{in vitro} was very similar to that of authentic lacticin 481 isolated from the producer strain, the E13A mutant did not show any zone of growth inhibition. Therefore, Glu13 is important for the antimicrobial activity of lacticin 481,\textsuperscript{32} similar to previous results for other lantibiotics containing the mersacidin-like lipid II binding motif.

In addition to inhibition of peptidoglycan biosynthesis, several lantibiotics are known to form pores in bacterial membranes using lipid II as a docking molecule, including nisin\textsuperscript{11,12} and the two-component systems lacticin 314\textsuperscript{26} and haloduracin.\textsuperscript{18,23} To investigate if lacticin 481 is similarly able to form pores once bound to lipid II, we used \textit{flow} cytometry to monitor changes in bacterial membrane polarization using the potential-sensitive fluorescent dye 3′,3′-diethylxoycarbocyanine iodide (DiOC\textsubscript{2}(3)). We chose to use \textit{Bacillus subtilis} ATCC 6633 in these experiments because lacticin 481 (IC\textsubscript{50} = 980 ± 110 nM) possesses antimicrobial potency relatively similar to that of nisin (IC\textsubscript{50} = 410 ± 170 nM)\textsuperscript{28} against this organism. As expected, nisin gave a marked concentration-dependent decrease in cell-associated mean fluorescent intensity (MFI), which is indicative of membrane depolarization due to pore formation (Figure 3). However, lacticin 481 did not decrease the MFI at concentrations up to 20 μM when compared to a control sample. We also probed potential membrane disruption by lacticin 481 in \textit{L. lactis} HP using the fluorescent dye propidium iodide (PI), which cannot cross intact cell membranes and enters cells only in the presence of a pore-forming agent. Nisin used at 0.2 μM (15-fold above its IC\textsubscript{50} value for this strain) gave a large increase in cell-associated MFI, consistent with pore formation and loss of membrane integrity (Figure 4). On the other hand, lacticin 481 was not able to increase the MFI above control levels at concentrations up to 20 μM, or 25-fold above its IC\textsubscript{50} value. Taken together, these data suggest that lacticin 481 is not able to form pores in bacterial membranes. This conclusion may partially explain the observations bode well for the use of non-proteinogenic amino acids to improve lantibiotics via molecular editing. Unlike nisin, lacticin 481 is not able to form pores in bacterial membranes, which may contribute to its modest activity against some bacterial strains compared to nisin.

\section*{METHODS}

\textbf{General Materials and Methods.} Cell culture media were purchased from BD Biosciences. The indicator strain \textit{Lactococcus lactis} subsp. \textit{crenaris} HP ATCC 11602 was obtained from American Type Culture Collection. Flow cytometry dyes 3′,3′-diethylxoycarbocyanine iodide (DiOC\textsubscript{2}(3)) and propidium iodide (PI) were purchased from Invitrogen. Nisin was purified from Nisaplin, purchased from Danisco A/S, as previously described.\textsuperscript{33} Lacticin 481 was purified from cultures of the producing organism \textit{L. lactis} subsp. \textit{lactis} CNRZ 481 as previously described.\textsuperscript{15}

\textbf{Chemoenzymatic Synthesis of Lacticin 481 Analogues.} Lacticin 481 analogues were prepared as previously described.\textsuperscript{17} Briefly, a constitutively active leader-LctM fusion enzyme (LctCE-GS\textsubscript{15}) was expressed in \textit{Escherichia coli} Rosetta 2 (DE3) and purified by immobilized metal ion affinity chromatography and gel filtration chromatography. Linear lacticin 481 core peptide analogues were prepared \textit{via} Fmoc-based solid-phase peptide synthesis and purified to homogeneity by reversed-phase high performance liquid chromatography (RP-HPLC). These core peptide analogues (20 μM) were incubated with LctCE-GS\textsubscript{15} (2 μM) in a buffer containing tris(hydroxymethyl)aminomethane (50 mM, pH 7.5), MgCl\textsubscript{2} (10 mM), and ATP (2 mM) for 5–12 h and purified by RP-HPLC to yield Figure 3. Membrane depolarization activities of nisin and lacticin 481 against \textit{Bacillus subtilis} measured using DiOC\textsubscript{2}(3) fluorescence. (a) Average mean fluorescence intensity (MFI) of triplicate measurements for different concentrations of lacticin 481 (blue) and the known pore-forming lantibiotic nisin (red). At each concentration, the difference in MFI between the compounds was statistically significant (P < 0.05). (b) Representative histogram of cell count versus DiOC\textsubscript{2}(3) fluorescence intensity at various lacticin 481 and nisin concentrations.

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\caption{Membrane depolarization activities of nisin and lacticin 481 against \textit{Bacillus subtilis} measured using DiOC\textsubscript{2}(3) fluorescence. (a) Average mean fluorescence intensity (MFI) of triplicate measurements for different concentrations of lacticin 481 (blue) and the known pore-forming lantibiotic nisin (red). At each concentration, the difference in MFI between the compounds was statistically significant (P < 0.05). (b) Representative histogram of cell count versus DiOC\textsubscript{2}(3) fluorescence intensity at various lacticin 481 and nisin concentrations.}
\end{figure}
Flow Cytometry Analysis of Membrane Disruption. For membrane potential assays using the dye 3,3’-diethyloxocarbocyanine iodide (DiOC6(3)), cultures of Bacillus subtilis ATCC 6633 were grown overnight at 37 °C in LB medium and then diluted with fresh LB to an OD600 of 0.1. Cells were combined with DiOC6(3) (final concentration 2 μM), HEPES (1 mM) and glucose (1 mM) and incubated for 20 min at RT. Stock solutions of nisin or lacticin 481 were added to final concentrations of 0.2, 2.0, and 20 μM and incubated for an additional 15 min prior to analysis; H2O was added instead of antibiotic as a negative control. Changes in cell-associated DiOC6(3) fluorescence were measured with a BD Biosciences LSR II flow cytometer, using excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 530/30 nm. A minimum of 50,000 events were detected for each sample, and experiments were performed in triplicate. Data analysis to calculate the geometric mean fluorescence intensity (MFI) of gated cell populations was performed using FCS Express 3.0.0.311 V Lite Stand-alone software. For membrane permeability assays using the dye propidium iodide (PI), cultures of Lactococcus lactis subsp. cremoris HP were grown overnight at 30 °C in GM17 medium (40 g L−1 M17, 0.5% glucose (w/v)) and then diluted with fresh GM17 to an optical density at 600 nm (OD600) of 0.1. Cells were combined with PI (final concentration 25 μM), HEPES (1 mM), glucose (1 mM), and lacticin 481 (0, 0.2, 2.0, 20 μM) or nisin (0.2 μM), incubated for 15 min at RT, and analyzed. Data acquisition and analysis were performed as for membrane potential assays, except emission was measured through a band-pass filter at 695/40 nm.

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Notes
The authors declare no competing financial interest.

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■ REFERENCES


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Figure 4. Unlike nisin, lacticin 481 does not alter the membrane permeability of Lactococcus lactis HP as measured by propidium iodide (PI) uptake. (a) Average MFI of triplicate measurements for nisin at a concentration 15-fold above its IC50 value and a range of lacticin 481 concentrations up to 25-fold above its IC50 value. (b) Representative histogram of cell count versus PI fluorescence intensity at antibiotic concentrations shown in panel a.

The pure, fully modified peptides as determined by MALDI-TOF MS analysis. HPLC solvent compositions: solvent A was 0.1% trifluoroacetic acid in H2O (v/v); solvent B was 4:1 acetonitrile/H2O (v/v) with 0.087% trifluoroacetic acid.

LctA core wild type. Sequence: H-KGGSGVIHTISH-

References


(32) In the previous study (ref 31), the amount of compound used for the bioassay was not quantified, and it is possible that the observed activity was caused by a relatively high concentration of material. Indeed, although the MIC value of the corresponding E22Q mutant of haloduracin α increased 40-fold compared to wild type, the haloduracin mutant did possess antimicrobial activity (ref 23).
