

Ring A of Nukacin ISK-1: A Lipid II-Binding Motif for Type-A(II) Lantibiotic

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S Supporting Information

ABSTRACT: Ring A of nukacin ISK-1, which is also present in different type-A(II) lantibiotics, resembles a lipid II-binding motif (TxS/TxD/EC, x denotes undefined residues) similar to that present in mersacidin (type-B lantibiotics), which suggests that nukacin ISK-1 binds to lipid II as a docking molecule. Results from our experiments on peptidoglycan precursor (UDP-MurNAc-pp) accumulation and peptide antagonism assays clearly indicated that nukacin ISK-1 inhibits cell-wall biosynthesis, accumulating lipid II precursor inside the cell, and the peptide activity can be repressed by lipid I and lipid II. Interaction analysis of nukacin ISK-1 and different ring A variants with lipid II revealed that nukacin ISK-1 and nukacin D13E (a more active variant) have a high affinity ($K_D = 0.17$ and $0.19 \mu\text{M}$, respectively) for lipid II, whereas nukacin D13A (a less active variant) showed a lower affinity, and nukacin C14S (a negative variant lacking the ring A structure) exhibited no interaction. Therefore, on the basis of the structural similarity and positional significance of the amino acids in this region, we concluded that nukacin ISK-1 binds lipid II via its ring A region and may lead to the inhibition of cell-wall biosynthesis.

Lantibiotics are ribosomally synthesized peptide antibiotics that contain unusual amino acids, lanthionine, 3-methyl-lanthionine, and dehydroamino acids.¹ These peptides have traditionally been subdivided into two major groups: type-A and type-B lantibiotics, which comprise peptides with straight-chain and globular structures.² Type-A lantibiotics are further divided into two subtypes, namely, type-A(I) and type-A(II), based on their biosynthetic enzymes. In addition, a third class, two-component lantibiotics, is now gaining recognition, wherein two structurally different peptides act synergistically to kill the target bacteria. Nukacin ISK-1 is a type-A(II) lantibiotic with 27 amino acids, including four unusual residues, and is produced by *Staphylococcus warneri* ISK-1.³

The modes of action of different lantibiotics have been extensively studied for a long time. The prototype lantibiotic nisin from the type-A(I) group targets the membrane-bound

cell-wall precursor lipid II, resulting in the inhibition of peptidoglycan biosynthesis⁴ and pore formation.⁵ It has also been shown that the pores formed by nisin in the membranes containing lipid II are much more stable than those formed in the absence of the receptor.⁶ The binding motif of nisin to lipid II involves only the first 10 N-terminal amino acids, containing the lanthionine rings A and B, termed the “pyrophosphate cage”. A similar binding motif was also observed for other type-A(I) lantibiotics such as subtilin, epidermin,⁷ and gallidermin.⁷ Another lipid II-binding motif for mersacidin and other lantibiotics has been described. The ability of mersacidin to specifically bind lipid II is well documented.⁸ Mersacidin and similar type-B lantibiotics possess a conserved sequence (CTLT/SHEC, underlined residues are the ones modified during their maturation) comprising residues 12–18, suggesting that these residues may form the core lipid II-binding site.⁹ A sequence identical to that of mersacidin is found in plantaricin C,¹⁰ LtnA1 of lactacin 3147,¹¹ Hal α of haloduracin,¹² and Lch α of lichenicidin,¹³ which are predicted to be involved in lipid II binding.

The mode of action of type-A(II) lantibiotics, comprising a large number of peptide members, is still unknown. Specifically, their interaction with lipid II has not been investigated, though they possess TxS/TxD/EC (x denotes undefined residues) sequence in their ring A structure, which suggests that they can bind lipid II as a docking molecule (Figure 1; Figure S1).

Here we focused on a potential lipid II-binding motif in nukacin ISK-1, with particular emphasis on the similar ring A structure, by using different variants in this region. We investigated their interaction with different peptidoglycan precursors.

The positional significance of the individual residues in the nukacin ISK-1 ring A region has been previously investigated using site saturation systematic mutagenesis (NNK scanning), and different ring A variants have been generated.¹⁴ In this study, we purified the ring A variants, namely, H12A, D13A, D13E, and C14S, and determined their minimum inhibitory concentrations (MICs) against different indicator strains (Table 1). As anticipated, H12A and D13A variants showed

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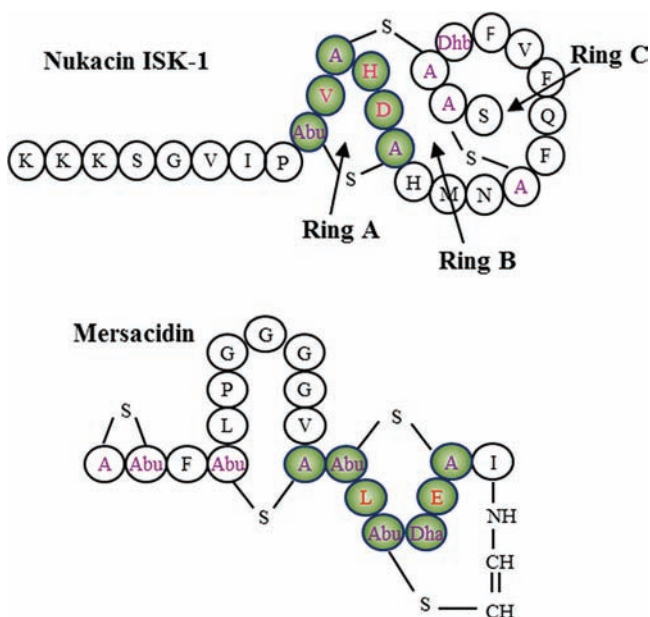


Figure 1. Primary structure of nukacin ISK-1 and mersacidin. The lipid II-binding motif of mersacidin and similar amino acids in nukacin ISK-1 is highlighted in green shad. Different rings of nukacin ISK-1 are indicated by arrows. Unusual amino acids are as follows: Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methylanthionine.

Table 1. Specific Activities (MIC, μM)^a of Nukacin ISK-1 Ring A Variants

indicator strain ^b	peptide ^c				
	ISK-1	H12A	D13A	D13E	C14S
<i>Lb. sakei</i>	0.62	80.0	80.0	0.12	>120.0
<i>L. lactis</i>	10.00	>80.0	>80.0	0.23	>120.0
<i>B. coagulans</i>	1.25	80.0	>80.0	0.31	>120.0
<i>L. lactis</i> HP	2.50	>80.0	>80.0	0.62	>120.0
<i>E. faecalis</i>	20.00	>80.0	>80.0	5.00	>120.0
<i>S. simulans</i> 22	5.00	>80.0	>80.0	0.62	>120.0

^aSpecific activity was determined twice for each peptide and the average value is shown. ^b*Lactobacillus sakei* JCM 1157^T, *Lactococcus lactis* NZ9000, *Bacillus coagulans* JCM 2257^T, *Lactococcus lactis* HP, *Enterococcus faecalis* JCM 5803^T, *Staphylococcus simulans* 22. ^cISK-1, nukacin ISK-1; H12A, nukacin H12A; D13A, nukacin D13A; D13E, nukacin D13E; C14S, nukacin C14S.

significantly reduced antimicrobial activity, while the ring-disrupted C14S variant was found completely inactive at the concentrations used. Replacement of H12 and D13 with Ala might obstruct in ring A formation, as Ala is not preferable at the N-terminal of the ring closing C14 residue.¹⁵ The D13E variant showed markedly higher activity (4-fold to 40-fold) than the wild type, as assessed by observing the inhibition of the growth of the indicator in the liquid medium by using different peptide concentrations, as described by Wiedemann et al.¹¹ This result indicates that the ring A structure of nukacin ISK-1 is critically important for maintaining the structural integrity and effectiveness of this peptide. This result also correlates with the LtnA1 of lactacin 3147¹⁶ and mersacidin,¹⁷ where a negatively charged Asp/Glu residue in this region was found to be essential.

The mechanism underlying the antimicrobial activity of nukacin ISK-1 has not been extensively studied. Recently,

Asaduzzaman et al. showed that nukacin ISK-1 significantly reduced cell-wall thickness, and the treated cells did not show complete septum formation in *Bacillus subtilis*.¹⁸ These data suggest that nukacin ISK-1 might inhibit cell-wall biosynthesis by targeting the cell-wall components. Antibiotics such as vancomycin or bacitracin, which interfere with the membrane-bound stages of peptidoglycan synthesis, trigger the accumulation of the final soluble peptidoglycan precursor, UDP-*N*-acetylmuramyl pentapeptide (UDP-MurNAc-pp), in the cytoplasm (Figure S2).¹⁹ Therefore, in this study, we determined the cytoplasmic levels of the lipid II precursor, UDP-MurNAc-pp, in *Staphylococcus aureus* SG511 by using nukacin ISK-1 and 2 nukacin D13 variants (D13E and D13A). Both nukacin ISK-1- and nukacin D13E-treated cells showed substantial precursor accumulation, similar to vancomycin (Figure 2). The less active variant, namely, nukacin D13A,

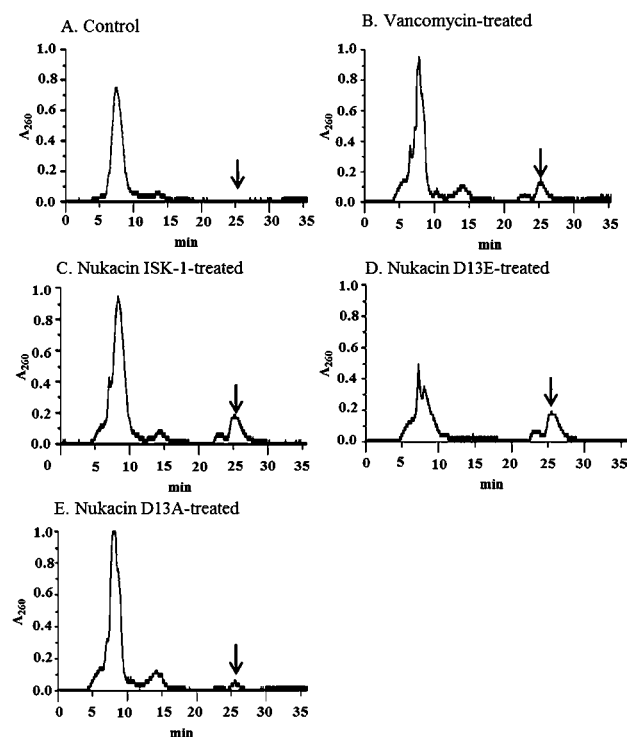


Figure 2. Intracellular accumulation of the soluble cell-wall precursor UDP-*N*-acetylmuramyl pentapeptide (UDP-MurNAc-pp) in *Staphylococcus aureus* SG511. Cells were treated with 10 \times MIC of the respective antibiotic compound, incubated for 30 min, and subsequently extracted with boiling water. Nukacin D13A was added at 30 \times MIC of nukacin ISK-1. The intracellular nucleotide pool was analyzed by applying standardized aliquots to reverse-phase HPLC. UDP-MurNAc-pp was eluted at 25–26 min and is indicated by the arrow. (A) Control cells, untreated, (B) vancomycin-treated, (C) nukacin ISK-1-treated, (D) nukacin D13E-treated, and (E) nukacin D13A-treated cells.

did not lead to significant accumulation of the precursor. This result suggests that nukacin ISK-1 and nukacin D13E might interfere with one of the later membrane-associated or extracellular processes of peptidoglycan biosynthesis (Figure S2).

To investigate the possibility of nukacin ISK-1 interacting with lipid II, an antagonization assay was carried out with nukacin ISK-1 and the highly potent variant nukacin D13E in the presence of different peptidoglycan precursors (*C*₅₅-P, lipid

I, and lipid II). Nisin and mersacidin, which are known to tightly bind lipid II, were used as positive controls. Thus, strong inhibition of nukacin ISK-1 and nukacin D13E activities was observed in the presence of lipid I and lipid II, and the growth of the indicator strain was similar to that observed in the positive control (Table 2). This experiment presents the first indication that nukacin ISK-1 could specifically bind to lipid I and lipid II.

Table 2. Antagonization of Lantibiotic Activity by Peptidoglycan Precursors

lantibiotic	growth with antagonists ^a		
	C ₅₅ -P	lipid I	lipid II
nisin Z	–	+	+
mersacidin	–	+	+
nukacin ISK-1	–	+	+
nukacin D13E	–	+	+

^a*Lactococcus lactis* HP was incubated with 4 × MIC of the corresponding peptides. Antagonists were added in 8-fold higher molar concentration of individual peptide MIC. Growth with precursors: +, lantibiotic activity antagonized; –, lantibiotic activity unaffected.

Direct interaction of nukacin ISK-1 and different ring A variants with lipid II was analyzed by isothermal titration calorimetry (VP-ITC). To investigate whether lipid II has an impact on the ITC traces of nukacin ISK-1 as well as its variants, we loaded 150 μM lipid II solutions in the syringe that was titrated into the peptide solutions in the reaction cell. As indicated in the titration profiles of nukacin ISK-1 (3.4 μM) and nukacin D13E (3.4 μM), the exothermic heat flow decreased with an increase in the number of injections, and free peptide concentration in the reaction cell decreased simultaneously (Figure 3A,B). The binding appeared to be very strong for both peptides, as observed in the ITC chromatograms. For nukacin ISK-1 and nukacin D13E, the saturation of heats was achieved after 14 and 10 injections, respectively (Figure 3A,B). The early saturation of heats for nukacin D13E might be because of a stronger affinity for lipid II, although the binding constant did not significantly differ. The apparent dissociation constants (K_D) for nukacin ISK-1 and nukacin D13E for lipid II were estimated to be 0.17 and 0.19 μM, respectively. The binding affinity of nukacin D13A at 3.4 μM for lipid II was significantly lower (Figure 3C), whereas at a higher concentration (7.1 μM) of the peptide, the K_D was measured to be 3.6 μM (data not shown). These results suggest that the decreased potency of nukacin D13A could be because of a decreased affinity for lipid II. Nukacin C14S (5.6 μM) did not show any interaction with lipid II (Figure 3D). The affinity of nukacin ISK-1 for lipid II is similar to that for other lipid II-binding lantibiotic peptides, like nisin,²⁰ bovicin HCS,²¹ and clausin.²² The thermodynamic parameters of the interaction between lipid II and different peptides are provided in Table S1. The binding of nukacin ISK-1 and its variants (nukacin D13E and nukacin D13A at 7.1 μM) to lipid II is predominantly enthalpy-driven, as observed from the parameters. In summary, ITC data clearly indicate that nukacin ISK-1 plausibly interacts with lipid II, and the interaction is mainly facilitated by the ring A structure where Asp13 plays a critical role.

In conclusion, the results presented here show that nukacin ISK-1 acts on the cell wall by initially interacting with lipid II as

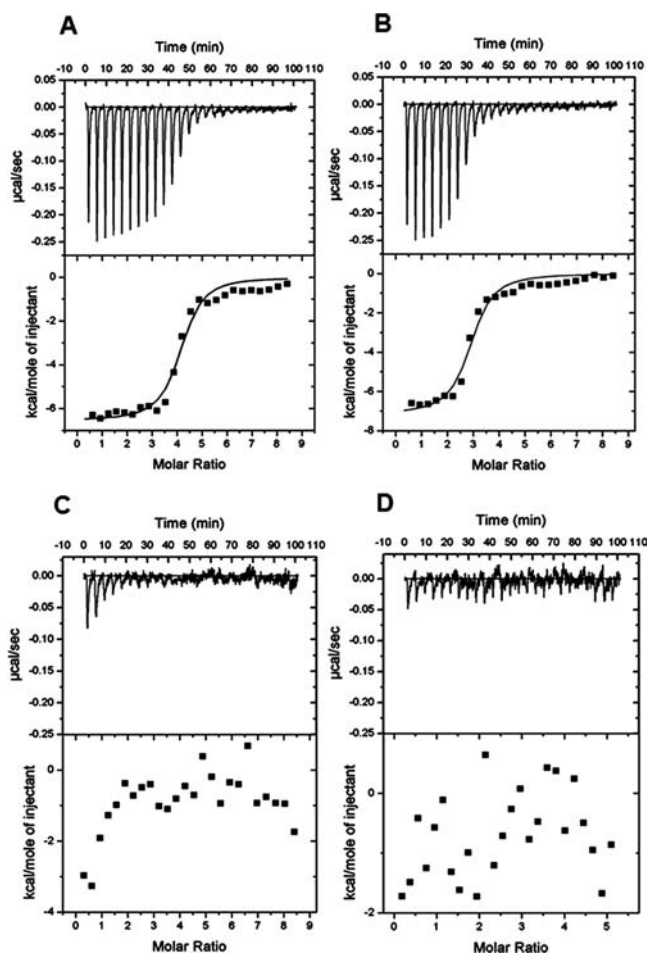


Figure 3. Calorimetric titration of nukacin ISK-1 and different ring A variants with lipid II. The titration cell was loaded with peptides, and 25 injections of 10 μL of lipid II (150 μM) were administered at 240-s intervals. The temperature was adjusted to 25 °C. The top graph shows the heat peaks after each injection. The bottom graph displays the integrated heat per injection, normalized to the injected amount of moles of lipid II, which is displayed against the molar ratio of lipid II versus the different peptides used. The corresponding chromatograms are (A) nukacin ISK-1 (3.4 μM), (B) nukacin D13E (3.4 μM), (C) nukacin D13A (3.4 μM), and (D) nukacin C14S (5.6 μM). The results were analyzed using the Origin software ver. 7 provided by MicroCal Inc.

the docking molecule, and then inhibits cell-wall biosynthesis, which might lead to the cessation of cell growth. This is the first report discussing the interaction of type-A(II) lantibiotics with lipid II, and it presents the first evidence that type-A(II) lantibiotics possess the lipid II-binding motif in their ring A structure. Therefore, our present findings will contribute to our understanding of the mode of action of this group of lantibiotics and their application to lantibiotic bioengineering.

■ ASSOCIATED CONTENT

📄 Supporting Information

Detail experimental procedures, Figures S1 and S2, and Table S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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