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# Haloduracin $\alpha$ Binds the Peptidoglycan Precursor Lipid II with 2:1 Stoichiometry

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Supporting Information

**ABSTRACT:** The two-peptide lantibiotic haloduracin is composed of two post-translationally modified polycyclic peptides that synergistically act on Gram-positive bacteria. We show here that Hal$\alpha$ inhibits the transglycosylation reaction catalyzed by PBP1b by binding in a 2:1 stoichiometry to its substrate lipid II. Hal$\beta$ and the mutant Hal$\alpha$-E22Q were not able to inhibit this step in peptidoglycan biosynthesis, but Hal$\alpha$ with its leader peptide still attached was a potent inhibitor. Combined with previous findings, the data support a model in which a 1:2:2 lipid II:Hal$\alpha$:Hal$\beta$ complex inhibits cell wall biosynthesis and mediates pore formation, resulting in loss of membrane potential and potassium efflux.

Inhibition of peptidoglycan biosynthesis is a common mode of action of many natural product antibiotics. Among the various ways of disrupting cell wall biosynthesis, sequestration of lipid II (Figure 1A) is particularly powerful. Lipid II is the substrate for the polymerases that generate the oligosaccharide chains of peptidoglycan. Bacterial resistance to compounds that bind to lipid II, such as nisin,[1] vancomycin,[2] and ramoplanin,[3−6] has been slow to develop, possibly because in comparison with other resistance mechanisms such as efflux pumps and enzyme mutations, it is more challenging to change the structure of an advanced intermediate that is biosynthesized in 10 steps.[7,8]

Several structurally diverse members of the lantibiotics have been reported to bind to lipid II.[1,9−13] Lantibiotics are ribosomally synthesized and post-translationally modified peptides characterized by thioether cross-links.[14] Two-peptide lantibiotics consist of two compounds that function synergistically to kill a range of Gram-positive bacteria.[15] In a recently proposed model for their synergistic activity, the $\alpha$-peptide binds to lipid II in stoichiometric fashion, generating a binding site for the $\beta$-peptide.[12,16] A 1:1:1 trimeric complex is then believed to form pores in the cell membrane, which results in the efflux of potassium and disruption of the membrane potential.[12] In this work, we evaluated this model with the two-peptide lantibiotic haloduracin and carried out structure−activity studies with haloduracin analogues. We show that the stoichiometry of binding lipid II by the $\alpha$-peptide of haloduracin is 1:2 (lipid II:Hal$\alpha$).

The two peptides that make up haloduracin are shown in Figure 1B.[17,18] Hal$\alpha$ contains several overlapping rings, including the B ring (residues 18−23) that is present in a variety of lantibiotics (including mersacidin[10] and lacticin 3147[19]) and has been proposed to be important for lipid II binding.[20,21] Hal$\beta$ has a more elongated structure and does not contain any overlapping rings (Figure 1B). To evaluate binding to lipid II, we used a previously reported in vitro assay that monitors the catalytic activity of PBP1b from *Escherichia coli*.[22] PBP1b uses lipid II as a substrate for glycan polymerization. Hal$\alpha$ inhibited PBP1b-catalyzed peptidoglycan formation using 4 $\mu$M heptaprenyl lipid II **1** (Figure 1A) with a half-maximal inhibitory concentration ($IC_{50}$) of 9.6 ± 0.4 $\mu$M (Figure 2). In contrast, Hal$\beta$ did not inhibit lipid II polymerization at concentrations up to 100 $\mu$M. We also tested a series of other post-translationally modified peptides as potential inhibitors of the polymerization process. The lantibiotics epilancin 15X,[23] lactocin S,[24,25] and cinnamycin[26,27] did not demonstrate any inhibitory activity at concentrations up to 200 $\mu$M. Similarly, the S-linked glycopeptide sublancin[28] did not inhibit lipid II polymerization at these levels. We therefore concentrated our further efforts on haloduracin.

A Hal$\alpha$ mutant in which Cys23 was mutated to Ala,[29,30] thereby disrupting the B-ring structure, still inhibited lipid II polymerization, albeit with a 5-fold increase in the $IC_{50}$ value (50.7 ± 1.7 $\mu$M) relative to wild-type (wt) Hal$\alpha$ (Figure S1 in the Supporting Information). Mutation of the highly conserved Glu22 within the B ring to Gln abolished inhibition at concentrations up to 100 $\mu$M. However, a C-ring Cys27 → Ala mutant did inhibit polymerization, but in a less potent manner than wt Hal$\alpha$ ($IC_{50}$ = 29.5 ± 3.5 $\mu$M; Figure S1). The B- and C-ring mutants were previously evaluated for their antimicrobial activity against *Lactococcus lactis* HP.[29,30] The combination of wt Hal$\alpha$ and Hal$\beta$ resulted in a minimum inhibitory concentration (MIC) of 0.039 $\mu$M, whereas the use of wt Hal$\beta$ with Hal$\alpha$-C23A or Hal$\alpha$-C27A yielded MIC values of 0.39 and 1.56 $\mu$M, respectively. It is not possible to compare directly the effects of these mutations on the antimicrobial activity and in vitro inhibition of lipid II polymerization because of different components that are present in each assay, including the membrane of whole cells in

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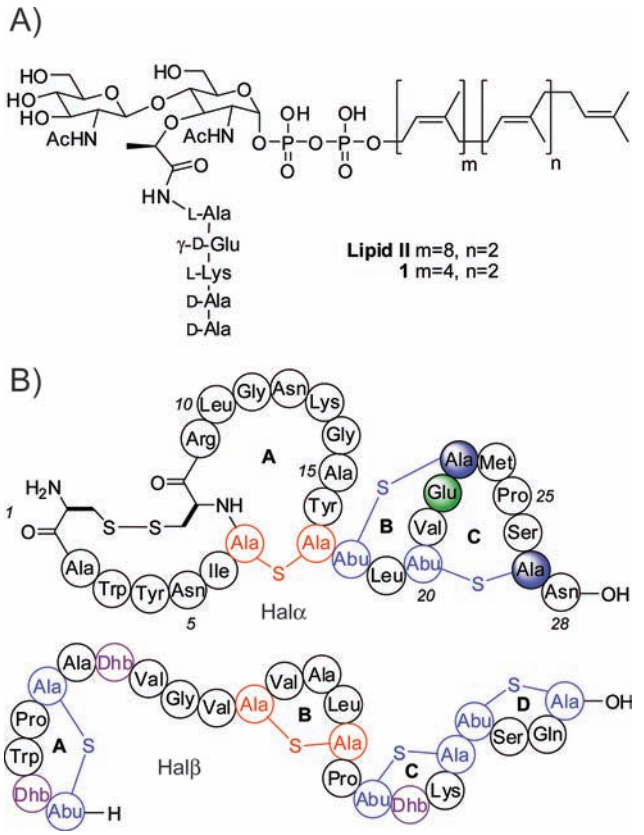


**Figure 1.** (A) Structures of lipid II and an analogue **1** used in this study with a shortened prenyl chain. (B) Structures of Hal$\alpha$ and Hal$\beta$. Shaded circles indicate residues mutated in this study. Abu, 2-aminobutyric acid; Dhb, dehydrobutyrine.

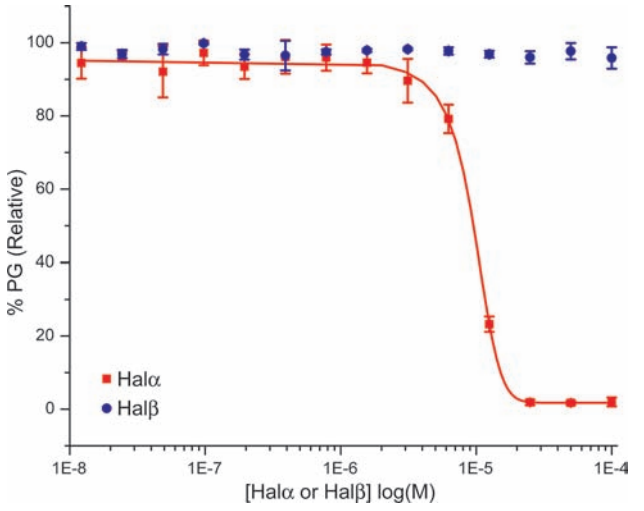


**Figure 2.** Inhibition of PBP1b-catalyzed formation of peptidoglycan (PG) by Hal$\alpha$ and Hal$\beta$. The lipid II concentration was 4 $\mu$M.

the antimicrobial assay. Nevertheless, the relative effects can be compared for each assay type. The larger deleterious effect on antimicrobial activity of the C27A mutation compared to the C23A mutation despite its higher affinity for lipid II suggests that disruption of the C ring has an additional deleterious effect on the interaction with Hal$\beta$ compared with disruption of the B ring.

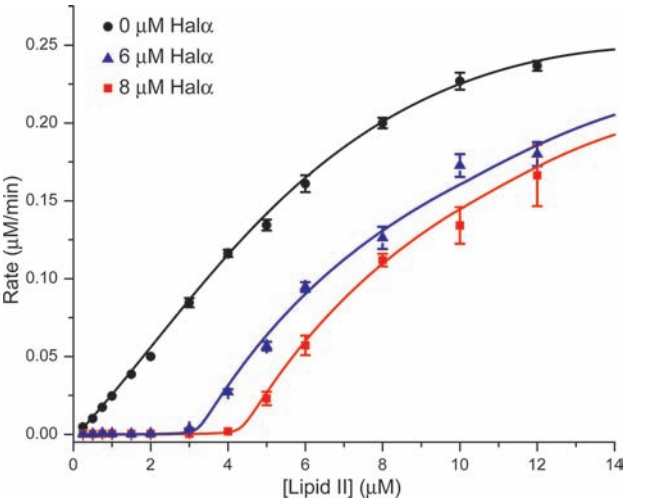


**Figure 3.** Kinetics of lipid II polymerization by PBP1b and inhibition of this process by Hal$\alpha$.

Conversely, the Hal$\alpha$-E22Q MIC of 1.56 $\mu$M when combined with wt-Hal$\beta$[30] was not expected given that the peptide did not inhibit in vitro polymerization. In the context of the membrane of whole cells and the presence of Hal$\beta$, the compound may regain some of its binding activity. Binding is still very weak, however, because the MIC of the combination treatment is only 4-fold lower than that of Hal$\beta$ by itself (6.25 $\mu$M).

The kinetics of the inhibition of the polymerization reaction catalyzed by PBP1b were examined next with wt Hal$\alpha$. As shown in Figure 3, the dependence of the reaction rate on the lipid II concentration exhibits Michaelis−Menten-like kinetics. In the presence of 6 $\mu$M Hal$\alpha$, the reaction was fully inhibited until the lipid II concentration exceeded 3 $\mu$M. Similarly, at a Hal$\alpha$ concentration of 8 $\mu$M, the reaction was completely inhibited until the lipid II concentration exceeded 4 $\mu$M. This type of behavior is similar to the inhibition of this process by ramoplanin[31] and indicates that Hal$\alpha$ forms a tight complex with lipid II with a 2:1 stoichiometry (Hal$\alpha$:lipid II).[32] This stoichiometry is reminiscent of the 2:1 ratio of nisin to lipid II in pores formed in bacterial membranes.[33] The data do not allow a precise determination of a $K_D$ for Hal$\alpha$ binding to lipid II,[34] but the inhibition curves in Figure 3 imply a nanomolar binding constant. Because previous work has demonstrated that like other two-peptide lantibiotics,[16] Hal$\alpha$ and Hal$\beta$ act in 1:1 stoichiometry,[30] the data further suggest that haloduracin inhibits peptidoglycan formation and causes pore formation by forming a lipid II:Hal$\alpha$:Hal$\beta$ complex with 1:2:2 stoichiometry.

The Hal$\alpha$ mutants used in this work were made using a previously described in vitro reconstituted biosynthesis.[17,29] In this process, the lanthionine synthetase HalM1 carries out a series of post-translational modifications on the HalA1 precursor peptide that result in the thioether cross-links shown in Figure 1B. The precursor peptide has an additional N-terminal extension of 41 amino acids called the leader peptide that is important for recognition by HalM1. In addition, the leader peptides of lantibiotic precursor peptides are generally believed to keep their products inactive while they are synthesized in the cytoplasm.[35−39] For haloduracin, the bifunctional protease/transporter HalT is believed to remove the leader peptide. HalT has not been investigated to date, but in a related system for the lantibiotic lacticin 481, the dedicated protease domain of the


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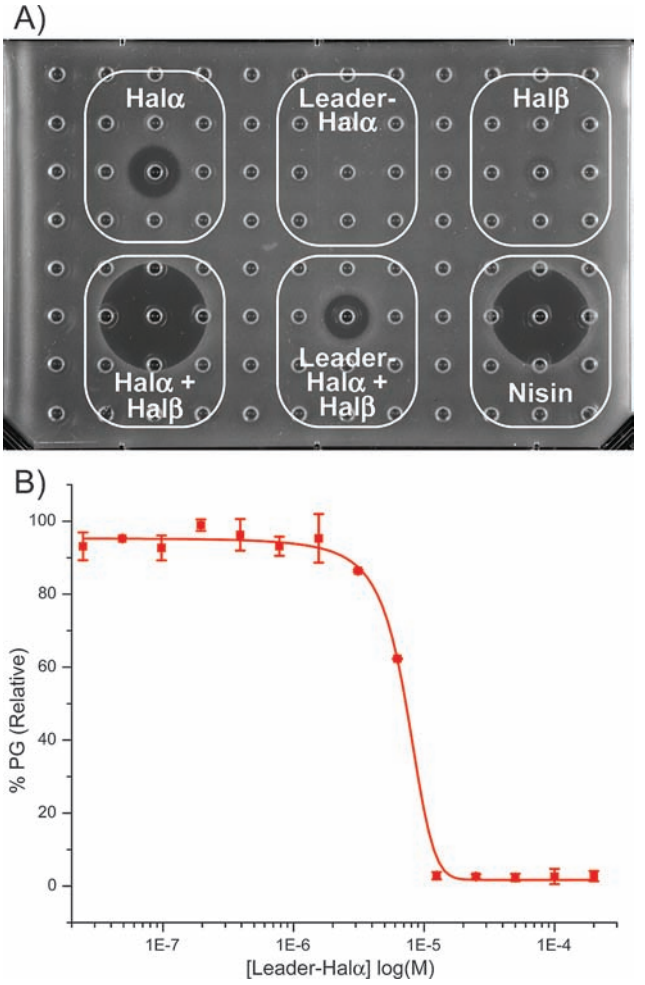


**Figure 4.** Evaluation of the antimicrobial activity and enzyme inhibitory activity of Hal$\alpha$ with its leader peptide attached. (A) Agar diffusion growth inhibition assay against *L. lactis* HP. Inhibitory activity was assessed using the individual peptides alone (50 $\mu$M Hal$\alpha$, 50 $\mu$M Leader-Hal$\alpha$, and 50 $\mu$M Hal$\beta$, upper row) and in combination (at 50 $\mu$M) with 50 $\mu$M Hal$\beta$ (lower row, left and center). Nisin was used as a control at a 50 $\mu$M concentration (lower right). (B) Inhibition of the PBP1b-catalyzed formation of peptidoglycan (PG) by leader-Hal$\alpha$.

transporter LctT removes the leader peptide of modified LctA precursor peptide and secretes the final product.[37,40] Given the common belief that lantibiotics with their leader peptides still attached are inactive, we were surprised to find that Hal$\alpha$ containing its leader peptide (leader-Hal$\alpha$) appeared to have antimicrobial activity against *L. lactis* HP when combined with Hal$\beta$ (Figure 4A). The activity is low relative to Hal$\alpha$ without its leader peptide attached, and antimicrobial activity of leader-Hal$\alpha$ was seen only in the presence of Hal$\beta$. We speculated that the indicator strain may secrete a protease that removes all or part of the leader peptide from a small subset of Hal$\alpha$ molecules, resulting in the observed activity. Alternatively, Hal$\alpha$ with its leader peptide attached may still engage with lipid II. To test the latter explanation, the polymerization assay was conducted in the presence of leader-Hal$\alpha$. Indeed, this peptide proved to be a potent inhibitor of lipid II polymerization with an $IC_{50}$ of 7.1 $\pm$ 0.2 $\mu$M (with 4 $\mu$M lipid II; Figure 4B), similar to the activity of wt-Hal$\alpha$. The weaker antimicrobial activity of leader-Hal$\alpha$ with wt Hal$\beta$ relative to Hal$\alpha$ combined with Hal$\beta$ (Figure 4A) is likely a consequence of the less optimal synergy between the two peptides when the leader peptide is still attached to Hal$\alpha$. Furthermore, the leader peptide of Hal$\alpha$ is highly negatively charged (four Glu, three Asp, one Arg, one Lys)[17,18] with a stretch of four negatively charged residues near the junction between the leader peptide and the core peptide. These negative charges are likely to significantly weaken the binding of leader-Hal$\alpha$ to lipid II in the context of a negatively charged membrane, explaining why the antimicrobial activity in Figure 4A is weaker than anticipated on the basis of the strong inhibition of the polymerization process by leader-Hal$\alpha$.

In summary, this study has shown that Hal$\alpha$ inhibits PBP1b by binding to its substrate lipid II in 2:1 stoichiometry. Glu22 is essential for this interaction, and the B and C rings are important but not critical. Attachment of the leader peptide does not prevent Hal$\alpha$ from binding to lipid II, but because the leader is removed during secretion, lipid II does not encounter leader-Hal$\alpha$ in the context of the producer strain. In combination with previous studies, the results presented here suggest that halodur-acin's antimicrobial activity is achieved in 1:2:2 lipid II:Hal$\alpha$:Hal$\beta$ stoichiometry.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Procedures for preparation of Hal$\alpha$ and Hal$\beta$ and their derivatives, preparation of the lipid II substrate **1** and PBP1b, and procedures for the transglycosylase and antimicrobial assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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