General Discussion

Liang Zhou¹, Auke J. van Heel¹, Oscar P. Kuipers¹

¹Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, the Netherlands
The rising resistance of specific pathogens to existing antimicrobial agents has made the search for new antibiotics essential. Currently, 24 new anti-Gram-negative agents (some are also active against MRSA) are in clinical trials and might reach the market in the near future (Bush, 2015). Of these agents, some are novel to the known antibiotic classes, such as mimetics of host defense peptides (in phase 2 trial), bridged diazabicyclooctane β-lactamase inhibitors (in phase 1, 2 and 3 trial) and unique bacterial topoisomerase inhibitors (in phase 2 trial). However, more new agents are still needed to fight resistant pathogens. Lantibiotics are posttranslationally modified peptides, with a broad inhibition spectrum against Gram-positive bacteria (Bierbaum and Sahl, 2009). The inhibition mechanism of most lantibiotics includes binding to the lipid II molecule to inhibit the cell wall synthesis and forming a pore complex together with lipid II in the membrane (Breukink and de Kruijff, 2006). The inhibition is efficient, as a minimal inhibition concentration at the nanomolar range has been observed. Specific resistances to lantibiotics are rarely reported (Kramer et al., 2006; Majchrzykiewicz et al., 2010a; Draper et al., 2015). For these reasons, lantibiotics are good candidates to expand our antimicrobial arsenal. The safety of nisin as food preservative has been demonstrated in practice. Cytotoxicity of nisin was shown to be low towards intestinal epithelial cells, as the concentration that caused hemolysis (89.9-132.2μM) was much higher than the antimicrobial concentration needed (Maher and McClean, 2006). It was demonstrated recently that nisin can efficiently inhibit the growth of oral bacteria and disrupt biofilm formation without causing cytotoxicity to human oral cells (Shin et al., 2015). This indicates that nisin is an efficient but low-toxicity antimicrobial agent, and engineering of nisin can be of great value. This thesis consists of two parts; the first describes an in vitro study of NisB and
the prenisin complex to gain mechanistic insight in the biosynthesis machinery, the second part describes three different engineering studies of nisin.

The nisin modification- and production system is the most studied system compared to other lantibiotics synthesis systems. Fully understanding the mechanism behind the modification machinery and developing this system to be used to produce more lantibiotics or other lanthipeptides are of great value. NisB is the first enzyme needed for the maturation of nisin. The catalytic mysteries of NisB and NisC and the interactions between the nisin leader peptide and NisB were clearly revealed when the structures of NisAB and NisC were published (Ortega et al., 2015; Li et al., 2006). However, the interactions between NisA, NisB and NisC, i.e. how NisB and NisC cooperate to complete the modifications are not totally clear. Moreover, NisT is an ABC transporter located in the membrane (Kuipers et al., 1993a, Kuipers et al., 2004), but whether there are interactions between NisB/C and NisT during modification and the location of these enzymes in the cell is still an open question. In Chapter 2, a NisB and prenisin complex was purified from the cell extract of L. lactis, by a nisin precursor his-tag and NisB-strep-tag co-purification strategy. Cryo-EM was used for analysis of the structure, as previously many attempts for crystallization failed. This purification method might also be used to purify the prenisin and NisC complex or prenisin, NisB and NisC complex. These complexes could then also be analyzed by cryo-EM, although the small size of the complex is still a challenge in this. It would be very handy if the nisin modification system could be constructed outside the cell to perform the synthesis of lantibiotics in vitro. This needs a large amount of purified NisB and NisC. However, the bulkiness and instability of NisB might make the purification of NisB difficult. If NisB could be engineered to remove the unstable regions (e.g. the regions in front of the last 220 or 100 residues) or if NisB could be divided into several active parts (e.g. the glutamylation part and
the glutamate elimination part), the reconstituted activity of NisB might be applied for *in vitro* biosynthesis, if also the important role of the leader binding is taken care of. Studies have shown that if the leader peptide of lacticin 481 was added *in trans*, the core peptide was almost fully modified. And when the leader peptide was attached to LctM by a long linker, the substrate could be fully modified (Oman et al., 2012). The leader peptide of nisin was also added *in trans* with the core peptide, and the core peptide was partially dehydrated (5 times) (Khusainov and Kuipers, 2012). This indicates that the leader peptide of nisin might also be linked to NisB (or only the glutamylation part of NisB) and NisC to perform the modifications on peptides *in vitro*.

Nisin binds to lipid II in the cell membrane to inhibit cell wall synthesis and bends into the membrane to form pores, which causes leakage of essential compounds out of the cell and kills the bacteria (Breukink et al., 1999). The amino acid composition of the hinge region and the C-terminus of nisin are very crucial for the pore forming activity. In Chapter 3, the hinge region of nisin was either truncated or elongated. The one or two amino acids elongated variants exhibited only modestly reduced activity in liquid culture but showed better activity in agar well diffusion assay. If the hinge region could be elongated with more suitable amino acids, higher activity against specific indicators might be obtained. For example, the original amino acids NMK could be repeated to create new hinge regions. Moreover, the intertwined rings of nisin might be modified (*e.g.* elongated to enlarge the ring D or/and ring E) to test the effects on the activity. The last six amino acids of nisin could also be shortened or elongated to kill bacteria with different membrane thicknesses. Furthermore, the pore formation activity of other lantibiotics could also be modified by changing the length of the peptides. For instance, epidermin and gallidermin (Bonelli et al., 2006) possess a shorter C-terminus and they could only form pores when the target bacteria have a thinner membrane. If these peptides could be elongated,
their pore formation activity against bacteria with a thicker membrane might be improved.

Improving the activity of lantibiotics to inhibit the growth of Gram-negative bacteria more efficiently could be a novel method to control Gram-negative pathogens. In Chapter 4, several anti-Gram-negative peptides were successfully fused to nisin or a part of nisin, and one fusion (GNT16) showed increased activity compared to nisin against Gram-negative pathogens. Although the outer membrane traversing activity of GNT16 might be increased, the inhibitory activity (lipid II binding and/or pore formation activity) was reduced. GNT7 showed higher activity against *L. lactis* than GNT16-3rings, which indicates that the tail from oncocin can perform better pore formation activity than the tail from apidaecin 1b. However, the trans-outer membrane efficiency of GNT7 is lower than that of GNT16. This indicates that to create new lantibiotics that are active against Gram-negative pathogens, increasing the outer membrane traversing activity and maintaining the inhibitory activity in the inner membrane are both important. To create more positive variants in the future, both activities have to be considered. Figure 1 shows different designs of the nisin and anti-Gram-negative peptides fusions. Design A is a successful design with the GNT16 as an example. The only drawback of this design is that the tails were easily degraded or cleaved off from the nisin part, and a full length peptide was rarely obtained. In Chapter 4, the anti-Gram-negative tail was directly added behind the hinge region (Group 1 peptides), but the activity of the fusion peptide (*e.g.* GNT16-3rings) was lower than that of design A (*e.g.* GNT16); this might be because of a low pore formation activity. Design B has added the ring D of nisin and an extra glycine to the Group 1 peptides, which might be better for the pore formation activity. To increase the pore formation activity of the fusions, the C-terminal region of nisin might be maintained. In this case, the anti-Gram-negative peptide could be inserted inside nisin, either behind the
hinge region (design C) or in front of the last six amino acids of nisin (design F). This might also reduce the chance of being cleaved by proteases. To make the peptides shorter and easier to be overproduced, the intertwined rings could be shortened (design D, G and H), or deleted (design E).

Figure 1. Design of the fusions of nisin and anti-Gram-negative peptides. Dark blue solid circle indicates a modified threonine; light blue solid circle indicates a modified serine; the green rectangle indicates an anti-Gram-negative peptide.

Incorporation of non-canonical amino acids into lantibiotics is a novel method to diversify the structure of lantibiotics and find variants with improved
antimicrobial activities and other properties. As the analogues have one or more
different groups compared to normal amino acids, they normally have special
physicochemical properties. When these analogues are incorporated into
lantibiotics, the characters of lantibiotics could be modified. And this could be
used, when there is any special need for particular properties. Compared to the
normal mutations between different amino acids, making use of the analogues
could adjust the composition of the peptide in a more refined way. This could
slightly change the structure of lantibiotics to modify the interactions between
peptides and target molecules. The incorporation system constructed in Chapter
5 can introduce various tryptophan analogues into nisin or nisin variants. The
tryptophan auxotrophic strain used is also methionine auxotrophic, so Met
analogues could also be incorporated. Furthermore, more different auxotrophic
strains could be constructed to incorporate diverse non-canonical amino acids
into lantibiotics. In the meantime, this system is also a lantibiotic production
system, so a synthetic biology strategy could be applied to combine other
modification enzymes into this system and make sure different types of
modifications and incorporations work efficiently. This strategy could also be
applied beyond the scope of antimicrobial peptides, such as other medical
peptides or industrial enzymes to obtain better activity or stability.

In summary, the engineering of nisin and other lantibiotics can be conducted in
various ways. The goal would be to predict the effect certain modification or
mutation have in terms of relevant properties and to further improve their
activity and alter their spectrum. For instance, fusing lantibiotic core parts to
anti-Gram-negative peptides could aid the activity of lantibiotics against
Gram-negatives; introducing a hydroxyl to the peptide could reduce the affinity
of the peptide to a chromatography column; mutations containing more lysines
possess higher solubility (Rollema et al., 1995; Yuan et al., 2004). However,
more engineering is needed to fully understand the structure and activity
relationship of these antimicrobials, to improve the properties of existing lantibiotics, and design and produce new highly potent antimicrobial peptides.