Lipid II as a target for antibiotics

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Abstract | Lipid II is a membrane-anchored cell-wall precursor that is essential for bacterial cell-wall biosynthesis. The effectiveness of targeting Lipid II as an antibacterial strategy is highlighted by the fact that it is the target for at least four different classes of antibiotic, including the clinically important glycopeptide antibiotic vancomycin. However, the growing problem of bacterial resistance to many current drugs, including vancomycin, has led to increasing interest in the therapeutic potential of other classes of compound that target Lipid II. Here, we review progress in understanding of the antibacterial activities of these compounds, which include lantibiotics, mannopeptimycins and ramoplanin, and consider factors that will be important in exploiting their potential as new treatments for bacterial infections.

Since the discovery of penicillin more than 75 years ago, antibiotics have had an immense impact on the treatment of infections caused by bacteria. However, the widespread, and sometimes inappropriate, use of antibiotics has generated a strong evolutionary pressure for the emergence of bacteria that either have an inherent resistance to a particular antibiotic or have the capacity to acquire such resistance. Consequently, today there is no antibiotic in clinical use to which resistance has not developed, and so there is a pressing need to discover and develop new agents that are active against resistant bacteria.

However, the recent strategy for finding new classes of antibacterial compounds based on targets identified from bacterial genomics has not yet proved successful. This has led to renewed interest in natural products, which have historically been invaluable as a source of antibacterial drugs, such as penicillins, macrolides and glycopeptides, reflecting their evolutionary origin as 'weapons' that bacteria use against each other. One glycopeptide, vancomycin, had long been reliable in treating infections caused by bacteria resistant to several other antibiotics, such as the 'super bug' methicillin-resistant *Staphylococcus aureus* (MRSA), but even vancomycin resistance has now become quite common[1], further underlining the need for new antibiotics.

Vancomycin was the first discovered example of a compound that kills bacteria by targeting Lipid II, a bacteria-specific membrane component that is essential in bacterial cell-wall synthesis (BOX 1). It has recently been demonstrated that Lipid II is the target of several other classes of natural products, including the lantibiotics, the mannopeptimycins and ramoplanin, which intriguingly all act differently on Lipid II. These compounds are attracting increasing attention given the need for novel antibacterial drugs, and here we review their mode of action and their antibacterial activities, and use this as a basis to discuss their potential as novel drugs for combating antibiotic-resistant bacteria.

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doi:10.1038/nrd2004
Published online 10 March 2006

The role of Lipid II in cell-wall synthesis

The cell wall (FIG. 1) of all bacteria comprises a polymer of alternating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc)[2]. These glycan polymer chains are cross-linked by a pentapeptide typically with the sequence L-alanyl-γ-D-glutamyl-diaminopimelyl (or L-lysyl)-D-alanyl-D-alanine that is attached to the MurNAc sugar, and it is this cross-linking that gives the cell structural rigidity and mechanical strength.

The assembly of the cell-wall subunit begins on the cytoplasmic side of the plasma membrane of the cell, where UDP-MurNAc-pentapeptide is coupled to bactoprenyl-phosphate to yield Lipid I. Next, coupling of the GlcNAc sugar by the peripherally membrane-associated protein MurG produces Lipid II, which therefore contains the complete peptidoglycan subunit linked via a pyrophosphate to the membrane-embedded polyisoprenoid anchor. Lipid II is then translocated to the periplasmic or exterior side of the plasma membrane via an unknown mechanism.

The cell wall in Gram-positive bacteria is generally around 20 layers thick, with each layer comprising vast amounts of peptidoglycan subunits. By contrast, only a small amount of bactoprenyl phosphate molecules are present (about 2×10^5 molecules per cell[3,4]), so the amount of Lipid II that can be synthesized is limited. Although the peptidoglycan layer of Gram-negative bacteria is much smaller (average thickness of about 1.5 layers[5]), only 2,000 molecules of Lipid II per cell are

Box 1 | **Glycopeptide antibiotics**

Vancomycin, the first glycopeptide antibiotic discovered, was isolated from the soil bacterium *Amycolatopsis orientalis* in 1956 (REF. 84). The peptide backbone of vancomycin is synthesized non-ribosomally and comprises some non-proteinogenic amino acids, such as hydroxyphenylglycine (Hpg) and dihydroxyphenylglycine (Dhpg). The structural rigidity of vancomycin is afforded by the cross-linking of the heptapeptide core (Leu-Tyr-Asn-Hpg-Hpg-Tyr-Dhpg). Oxidative phenolic coupling of the aromatic rings of residues 2, 4 and 6 (REFS 85–87; FIG. 2) forms a triphenyl ether functionality; the aromatic rings of residues 5 and 7 are also linked. Vancomycin is also glycosylated, and has a disaccharide chain of glucose and vancosamine attached at position 4. These sugar units are important for the dimerization of this antibiotic, which is believed to be important in the mode of action of this class of antibiotic.

In 1965, Strominger[88] and colleagues showed that vancomycin elicits its bactericidal response by targeting bacterial cell-wall synthesis. Later, vancomycin was shown to specifically interact with the D-Ala-D-Ala of the pentapeptide chain of cell wall precursors[89,90]. Taken together with the finding that radiolabelled vancomycin does not enter bacterial cells (the size of vancomycin is thought to preclude its entry into cells)[91], these results suggested that vancomycin binds to Lipid II. Definitive proof that vancomycin binds to D-Ala-D-Ala came from NMR studies on the vancomycin-D-Ala-D-Ala complex[86,92]. However, these structural studies used synthetic penta- or tripeptide derivatives of the Lipid II molecule. A recent study using water-soluble Lipid II, which is a closer model of the cellular target, showed that glycopeptide antibiotics interact differently with this synthetic target compared with D-Ala-D-Ala[93].

The emergence of vancomycin-resistant bacteria has highlighted the need for the development of second-generation glycopeptide antibiotics. Three new glycopeptide antibiotics — dalbavancin[94], telavancin[95] (TD-6424) and oritavancin[96] — have been developed. These antibiotics have been generated by the addition of a hydrophobic moiety to the disaccharide functionality of vancomycin, with oritavancin and telavancin being modified at the vancosamine nitrogen, and dalbavancin at the glucosamine nitrogen. This structural modification renders these antibiotics active against vancomycin-resistant bacteria. The FDA has designated the New Drug Application (NDA) of dalbavancin as approvable (see Pfizer dalbavancin approval, Further information), and telavancin is being 'fast-tracked' and is in Phase III development (see Theravance telavancin development, Further information). The rights for oritavancin are now owned by Targanta Therapeutics and an NDA filing is being planned for late 2007 (see Targanta pipeline, Further information).

involved in cell-wall synthesis. Therefore, the Lipid II cycle described above should be conceived of as a dynamic process, with each Lipid II molecule in the cell having a high turnover rate (a rate of 1–3 transits per second per prenyl chain has been estimated[6]) in both Gram-positive and -negative bacteria. The Lipid II cycle can therefore be regarded as a bottleneck in bacterial cell-wall synthesis, and so Lipid II is an ideal target for antibiotics. However, targeting this cycle is hindered by the development of resistance to the lantibiotics, the key mechanism of which seems to be modifications in cell-wall structure that 'protect' Lipid II from antibiotic action (see section on resistance).

Different classes of antibiotics form various interactions with Lipid II. For example, the glycopeptide vancomycin (FIG. 2), which was the first antibiotic identified as targeting Lipid II, binds to the carboxy (C)-terminal Lys-D-Ala-D-Ala of the pentapeptide, thereby blocking cell-wall biosynthesis (FIG. 1). However, this is not the only type of interaction formed by antibiotics with Lipid II. Lantibiotics, mannopeptimycins and ramoplanin (FIG. 2) each interact with Lipid II in different ways, and the current understanding of these interactions, and the implications of these interactions, for the mode of action of these agents will be considered.

The lantibiotic family

The lantibiotics are a large family of peptide antibiotics. Their precursors are biosynthesized ribosomally, and the unusual amino acids, such as lanthionine and dehydrated serine residues, present in their structures are the result of post-translational modifications and proteolytic processing of the gene-encoded precursor peptides[7] (FIG. 3).

Since the discovery of the first lantibiotic, nisin (FIG. 2), in the 1920s, almost 50 different lantibiotics have been characterized from about 30 different bacteria, including lactic-acid-producing bacteria and *Streptomyces* spp., and new lantibiotics continue to be identified[8]. Screening of natural products typically identifies members of the lantibiotic class as having potent antibacterial activity, but so far none has made it to the clinic, for reasons such as poor pharmacokinetics that will be discussed in more depth later.

The lantibiotic family is subdivided into two groups — the type A and B lantibiotics — that comprise peptides with straight-chain and globular structures, respectively. Most, if not all, lantibiotics target components of the plasma membrane of bacteria, with more than one-third of the known lantibiotics targeting Lipid II.

Nisin and Lipid-II-interacting type A lantibiotics. Nisin (FIG. 2) is the oldest known and most extensively studied lantibiotic[9,10]. Produced by some strains of *Lactococcus lactis*, this lantibiotic is 34 amino acids in length and has an overall positive charge (+4). Of the five lanthionine-based rings in the peptide core of nisin, the first three are separated from the intertwined rings four and five by a relatively more flexible hinge region of three residues. Nisin is produced in an inactive precursor form that has an amino (N)-terminal leader peptide that is cleaved during the secretion of nisin out of the cell (FIG. 3). Because of its potent bactericidal activity and low toxicity in humans, nisin is used as a natural preservative in the food industry[11].

There has been controversy about the mode of action of nisin in the past. At first, it was suggested that nisin killed bacteria by inhibiting cell-wall synthesis via binding to Lipid II[12,13]. However, the treatment of Gram-positive bacteria with nisin resulted in the rapid outflow of small cytoplasmic compounds such as amino acids, ATP or pre-accumulated rubidium from cells, as well as the collapse of vital ion gradients[14,15], showing that this compound acts by perturbation of the plasma membrane.

In attempts to explain these phenomena, the nisin–membrane interaction was extensively studied, with a focus on the nature of the interaction of this cationic peptide with the abundant anionic lipids of the plasma membrane of Gram-positive bacteria[16–19]. However, these *in vitro* studies with model membrane systems failed to bridge the gap between nisin activity in these experiments, which was in the micromolar range, and the *in vivo* activity of nisin, which is in the nanomolar range. It is only more recently, since it has been shown that nisin uses Lipid II as a 'docking molecule' to form pores in a targeted manner with high efficiency[20,21], that the findings have been reconciled. In the presence of Lipid II, the

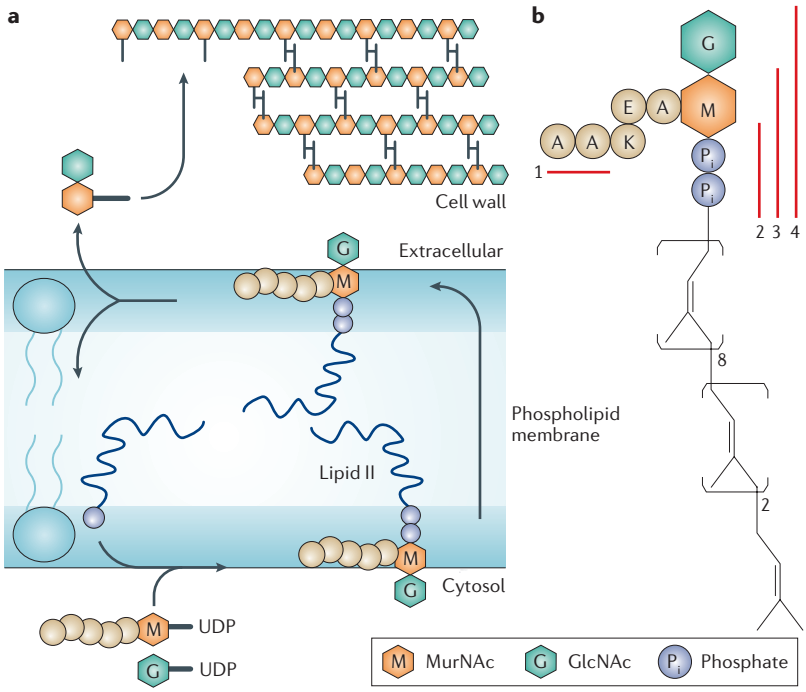

Figure 1 | **Schematic presentation of the cell-wall synthesis cycle. a** | Assembly of the cell-wall subunit takes place on the cytosolic side of the bacterial plasma membrane. UDP-activated precursor sugars are assembled on a polyisoprenoid carrier, the coupling of which produces Lipid II, which is then transported across the membrane by an unknown mechanism. Next, the peptidoglycan subunit is transferred to the growing peptidoglycan chain and the polyisoprenoid carrier is recycled back to the cytoplasmic side to complete the cycle. **b** | Structure of Lipid II. The polyisoprenoid anchor consists of eight isoprene units in the *cis*-conformation followed by two units in the *trans*-conformation and the terminal isoprene unit. Depicted here is a Lipid II carrying a pentapeptide with a lysine at the third position. Together with diaminopimelic acid, this is the most common residue at this position. The third amino acid of the pentapeptide is coupled to the glutamate at position two via the side chain carboxylate. The red bars indicate the minimal binding sites in Lipid II of glycopeptide antibiotics (1), nisin (2), ramoplanin (3) and mersacidin (4). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

activity of nisin in model membrane systems increased by three orders of magnitude, which is comparable to the activity of nisin against susceptible bacteria.

So, nisin can permeabilize membranes by two different mechanisms: through a low-affinity permeation mechanism that is only observed in model systems; and through a much more efficient Lipid-II-dependent targeted pore-formation mechanism. In the first mechanism, which requires micromolar concentrations of nisin and the presence of anionic lipids in the target membrane, nisin binds to the anionic lipids, and then inserts between the phospholipid head groups. The accumulation of nisin in the outer lipid monolayer of the target membrane drives aggregation of nisin monomers, which is followed by formation of short-lived pore-like structures. Relaxation of the pore-like structure can lead to translocation of the peptides across the lipid bilayer[22,23]. This is similar to the mechanism used by other positively charged antimicrobial peptides of animal origin, such as magainin (for a recent review see REF. 24).

The second mechanism of action of nisin, which operates in the presence of Lipid II in the membrane (as in Gram-positive bacteria), can be described as follows: specific recognition and binding of Lipid II, followed by assembly and pore formation (FIG. 4). It should be stressed that if nisin can interact with Lipid II in the target membrane of a bacterium, it will follow the Lipid-II-dependent pathway for pore formation, as the affinity of nisin for Lipid II is much higher (2×10^7 M^{-1} (REF. 21)) than the affinity of nisin for membranes containing anionic lipids (about 1,800 M^{-1} (REF. 18)). Support for this comes from the observation that in the presence of Lipid II, nisin activity was not dependent on the membrane lipid composition with respect to anionic lipid content over a broad concentration range[25]. Furthermore, several experiments have shown that the pores formed by nisin in the presence of Lipid II are much more stable than pores formed in the absence of the receptor[26–29]. Nisin has a stable transmembrane orientation in the presence of Lipid II, indicating that stable pores are formed[28] and also indicating a role for Lipid II in the nisin-mediated pore-formation that is not restricted to a receptor function. Indeed, two independent approaches have shown that Lipid II is a constituent of the pore complex[30,31], the stoichiometry of which is four Lipid II molecules and eight nisin molecules[32] (FIG. 4).

The recent solution of the NMR structure of a nisin–Lipid II complex at atomic resolution[33] revealed that nisin contains a new motif — a pyrophosphate cage (FIG. 5) — that binds the pyrophosphate of Lipid II; the structure also clarified the purpose of the post-translational modifications of nisin and related lantibiotics. The pyrophosphate cage, involving hydrogen bonds between the amide groups of the nisin backbone and the pyrophosphate moiety of Lipid II, is formed by rings A and B of nisin, with the N terminus folding back towards ring B, allowing the side chain of the first residue to interact with the B ring (FIG. 5).

The first two rings of several other type A lantibiotics are highly homologous to those in nisin, suggesting that these peptides also interact with Lipid II. The closest relative of nisin, subtilin, differs at three places in the first eleven residues of the sequence. However, these three places are not key positions, and so the cage structure remains intact and only the side chains differ. Members of the epidermin family are much shorter, but have an almost fully conserved B ring and an A ring of similar size. Also noticeable is that two amino acids are always present before the first lanthionine ring, which seem essential to form the cage structure: the first amino acid is required for formation of a side-chain interaction with ring B, and the backbone NH of the second is part of the pyrophosphate cage. Indeed, removal of the first two residues resulted in an almost 700-fold loss of activity of subtilin towards *Micrococcus luteus*[34]. For two lantibiotics of the epidermin family, it has been shown that they indeed interact with Lipid II[20,33]. Any newly discovered lantibiotic containing a similar A/B ring structure to nisin can therefore be classified as a Lipid-II-targeting lantibiotic.

Besides binding of the pyrophosphate via hydrogen bonds, the crystal structure of the nisin–Lipid II complex also revealed minor interactions of nisin with the first isoprene unit and the MurNAc sugar. No interaction

of nisin with the GlcNAc moiety of Lipid II could be observed, which explains why nisin does not discriminate between Lipid I or II[20,31]. Nisin could therefore potentially target both Lipid I and Lipid II, but only Lipid II is present on the extracellular side of the plasma membrane and therefore available for binding by nisin. In addition, no interaction of nisin with the amino acids of the pentapeptide was observed, which explains why nisin is still active against bacteria that express the *vanA* type of resistance, which changes the terminal D-Ala of the pentapeptide of Lipid II into a D-Lac, resulting in high levels of vancomycin resistance[35].

Whereas the N terminus of nisin is important for the interaction with Lipid II, the hinge region (and most likely also the C terminus) seems to have an important function in the pore-formation process, because mutations in the hinge region primarily affect the ability of these variants to form pores[29,32]. Experiments with pyrene-labelled Lipid II showed that a hinge-region mutant of nisin was trapped in a pre-pore-like complex, which is an assembly of multiple nisin and Lipid II molecules (probably with the same stoichiometry as the pore complex), but which is not membrane-embedded[32] (FIG. 4).

The targeted pore-formation mechanism of action of nisin and similar lantibiotics is the only example of its type described so far. However, lantibiotics could also exert their bactericidal effects through another Lipid-II-dependent mechanism. Studies on the effects of mutations in the hinge region on the activity of nisin showed that, although the mutated antibiotic was no longer able to initiate pore formation, the minimum inhibitory concentration (MIC) values of these compounds were only reduced by a factor of 3–8 with respect to wild-type

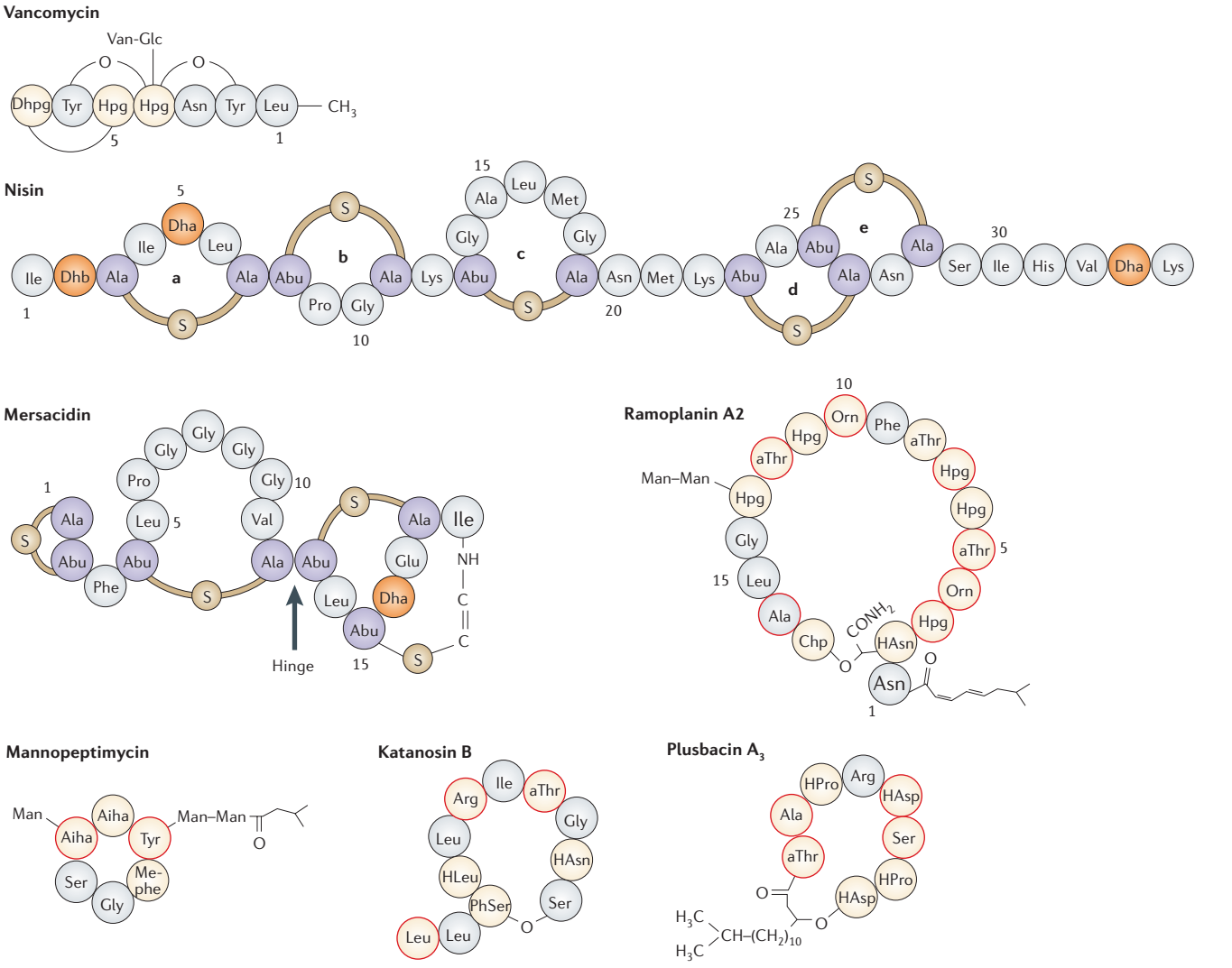

Figure 2 | **Structures of antibiotics that target Lipid II.** For all antibiotics shown, D-amino acids are colour-coded red. In the structure of nisin, the five lanthionine rings are labelled **a**–**e** from the amino terminus. In the structure of mersacidin, the arrowhead indicates the location of the hinge region that was revealed by NMR. Abu, aminobutyric acid; Aiha, α-amino-β-[4′-(2′-iminoimidazolidinyl)]-β-hydroxypropionic acid; aThr, *allo*-threonine; Chp, 3-chloro-4-hydroxyphenylglycine; Dha, didehydroalanines; Dhb, didehydrobutyrines; Dhpg, dihydroxyphenylglycine; Glc, glucose; HAsn, β-hydroxyasparagine; HAsp, β-hydroxy-aspartic acid; Hleu, β-hydroxyleucine; Hpg, hydroxyphenylglycine; HPro, 3-hydroxyproline; Man, mannose; Me-phe, β-methylphenylalanine; Orn, ornithine; PhSer, β-phenylserine; Van, vancosamine.

nisin[25]. In addition, the epidermin lantibiotics bind Lipid II, but these antibiotics are of insufficient length to span the membrane. Mutacin 1140, a member of the epidermin class, cannot induce leakage from model membrane systems in the presence of Lipid II, but it does inhibit the Lipid II-dependent pore-forming activity of nisin: subsequent additions of nisin did not result in any leakage (E.B., unpublished data). However, the MIC values of mutacin 1140 are in the same nanomolar range as those of nisin. These observations point to an alternative Lipid-II-dependent mechanism of action that does not involve pore-formation that has yet to be characterized; so far no mechanism has been proposed for this action.

Mersacidin and related type B lantibiotics. Members of the type B lantibiotics also trigger bacterial cell death via an interaction with Lipid II, thereby inhibiting cell-wall synthesis[36]. However, this interaction does not lead to pore-formation. The paradigm for these lantibiotics is mersacidin[36] (FIG. 2). Because the binding site of mersacidin on Lipid II includes the terminal GlcNAc sugar, mersacidin does not bind to Lipid I[36]. The affinity of the interaction of mersacidin with Lipid II is also much lower than that of nisin[36,37]. Mersacidin does not contain a positively charged amino acid, and even has one negatively charged glutamate residue (position 17, see FIG. 2). Therefore, direct charge interactions with Lipid II

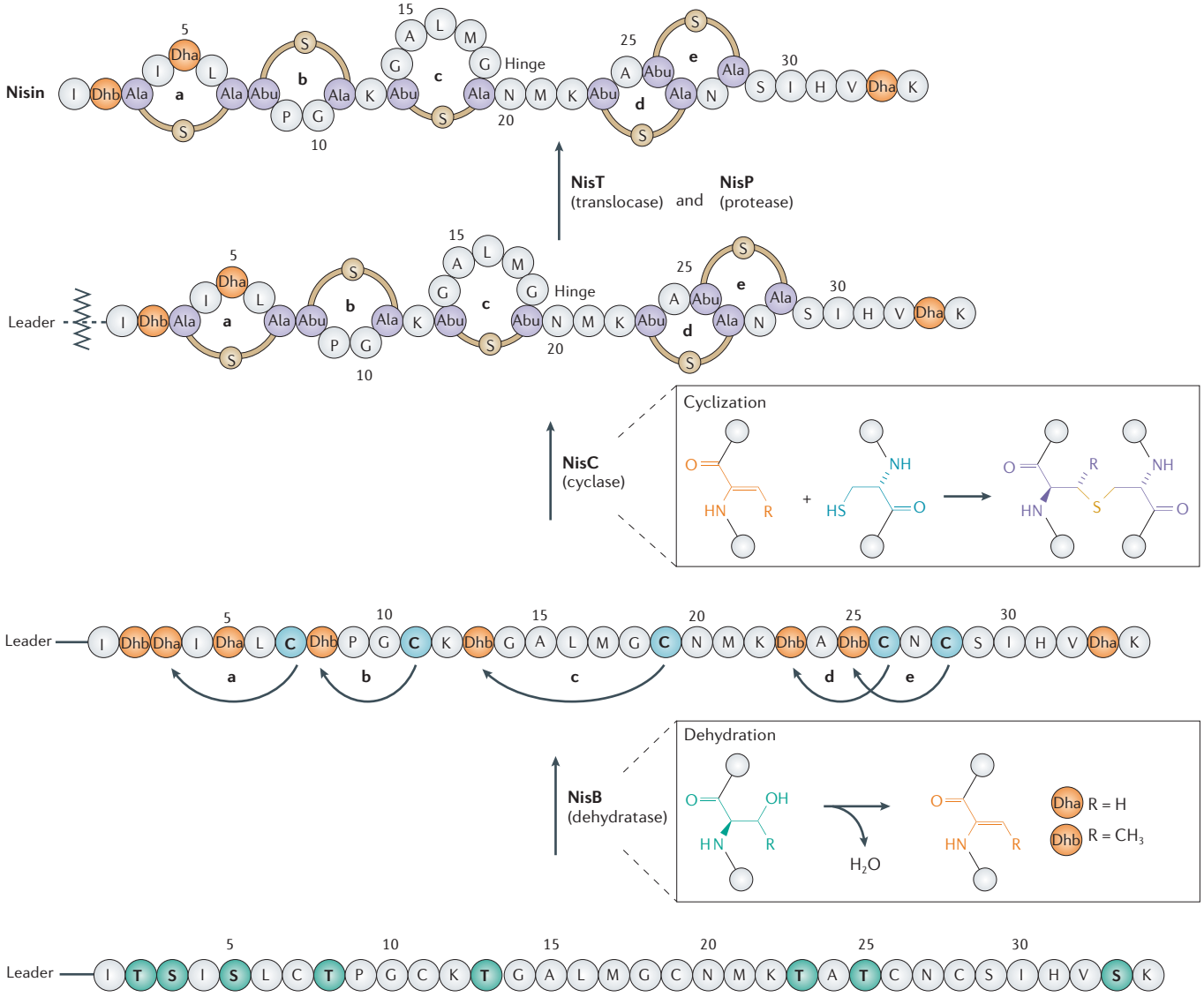

Figure 3 | **Post-translational modifications in nisin.** Like all other lantibiotics, nisin is produced in an inactive precursor form containing a leader peptide that directs it to the modification and transport machinery. The modifications are performed in two steps. First, dehydration of the serines and threonines (green) by NisB affords didehydroalanines (Dha) and didehydrobutyrines (Dhb), respectively (orange). Next, NisC catalyses the cyclization of the didehydro amino acids with cysteine residues that are located downstream (towards the carboxy terminus), which forms the lanthionine rings (Ala-S-Ala = lanthionine; Abu-S-Ala = methyl-lanthionine). During the dehydration and cyclization steps the stereochemistry of the serine (or threonine) residue is changed from the L- to the D-form. Only when the modifications are complete is the peptide exported (by NisT) from the cell and the precursor peptide cleaved (by NisP), rendering nisin. The five lanthionine rings of nisin are labelled **a**–**e** from the amino terminus. Abu, aminobutyric acid.

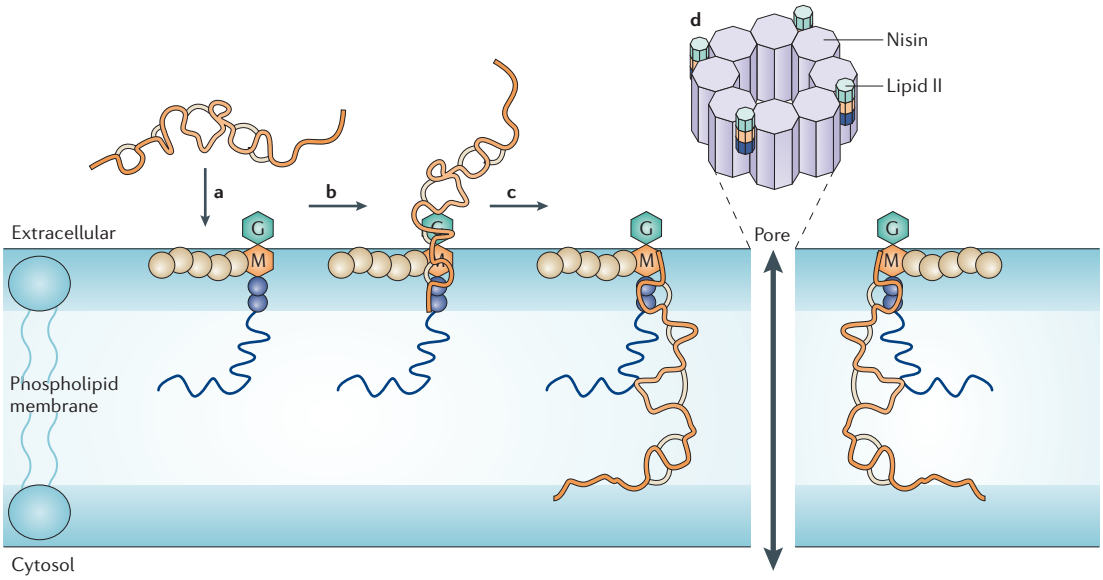

Figure 4 | **Model for the target-directed pore-formation mechanism of nisin.** First, nisin reaches the bacterial plasma membrane (**a**), where it binds to Lipid II via two of its amino-terminal rings (**b**). This is then followed by pore formation (**c**), which involves a stable transmembrane orientation of nisin. During or after assembly of four 1:1 (nisin: Lipid II) complexes, four additional nisin molecules are recruited to form the pore complex (**d**).

are unlikely. However, the glutamate at position 17 has been proposed to be important for Lipid II binding[20] — replacement of this residue with an alanine renders it inactive. Additionally, calcium ions have been shown to increase the MIC value of mersacidin, suggesting that these divalent ions form a bridge between the peptide and Lipid II. Comparison of mersacidin with similar lantibiotics reveals a conserved sequence that comprises residues 12–18, which suggests that these residues form the core site for binding to Lipid II. NMR studies showed that mersacidin has considerable conformational flexibility[37]. Surprisingly, it was discovered that mersacidin also has a definite but small hinge region located between the two lanthionine rings at residues 12 and 13 (arrow in FIG. 2). The flexibility in conformation ensures that mersacidin can modulate the accessibility of the charges. On binding of Lipid II, mersacidin, which has a hydrophobic character, exposes the charged groups, which suggests that electrostatic interactions have a dominant role in the interaction. Moreover, most of the spectroscopic changes were observed in this conserved region on binding of the antibiotic, providing further evidence in support of the importance of this sequence in Lipid II binding.

Lipid-II-interacting two-component lantibiotics. The two-component lantibiotics, such as lacticin 3147, are interesting because they seem to have split the Lipid-II-targeting and pore-forming activity between two different peptides, in effect combining different Lipid-II-dependent mechanisms. Data on the mode of action of these peptides are scarce; the best-studied example is lacticin 3147, which comprises the peptides A1 and A2. On the basis of the resemblance of the A1 peptide to mersacidin, it has been proposed that this peptide interacts with Lipid II[38]. The globular A1 peptide displays modular activity towards bacteria (probably, like mersacidin, by inhibiting cell-wall synthesis), yet full nanomolar activity is displayed in the presence of the more elongated A2 peptide. The authors proposed that this two-component system has a similar mode of action to nisin: first the A1 peptide interacts with Lipid II (FIG. 4a), which allows the interaction of the A2 peptide, resulting in pore-formation (FIG. 4c). If this proposal is correct, then targeting and pore-formation are separable. This offers many possibilities for the design of new antibiotics, as once the basis of the mutual interaction between the A1 and A2 peptides is revealed, the A1 peptide could be directed to other more strain-specific targets than Lipid II.

Ramoplanin

In an industrial drug discovery programme aimed at the identification of antibiotics specifically targeted at cell-wall synthesis, screening the metabolites produced by the *Actinomycetes* spp. identified a mixture of ramoplanin analogues (A1–A3, FIG. 2)[39]. In contrast to the lantibiotics, these ramoplanin analogues are produced via non-ribosomal peptide synthesis (explained clearly in REF. 40), and so these peptides comprise unusual amino acids, such as amino acids in the D-configuration; in fact, seven out of a total of seventeen amino acids of the cyclic peptide are in the D-form. Like nisin, ramoplanin has an overall positive charge. The mode of action of ramoplanin has been somewhat controversial[40,41], but recently it has become clear that its main target is Lipid II. Binding experiments with short-chain variants of Lipid II have shown that ramoplanin binds any variant of Lipid II provided that it contains a pyrophosphate[41,42]. The observation that even farnesyl pyrophosphate was bound by ramoplanin pinpoints the central role of the pyrophosphate in the binding interface[41].

The successful complete chemical synthesis of ramoplanin made it possible to map the sites on the molecule that are important for its activity. The sugar moieties do not seem to be important for the *in vitro* activity of ramoplanin[43,44]. The ring structure is essential for activity, as a linear peptide is inactive and even a small increase in ring size by insertion of a methylene functionality decreased the activity 100-fold[43]. Although the positively charged ornithines at positions four and ten were also suggested to be important for activity, single alanine substitutions for these amino acids showed that only the positive charge of the ornithine at position ten is crucial for the activity of ramoplanin[45].

Another functional group that is important for activity is the lipid tail, and the length of the tail seems to be optimal, as analogues with shorter or longer tails have much reduced activities[46]. Interestingly, although the affinity for Lipid II did not change on removal of the lipid tail, the antibacterial activity decreased at least tenfold[47]. This suggests that the lipid tail has an important role in targeting of ramoplanin to the bacterial membrane, the location of Lipid II.

An interesting observation has been made by McCafferty and coworkers[48]. They constructed a ramoplanin variant of only ten residues that was cyclized via a disulphide bond between cysteines at positions three and nine that could still bind to peptidoglycan precursors, suggesting that a large part of the Lipid-II-binding interface is located in this region. Considering the recent structural data on the nisin–Lipid II interaction (FIG. 5), and the idea that the alternating D- and L-form amino acids in this region are ideally suited for the nest–egg principle[49,50], it is tempting to speculate that hydrogen bonds from the backbone NH groups of ramoplanin are involved in the binding of the pyrophosphate moiety of Lipid II.

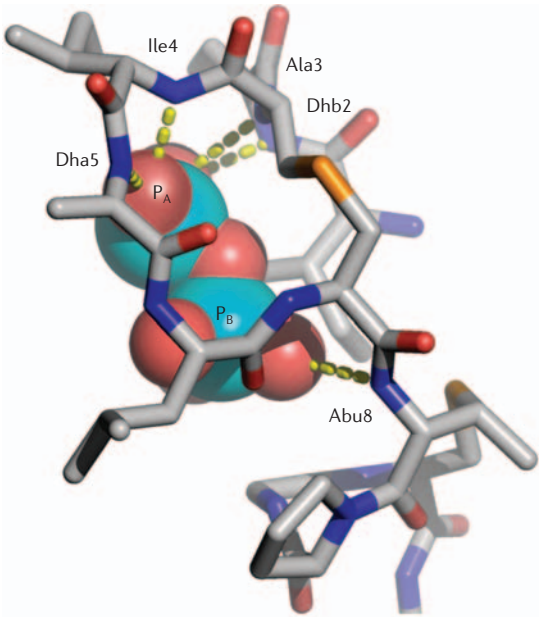

Figure 5 | **Interaction of nisin with Lipid II: the pyrophosphate cage.** The first two lanthionine rings of nisin (only the backbone is shown) form a cage-like structure around the pyrophosphate (spheres). The hydrogen bonds from the backbone amides that hold the pyrophosphate in place are depicted in yellow. The sulphur atoms of the thioether bonds are shown in orange. The first amino acid of lanthionine residues is always in the D-configuration as this ensures that the backbone NH bond of this amino acid points into the cage, which allows hydrogen-bond formation with the pyrophosphate. In addition, as the high affinity of nisin for Lipid II is likely to be predominantly determined by the affinity of the cage for the pyrophosphate and the pyrophosphate is bound via the backbone NH groups, changing side chains would not be expected to improve affinity — which is one explanation of why attempts to improve nisin via site-directed mutagenesis in the amino-terminal region have been unsuccessful so far. Introduction of an extra methyl group at the third residue of nisin through mutation of a serine into a threonine (S3T-nisin) decreased the activity of nisin by 12-fold[25]. The extra methyl group would occupy part of the cage-space, thereby hindering the formation of the hydrogen bonds between the backbone and the pyrophosphate. Reproduced, with permission, from REF. 33 © (2004) Macmillan Publishers Limited. Abu, aminobutyric acid; Dha, didehydroalanine; Dhb, didehydrobutyrine.

Mannopeptimycins

In the 1950s, screening of a complex mixture of molecules produced by a *Streptomyces* strain identified compounds that were effective against Gram-positive bacteria[51]. These compounds were re-examined at the beginning of this decade, which highlights the renewed interest in natural products as a source of new antibiotics. As a consequence, a new class of antibiotics — the mannopeptimycins — was discovered that has promising activity against MRSA. The mixture contained five different mannopeptimycins (α–ε), the structures of which were determined by spectroscopic analysis and chemical methods[52]. The mannopeptimycins are characterized by a cyclic ring structure formed by six amino acids in alternating D- and L-configurations (FIG. 2). The peptides are glycosylated with mannoses, and some variants have a lipophilic (isovaleryl) group attached to the terminal mannose that is crucial for activity (FIG. 2). Chemical modification at this position (mainly an increase in lipophilicity) has generated a variant with excellent activity against clinically important strains[53]. Only recently it has been shown that the mannopeptimycins specifically bind to Lipid II, and it has been suggested that this is the main cause of the inhibition of the cell-wall synthesis[54]. Unlike ramoplanin (and nisin), there is no evidence that the mannopeptimycins bind to Lipid I (or have only low affinity for Lipid I), as they do not inhibit the formation of Lipid II *in vitro*. However, a direct action of the mannopeptimycins on the high-molecular-mass penicillin-binding proteins responsible for transglycosylation cannot currently be ruled out.

Other potential Lipid-II-interacting antibiotics

Two other peptide antibiotics have been isolated and identified from different bacterial strains from the genus *Cytophaga* and *Pseudomonas*, called katanosins (FIG. 2) and plusbacins (FIG. 2), respectively, that have promising activity against MRSA and vancomycin-resistant *Enterococci* (VRE)[55,56]. Similar to ramoplanin and the

Nest–egg principle
The nest–egg principle applies to atoms or molecules with a full or partially negative charge (eggs) that sit in concave depressions ('nests') of a protein structure. The NH groups of the amino acids that form the nest point into the depression such that they can serve as a binding site for the atom or group of atoms.

mannopeptimycins, these peptides are cyclic and the peptide ring contains a stretch of alternating D- and L-form amino acids. It has been suggested that these peptides directly interact with Lipid II, although evidence to support this is lacking[57]. However, the presence of the alternating stretches of D- and L-amino acids could indicate that these peptides interact with the pyrophosphate of Lipid II. Interestingly, the katanosins lack a lipid tail, but are slightly more active than the plusbacins[57]. It is possible that the N-terminal hydrophobic leucines and phenyl-serine can substitute for the lipid tail.

All the peptides described are from different sources, and so it seems that the targeting of Lipid II by cyclic peptides is a general phenomenon. Perhaps there are considerably more such peptides that have yet to be discovered.

Resistance mechanisms

In general, the presence of antibiotics in bacterial populations will set up a selective pressure for the emergence of resistance to those antibiotics through evolution by natural selection. This problem is particularly acute in a clinical setting, in which the selective pressure for the evolution of antibiotic resistance is magnified because of the heavy use of antibiotics. Therefore, insights into the pathways that can lead to the development of resistance against a candidate antibiotic are crucial because these can result in the postponement or prevention of the occurrence of resistance.

There are several mechanisms by which bacteria can become resistant to an antibiotic. The first is destruction or modification of the antibiotic, thereby rendering it ineffective. For example, β-lactamases degrade the β-lactam ring of penicillins. A second is shielding of the target such that the antibiotic cannot get access to its target — for example, by cell-surface alteration (capsules, S-layers). Also the presence of multi-drug-resistance pumps can be regarded as shielding. This latter type of resistance can pose a problem once nisin is clinically used. As producer resistance is based on ATP-binding cassette transporters[7], there is a theoretical possibility that this resistance can be transferred to pathogenic strains. A third mechanism of resistance is modification of the bacterial target such that the antibiotic can no longer interact with its target. It is this last mechanism that is responsible for high-level bacterial resistance to vancomycin (called *vanA* or *van*B resistance) — five enzymes act together to effect a change in the sequence of the pentapeptide of Lipid II. Conversion of the terminal D-Ala to a D-Lac[35] leads to a considerable reduction in the binding affinity of vancomycin for Lipid II, rendering it inactive.

So far, no resistance has been reported for ramoplanin or the mannopeptimycins. However, instances of resistance to nisin have been described. Mechanisms of nisin resistance involve the degradation or modification of the antibiotic by specialized proteins. Several *Bacillus* spp. strains were shown to express a protein that specifically modified lantibiotics, including nisin[58], although this protein has never been characterized. It is thought that modification of the dehydrated residues leads to inactivation of the peptide[59]. A possible protease that was shown to be involved in nisin resistance was identified from *L. lactis* subspecies *diacetylactis* (a *L. lactis* strain that does not produce nisin, and therefore has no inherent resistance genes)[60]. Recent homology studies indicated that this protein might be a tail-specific protease[7]. However, cleavage of the C terminus would leave the binding site for Lipid II intact, and nisin is tolerant to C-terminal cleavage[34].

Nisin-resistance mechanisms are strain-specific. Repeated exposure of sensitive strains to increasing nisin concentrations has shown that most Gram-positive bacteria, including clinically relevant strains, can acquire nisin resistance under these conditions[3,61–63]. Often, this type of resistance is not stable, and is lost once the nisin pressure is removed[3]. Consequently, it can also be referred to as a physiological adaptation.

Because of the importance of Lipid II for the activity of nisin, decreasing the amount of Lipid II would be a simple mechanism for generating resistance to nisin. However, given the importance of Lipid II for cell-wall synthesis this is unlikely. Indeed, despite a 10–75-fold increase in resistance with respect to their parent strains, no differences in Lipid II levels were detectable in nisin-resistant *Listeria monocytogenes* or *Micrococcus flavus* strains[3]. If the level of Lipid II is not changed, another possible modification is to reduce the accessibility of Lipid II. Indeed, this is the most important mechanism for bacteria to become nisin-resistant, as most studies found that in the resistant strains the cell-wall composition was changed compared with the sensitive parent strains[3,61,64–67]. Normally, the cell wall of a Gram-positive bacterium is highly negatively charged. This charge is primarily generated by the phosphate groups of teichoic acid, a polymer of a polyol phosphate (glycerol or ribotol are the most common polyols), which is another major component of the cell wall. These teichoic acids are either covalently linked to the cell wall (wall teichoic acids or WTA) or membrane linked via a lipid anchor (lipoteichoic acids or LTA). The LTA and WTA can carry different substituents on the polyol group, one of which is D-alanine. Coupling of D-alanine to teichoic acid adds a positive charge to the overall negatively charged teichoic acid. This is used by the bacterium to regulate the charge of the cell wall[68], and it is this regulation of cell-wall charge that seems to be the most important mechanism leading to nisin-resistance.

Alteration of the amount of D-Ala in LTA has recently been shown to be a major cause of nisin resistance in a non-producing *L. lactis*[69]. Resistant strains of *L. lactis* were found to have LTA that contained almost twice as much D-Ala, and this coincided with a ninefold upregulation of the *dlt* operon (regulates incorporation of D-Ala into LTA)[69]. *B. subtilis* was shown to be able to regulate the charge of the cell wall via the *dlt* operon in defence against cationic antimicrobial peptides, such as nisin[70]. Similarly, the teichoic acids of a resistant *Streptococcus bovis* strain were more positively charged compared with LTA from sensitive strains, pointing to an increase in D-Ala substitution[61].

An alternative way to increase the positive charge of the cell wall, thereby rendering the bacterium resistant to nisin, has also been described[66]. The degree of resistance depended on the presence of divalent cations, and it was proposed that the accumulation of these divalent cations

Table 1 | **Activity spectrum and development stage of Lipid-II-interacting compounds**

Antibiotic	Important strains	MIC (mg l^{-1})	Development stage	Refs
Nisin	*Staphylococcus aureus* *Enterococcus faecalis/faecium* Vancomycin-resistant *Enterococci* (VRE) *Streptococcus pneumoniae*	1.5–83.6 8.4–33.4 1.5–16 0.03–0.25	Preclinical	72,74,79
Mutacin	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.1–18.1 1.6–25.6 6.4 0.03–6.4	Preclinical	74
Mersacidin	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.78–32 32–64 Not published 2–4	Preclinical	81,97
Ramoplanin	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.03–1.5 0.06–1 0.1–1.5 0.03–0.12	Phase III	71,72
Mannopeptimycin (AC98-6446)	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.03–0.06 0.06–0.25 0.06–0.12 ≤0.008	Preclinical	98
Katanosin B	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.39 0.78 0.78 Not published	Preclinical	57
Plusbacin A_3	*S. aureus* *E. faecalis/faecium* VRE *S. pneumoniae*	0.78–1.56 3.13 1.56–3.13 Not published	Preclinical	57

MIC, minimum inhibitory concentration.

and the consequent increase in positive charge of the cell wall prevented nisin from reaching the membrane[66].

All the data published so far and mentioned above indicate that the main mechanism leading to nisin resistance is a shielding mechanism: incorporation of positive charges in the cell wall whereby the positively charged nisin will be expelled from the cell, preventing it from reaching Lipid II.

Ramoplanin and the mannopeptimycins are also positively charged molecules. This raises the question as to why the above resistance mechanism does not seem to protect the bacteria from these antibiotics. For instance, there are only slight differences in the MIC value for ramoplanin for different strains[71], suggesting that the changes in D-Ala content of LTA does not affect the susceptibility of the bacteria to ramoplanin. A similar argument can be made for the mannopeptimycins. This is most likely due to the presence of lipophilic moieties on these molecules that direct the targeting of the antibiotics to the bacterial membrane, despite the non-ideal electrostatics.

Activity spectra and *in vivo* activity

If lantibiotics and the other classes of Lipid-II-interacting compounds described above are to enter clinical use, they must be active against clinically relevant strains. The activity spectra and current development status of the compounds discussed in this review are summarized in TABLE 1.

Isogenic
Bacteria (and other organisms) are isogenic if they are characterized by essentially identical genes.

Nisin showed promising activity towards clinical isolates of the MRSA bacterium and VRE, and from this it was suggested that nisin should be therapeutically used[72]. In another study, the efficacy of nisin displayed a wide and powerful effect towards several bacterial isolates that represented the most severe pathogens, among which were the multi-resistant *Streptococcus pneumoniae* and vancomycin-resistant *Enterococcus faecium* or *faecalis*, against which new antibiotics are most urgently needed[63]. Also, promising activity of nisin was reported towards *Streptococcus pyogenes*[73]. An additional study compared the activity of nisin against several bacterial pathogens with that of vancomycin and a member of the epidermin group of lantibiotics, mutacin B-Ny266. Nisin had equivalent activity to vancomycin, but considerable variation was observed for certain bacteria (for example, 1.5–83.6 μg per ml for *Staphylococcus* spp.)[74]. Interestingly, mutacin B-NY266 was in most cases more active than nisin or vancomycin.

In all these studies, it is noticeable that there is a large variation in the sensitivity to nisin within isogenic strains of Gram-positive bacteria, and some bacteria are inherently resistant to nisin. It is likely that the basis of this insensitivity is similar to the acquired resistance mechanism (or physiological adaptation) described above. *S. aureus* strains were shown to become more sensitive to positively charged antimicrobial peptides, including nisin and gallidermin (a lantibiotic resembling mutacin 1140), if the D-alanination pathway of

the teichoic acids was shut down[75]. This makes it tempting to speculate that the incorporation of D-Ala into the cell wall is the major defence system of bacteria towards the action of not only nisin but also other positively charged antibacterial peptides.

It has been reported that nisin has hardly any activity against Gram-negative bacteria such as *Escherichia coli* or *Salmonella* species[76,77]. Gram-negative bacteria possess an outer membrane, which acts as a permeability barrier. Only in combination with compounds that destabilize the outer membrane, such as ethylene diaminetetraacetic acid (EDTA) or citrate, does nisin show activity towards these strains[76]. However, nisin does elicit bactericidal responses from certain Gram-negative strains such as *Helicobacter pylori* or *Campylobacter jejuni*[74]. Mutacin B-NY266 was even more active against these strains and also considerably active against *Neisseria* spp., including strains that have multiple resistances towards penicillins. Recent studies have shown that modification of the hinge region of nisin increases efficacy against Gram-negative bacteria. Incorporating positively charged residues caused the variants to become considerably more active against *Salmonella*, *Shigella* and *Pseudomonas* strains[77]. Ramoplanin and the mannopeptimycins also have greater potency against most clinically relevant strains[71,78], including vancomycin-resistant strains, compared with vancomycin.

The above reports show that the lantibiotics possess promising activities *in vitro* against clinically relevant strains. So why are they not yet used in the clinic? A definite answer to this question is not possible without considering the activities of these peptides *in vivo*.

In the early 1950s, nisin was tested for the treatment of tuberculosis in mice and guinea pigs, but was not effective in the treatment of *Mycobacterium tuberculosis*. It was reported that nisin was cleared rapidly from the blood. Nevertheless, nisin could cure mice from *S. pyogenes* or *S. aureus* infections with similar effectiveness to penicillin[73]. However, the authors commented that nisin is "most unlikely to find a place in therapeutics", a conclusion that was based on the (at that time) high production costs of nisin and rapid clearance from the blood. More recently, the effectiveness of nisin against clinical isolates of *S. pneumoniae* was tested in a mouse infection model[79]. Nisin, which had similar MIC values against the *S. pneumoniae* strains compared with vancomycin, was 8–16 times more effective in curing the mice than vancomycin. Nisin therefore seemed to be potent despite the observed half-life time of 0.9 hours: after 3 hours nisin could not be detected when administered at an intravenous dose of 20 mg per kg. However, nisin does have the potency to be clinically used. As nisin specifically targets Lipid II, which is only present in bacteria, it is unlikely to be toxic to human cells. Indeed, nisin was shown to have low haemolytic activity and only a detectable effect on human lung fibroblast cells could be shown at excessively high concentrations (2.5 g per litre)[80].

Mersacidin has shown promise as a treatment for MRSA. Although *in vitro* tests showed that the activities of mersacidin against *S. aureus* and *Enterococci* are lower than that of vancomycin by about a factor of 10 and 16–128-fold, respectively[81], in a mouse infection model using several strains of *S. aureus*, mersacidin was almost twice as potent as vancomycin[81]. Mersacidin also eradicated MRSA from nasally infected mice[82].

Ramoplanin is not suitable for an intravenous formulation, as the lactone bond holding the ring together is rapidly hydrolysed in the bloodstream. However, the successful total synthesis of ramoplanin has now paved the road for variants that have an amide-linked ring system that is considerably more stable and could have a future as an intravenous therapeutic[47]. So far, clinical trials with ramoplanin have focused on the treatment of gastrointestinal tract infections by *C. difficile* or VRE (for an overview see REF. 71). Ramoplanin displayed excellent activity in these trials, with an activity against *C. difficile* similar to that of vancomycin, making it an attractive alternative to vancomycin for the treatment of infections caused by vancomycin-resistant species.

The hydrophobic mannopeptimycin derivative AC98-6446, which is currently the variant with the highest probability of being used as a therapeutic, has a much longer half-life than nisin (3–11 hours depending on the animal species[83]). This variant showed superior activity over vancomycin in an acute lethal infection model (mice), and also promising activity in an infective endocarditis model (rats)[83]. To date, it is not known whether any clinical trials are planned for this promising antibiotic.

Prospects

The lantibiotics bind to Lipid II in a unique fashion and have several modes of action. *In vitro* screening has shown that all of the tested lantibiotics show promising activity against multi-resistant and vancomycin-resistant strains. Because Lipid II production is restricted to bacteria, these antibiotics should have low toxicity in humans, which is another potential advantage for their use as clinical antibiotics in the treatment of vancomycin-resistant *S. aureus* infections.

However, there are obstacles that must be overcome before lantibiotics can be used in the clinic. In the case of nisin, its poor pharmacokinetic properties seem to be the key hindrance to its clinical development. For mersacidin, the low *in vitro* activity of this lantibiotic is more than compensated for by its *in vivo* characteristics. With the current structural knowledge of the mode of action of these peptides, it should be possible to modify the antibiotics to circumvent these barriers. The clinical development of the Lipid-II-targeting lantibiotics should therefore be considered. The clinical development of nisin could benefit from the considerable information gleaned from the use of nisin in the food industry as a food preservative — for example, on issues relating to formulation, conditions of use and stability of nisin.

Provided that they pass the clinical trials, both ramoplanin and the mannopeptimycins will be excellent alternatives to vancomycin. If used sparingly, resistance build-up towards these antibiotics will be slow, thereby extending the period of usefulness of these urgently needed antibiotics.

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Competing interests statement
The authors declare no competing financial interests.

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