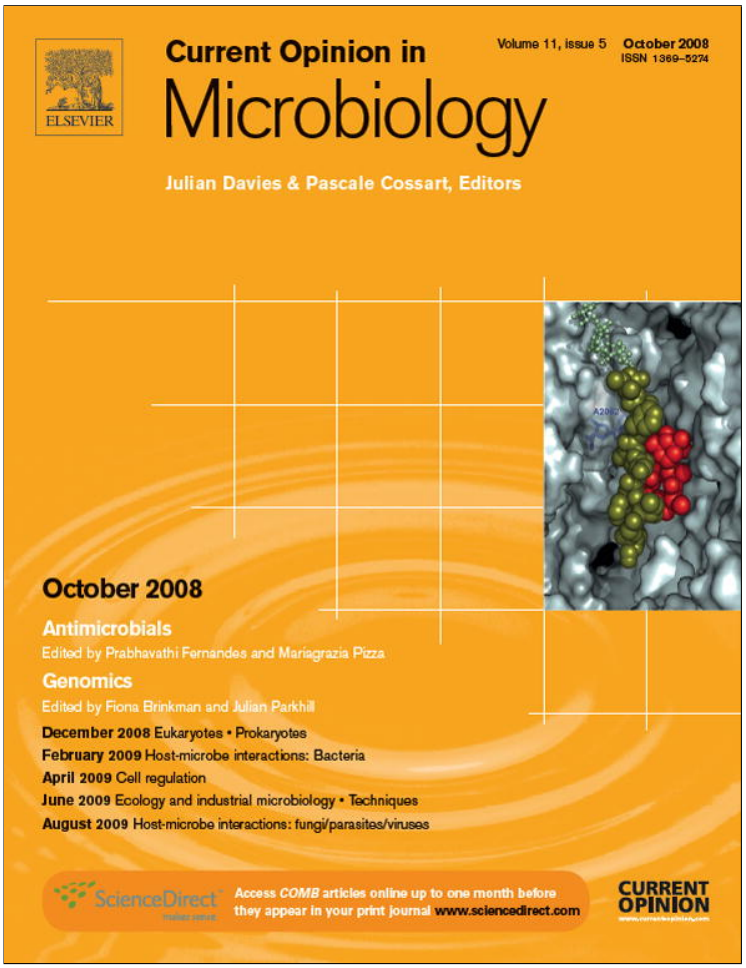

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Therapeutic potential of type A (I) lantibiotics, a group of cationic peptide antibiotics

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Type A (I) lantibiotics are cationic antimicrobial peptides that have a potential usefulness in treating infectious diseases. They are known to have a potent and broad spectrum of activity, an insignificant cytotoxicity, and demonstrated efficacy in animal infection models, suggesting therapeutic potential. In this review, topics pertaining to their basic structure, mode of bactericidal activity, pharmacology, and methods of manufacture are described.

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Introduction

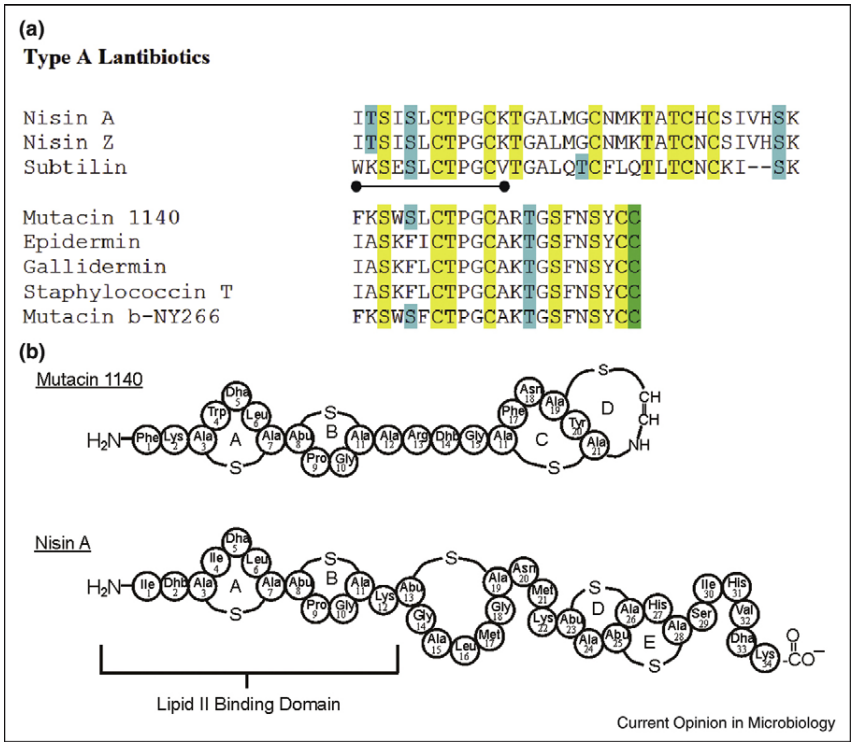
A group of cationic peptide antibiotics, called type A (I) lantibiotics, has received considerable attention because of the broad spectrum of activity, high potency, low frequency of antimicrobial resistance, and a cytotoxicity of two to three orders of magnitude higher than those required for antimicrobial activity. This class of antibiotics has been known for decades but has not been extensively tested for their potential usefulness in treating infectious diseases. The principal reason for this is the general difficulty of obtaining these molecules in sufficient, cost effective amounts to enable their testing and commercialization. Lantibiotic synthesis is a complex process involving multiple enzymes that leads to the formation of several unique amino acid residues. The post-translational modifications are believed to be partly responsible for low production by fermentation and make synthetic synthesis of these antibiotics difficult. In this review, before discussing topics pertaining to mode of bactericidal action, pharmacology, and manufacture of type A (I) lantibiotics, a general discussion of lantibiotic structure and synthesis is provided to give the reader the basic knowledge and understanding of these compounds.

Type A (I) lantibiotic structure and synthesis

There are 5 subclasses of lantibiotics based on differences in their chemistry and biosynthesis: type A(I), type A(II), type B, two-component, and those of unknown structures. Type A (I) lantibiotics (Class I bacteriocins) fall into two subgroups: those that are structurally similar to nisin A [1], which is produced by *L. lactis*, and those that share structural similarities to mutacin 1140 [2], which is produced by *Streptococcus mutans* (Figure 1). Gram positive bacteria are responsible for biosynthesis of the known lantibiotics. Lantibiotics are rich in the sulfur-containing amino acids, lanthionine (Lan, ala-S-ala) and, frequently, 3-methyl-lanthionine (MeLan, abu-S-ala). The occurrence of the unusual amino acids Lan and MeLan define lantibiotics and give them their name. In addition to the Lan and MeLan residues, there may be other post-translationally modified amino acids. Some of the other modified amino acids found in mutacin 1140 include 2,3-didehydroalanine (Dha), 2,3-didehydrobutyrine (Dhb), and the unsaturated lanthionine derivatives such as S-amino vinyl-D-cysteine (AviCys) (Figure 2).

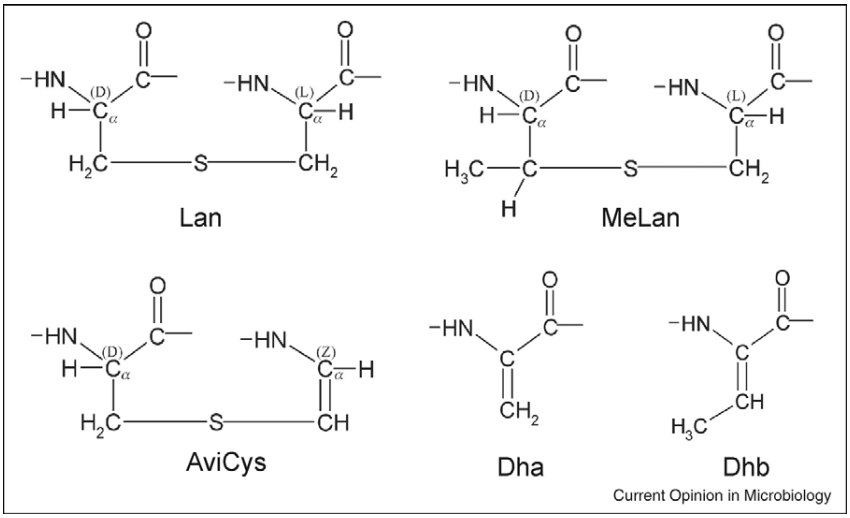
The mature lantibiotic molecule is made using a series of sequential enzymatic steps that act on a ribosomally synthesized prepropeptide (Figure 3, step 1) [3]. The genes responsible for encoding the modifying enzymes are typically clustered on an 8–10 Kb DNA fragment that may reside on the chromosome, a plasmid, or as part of a transposon. In type A lantibiotics, the serine and threonine residues in the ribosomally synthesized prepeptide encoded by the *lanA* gene are dehydrated by an enzyme encoded by the *lanB* gene and these dehydrated amino acids are involved in the formation of thioether linkages to a nearby cysteine residue that is situated more toward the carboxyl end of the molecule. This reaction is catalyzed by the protein expressed by the *lanC* gene (Figure 3, step 2). In the case of certain mutacin 1140-like type A (I) lantibiotics the C-terminal cysteine is decarboxylated by the enzyme expressed by the *lanD* gene and converted into an S-amino vinyl-D-cysteine (Figure 3, step 2). Following transport out of the cell by the product of the *lanT* gene, the leader sequence of the modified prepropeptide is then cleaved by an extracellular protease encoded by *lanP* to produce the mature antibiotic (Figure 3, step 3). Lastly, the production is regulated through the binding of the lantibiotic to a membrane bound kinase (Figure 3, step 4). The mechanism of regulating lantibiotic production is still not well understood.

Figure 1



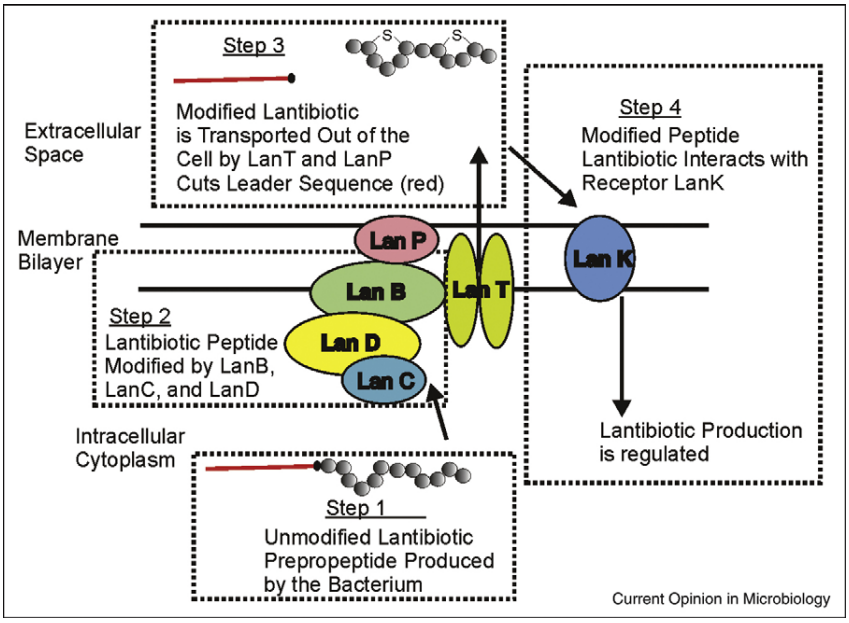
(a) Sequence alignment of type A (l) lantibiotics belonging to the nisin A and mutacin 1140 structural group. There is a considerable amount of similarity between the first eleven amino acids in the nisin A and mutacin 1140 group, represented by the dumbbell. Residues highlighted in yellow represent amino acids involved in thioether linkages. Residues highlighted in blue designate the location of the dehydrated residues 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb). The residues that are decarboxylated are highlighted in green. **(b)** Representation of the covalent structures of nisin A and mutacin 1140.

Figure 2



The structure of the modified residues in type A (l) lantibiotics of nisin and mutacin 1140 subgroup.

Figure 3



Schematic of the synthetase complex of type A (I) lantibiotics.

Modes of activity for type A (I) lantibiotics

Lantibiotics have multiple modes of bactericidal activity. Transmembrane pore formation, lipid II-mediated pore formation, and lipid II abduction from physiological domains have been reported [4•,5,6,7•,8•]. Given that a single lantibiotic monomer is too small to form a pore across a lipid bilayer, they must have a mechanism for lateral assembly in which they aggregate into complexes that can span the bilayer, an event that is followed by the disruption of the cytoplasmic membrane causing efflux of ions, ATP, and other essential cellular components. Two possible mechanisms for pore formation for nisin A have been reported, a barrel-stave model [9,10] and a wedge-like model [6,11]. Other cationic peptide antibiotics, including magainins, melittins, and protegrins, all appear to induce transmembrane pores that conform to the toroidal model [12,13]. Several good reviews have been written on these mechanisms of pore formation [14,15].

Electrostatic interactions between the positive charge of type A (I) lantibiotics and the negative charge of the bacterial membrane are believed to be required for initial binding. Experiments with nisin have demonstrated that the sensitivity of the host bacterium is dependent on the charged state of its cell wall and membrane [9,16–18]. However, the bacterium *Listeria monocytogenes* was shown to be relatively insensitive to nisin (MIC between 200–1000 μg/L) even though the negatively charged lipid composition of the membrane is relatively high (50–88%) [16,19]. Therefore, other factors besides anionic content are likely to affect the sensitivity of a bacterium to this class of lantibiotics. The thickness and composition of the cell wall and the accessibility of the peptidoglycan precursor lipid II are other potentially important determinants for bacterial susceptibility [4•,5,20].

Nisin's interactions with bacterial cells are enhanced by the presence of lipid II [5,20,21]. Early experiments with nisin showed an interference with cell wall biosynthesis of *in vitro* systems [22], and that it forms complexes with the lipid bound peptidoglycan precursors lipid I and lipid II [23,24]. These effects of inhibiting cell wall biosynthesis were once believed to be secondary to the primary mode of action of membrane disruption and pore formation. However, membrane pore formation by nisin and epidermin was shown to be inhibited by preincubation of *M. luteus* or *S. simulans* with ramoplanin, a lipopeptide that binds lipid II [25,26]. In addition, model membranes containing lipid II were shown to increase nisin activity threefold [5]. More recently, the mutacin 1140-like lantibiotics gallidermin and epidermin have also been shown to interfere with cell wall biosynthesis via lipid II binding as well as binding to lipid II precursor, lipid I [4•].

The formation of a highly stable complex of nisin with lipid II has been reported [27]. Recently, a novel lipid II binding motif for nisin has been characterized by NMR [28••], in which the N-terminal portion of nisin, lanthionine rings A and B, interact with the pyrophosphate, peptidoglycan MurNAc, and first isoprene of lipid II. The N-terminal portion of nisin A and mutacin 1140 share a high degree of similarity (Figure 1b), and it is

believed that this portion of mutacin 1140 is also the lipid II binding motif [7•,8•].

A novel mode of action for type A lantibiotics that interact with lipid II was recently published [7•]. Using confocal fluorescence microscopy, fluorescein labeled nisin was found to induce the clustering of lipid II into large patches in model membrane vesicles and in *in vivo* membrane studies. From these observations, it was clear that nisin A and presumably other type A (I) lantibiotics, have a novel mechanism of bactericidal activity that not only involves lipid II binding but also abduction of lipid II from its physiological domain in the bacterial membrane where active cell wall formation is occurring. This mechanism ensures that the peptidoglycan subunits carried by lipid II will not be available for cell wall synthesis, thereby inhibiting bacterial cell growth.

Moreover, the abduction of lipid II from the physiological domain by mutacin 1140 is sufficient to inhibit target cell growth, even in the absence of pore formation. Lipid II-containing model membrane vesicles revealed no detectable pore formation by mutacin 1140 indicating that lipid II abduction and not pore formation is responsible for its observed bactericidal activity [7•,8•]. Vancomycin also targets lipid II, although the interaction occurs at a different site on lipid II. This glycopeptide also does not abduct lipid II from its normal physiological locations in the bacterial cell membrane [29].

Differences in the lipid II-lantibiotic complex were observed in model membrane experiments between nisin A and mutacin 1140. Nisin has a distinctive pyrene excimer signal when added to model membrane vesicles containing pyrene labeled lipid II. Conversely, there was no induced excimer signal following the addition of mutacin 1140 to the model membrane vesicles containing pyrene labeled lipid II [8•]. The lack of an excimer signal is most probably attributed to differences in the way lipid II is oriented during formation of the mutacin-lipid II and nisin-lipid II complexes. Lipid II monomers must be located farther apart in the mutacin complexes than in the nisin complexes, and the observed nisin-induced excimer signal observed may actually be attributed to an actual pore complex. The peptide sequence variability outside of the lipid II binding domain (Figure 1b), in all probability, confers the functionality of the lantibiotic including whether or not it is a pore former and how it positions lipid II during complex formation. In addition to the lipid II binding domain, there must be a binding motif for lateral association of these lantibiotics to account for the large mutacin or nisin complexes observed in the florescence studies and transmission electron microscopy studies [7•,8•]. This dual activity of binding to lipid II followed by lateral assembly of the lantibiotic may explain the high potency of these antimicrobial compounds. The binding affinity for nisin to lipid II has been calculated to be in the order of 10^7 [5]. This binding affinity does not take into consideration the lateral assembly of the lantibiotic that would further trap lipid II into large lantibiotic islands in the bilayer. This bactericidal mechanism of activity ensures that lipid II will not become available for cell wall synthesis.

Pharmacology of type A (I) lantibiotics

This class of antibiotics has been known for decades but has not been extensively tested for their potential usefulness in treating infectious diseases even though many are known to be both potent and have a broad spectrum of activity. The principal reason for this is the general difficulty of obtaining these molecules in sufficient, cost effective amounts to enable their testing and commercialization. Numerous studies have shown that they have low MICs against many clinically relevant Gram positive bacterial spp., such as Enterococcus spp., Listeria spp., Staphylococcus spp., Streptococcus spp., Bacillus spp., and others [30–32]. As early as 1952, nisin A was shown to be as effective as penicillin in treating mice infected with *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* [33]. However, the authors commented that nisin most probably would not find a place in therapeutics given the cost of production and the rapid clearance of the antibiotic from the blood. The rise of antibiotic resistant pathogens like MRSA and the 8 billion dollar cost attributed to the treatment of this pathogen [34] counters the authors' remark, but this comment does illustrate the differences in the mindset of 1952 and that of today.

Cytotoxicity of gallidermin and nisin A on intestinal epithelial cells following 24 h incubation was determined by MTT assay and neutral red dye uptake assay [35]. The IC_{50} was greater than 210 μM in both assays for gallidermin and greater than 89 μM in both assays for nisin. Hemolytic potential of gallidermin and nisin A on live sheep erythrocytes, following a 1 h exposure to their respective IC_{50} concentration, showed $<2\%$ and $<5\%$ hemolysis for gallidermin and nisin, respectively, which are comparable to the activity seen with vancomycin. In a separate study, no hemolytic activity for nisin A or mutacin B-Ny266 was observed when incubated with sheep erythrocytes at concentrations 30–60 times greater than is required for *in vitro* bactericidal activity [32]. Nisin was also shown to have no significant cytoxicity on promyelocytic leukemia HL-60 cells after 24 h exposure [36] and no significant hemolytic activity against human lung fibroblast cells [37] and human erythrocytes [36].

Nisin was shown to be more effective than vancomycin in an *S. pneumoniae* mouse infection model [31]. Nisin was 100% effective with two intravenous doses of 0.16 mg/kg body weight, while vancomycin was only effective 80% of the times with two intravenous doses 1.25 mg/kg body weight. These authors also noted the rapid clearance of

nisin from the blood and provided a predicted half life of 0.9 h. However, they noted that nisin is very effective despite the short half life. The assumption was made that nisin may have a more rapid bactericidal action compared with vancomycin, since both antibiotics have comparable MICs against *S. pneumoniae*. Intraperotineal injection of 1 mg/kg body weight of Mutacin B-Ny266 in mice infected intraperitoneally with 6.2×10^7 CFU of *S. aureus* demonstrated efficacy, but the experimental design in which the drug was injected into the same compartment as the bacterial challenge makes interpretation of the results difficult [38].

Oni Biopharma Inc. is currently conducting preclinical trials on the lantibiotic mutacin 1140 (MU1140). A particularly interesting feature of mutacin 1140 is the relative difficulty of inactivating mutacin 1140 by trypsin or pronase treatment [39,40]. The potentially susceptible arginine at residue 13, was later found by 3-dimensional NMR structural analyses to be buried in a horseshoe-like confirmation [41], thus protecting it from protease cleavage. Presumably, bacterial resistance to mutacin 1140 by acquiring a protease activity would be difficult. Another interesting feature was noted when none of four sensitive species of bacteria tested were able to acquire genetically stable, spontaneous resistance. Incubation of 10^{11} cells of multidrug resistant (MDR) strains of *S. aureus* and *E. faecalis*, as well as *L. casei* and an *S. rattus* indicator strain, with a threshold killing concentration of mutacin 1140 failed to lead to the recovery of resistant mutants (unpublished data). By contrast, spontaneous mutants of *L. monocytogenes* that were resistant to nisin were isolated at frequencies that ranged from 10^{-6} to 10^{-8} [42,43]. Furthermore, there was only a slight (4-fold) increase in the MIC against *S. pneumoniae* or *S. aureus* following a 21 day sequential passage in subinhibitory concentrations of mutacin 1140. The producer strain, *Streptococcus mutans* JH1140, has no significant immunity to mutacin 1140 as demonstrated by the fact that its MIC is comparable to MICs of other viridans streptococci tested.

Time-kill studies against *S. aureus*, *S. pneumoniae*, and *E. faecalis* show that mutacin 1140 is bactericidal against *S. aureus* and *S. pneumoniae*, and bacteriostatic against *E. faecalis* (unpublished data). Vancomycin also exhibits this species-dependent difference in activity [44,45]. *In vitro*, the addition of human or rat serum causes a reduction in mutacin 1140 activity against *S. pneumoniae*, presumably due to significant (92%) binding to serum protein(s), but serum was found to cause an unexplained increase in activity against *S. aureus*. The half life of this lantibiotic was approximately 1.5 h in a rat model. Other aspects of safety and efficacy that have been tested, including maximum tolerated dose, immunogenicity, cytotoxicity, and efficacy in *S. aureus* sepsis models, all suggest the potential usefulness of mutacin 1140 for the treatment of Gram positive infectious diseases. Presumably, other lantibiotics will demonstrate potential for clinical application as well. The current challenge is to develop an approach for their production in cost effective amounts to enable their testing and commercialization.

Manufacture of lantibiotics

Several interesting approaches to manufacturing lantibiotics have been tried over the past few decades. These include the development of fermentation methods, semi-synthetic methods, and organochemistry synthesis approaches. Fermentation methods have been reported for gallidermin, in which the authors report improvements in its production [46,47] with a yield of 249 mg/L in a 200 L bioreactor [46]. Fermentation methods for mutacin B-Ny266 have also demonstrated higher yields of production [48]. Common components in their production appear to be 10% inoculums in late log growth, and the media component, yeast extract. These reports demonstrate the possibility of fermentation-based methods for the manufacture of lantibiotics and also demonstrate the efficiency of the lantibiotic modification machinery for a high yield production. Proof of principal for a semi-synthetic approach was demonstrated for a type A lantibiotic lacticin 481 [49•], which belongs to the A (II) subgroup of lantibiotics. An enzyme, LctM, characteristic of this subgroup contains both LanB and LanC enzymatic activities. Using purified *E. coli* recombinant expression products of the LctA protein and LctM, the authors demonstrated *in vitro* modification of the prepropeptide. The authors also demonstrated permissive substrate specificity for the LctM enzyme on several LctA mutant peptides, suggesting that the enzyme may also be useful in other antibiotic engineering experiments. By contrast, cell extracts and recombinant epidermin LanB and LanC enzymes have no *in vitro* activity [50–52]. The researchers postulate that the post-translational modifications brought about by LanB and LanC happen only if the lantibiotic synthetase complex is formed in the bacterial membrane.

Several organic synthesis schemes have been developed to produce the lanthionine rings found in the lantibiotics [53]. None of these methods have led to the synthesis of a completely functional lantibiotic. One reason for this is the complexity of the overlapping rings (e.g. rings D and E in nisin or C and D in mutacin 1140; Figure 1b). Using a ring closing metathesis approach, one group produced rings A, B, and C (ABC) mimics of nisin that contained alkene bridges instead of thioether linkages [54••]. The substitution of the thioether linkage for an alkene bridge was shown not to interfere with the lipid II binding activity. The ring ABC mimics did not induce CF leakage in model membrane studies, but did compete for lipid II in a competition study between nisin A and the ring ABC mimics. Presumably the synthesis of the complete nisin molecule will be necessary for CF leakage and biological activity. To accomplish a complete synthesis of nisin A or mutacin 1140, an approach that can synthesize the overlapping rings of these antibiotics is needed. One approach,

currently under development at Oni Biophama utilizes a novel technology called differentially protected orthogonal lanthionine technology (DPOLT). This technology uses two differentially protected orthogonal lanthionine residues in standard peptide synthesis chemistry (United States Patent Application), which will enable the synthesis of the overlapping rings found in type A (I) lantibiotics.

Another technology called functional enhancement of antimicrobials (FEAM) holds promise for the manufacture of novel lantibiotic analogs (United States Patent Application). The premise for this technology involves understanding that defined and undefined constraints on lantibiotics prevent Nature from making the most effective bactericidal compound. For instance, in the case of nisin, conformational constraints required for its interaction with the lantibiotic synthetase enzymes, immunity gene product [55–58], and membrane receptor for its autoinducing activity [56,59], may prevent it from evolving into the most effective antibiotic. A single addition of a functional group, such as a charge group, polar group, or a hydrophobic group can have significant effects on the bioactivities, pharmacokinetics, and/or pharmacodynamics of an antimicrobial compound. For instance, ring A of nisin showed mutational freedom, and the incorporation of a positive charge or a hydrophobic group by site directed mutagenesis had a profoundly positive effect on the spectrum of activity and level of antimicrobial activity against some target bacterial species when compared with wild-type nisin [60••]. As described above, 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) residues are commonly found in lantibiotics, as well as other ribosomally and non-ribosomally synthesized antimicrobials. FEAM makes use of the alpha, beta unsaturated carbonyl group found in these residues that lend themselves to the addition of thiol compounds containing novel functional groups in a highly selective fashion. The single step additions are easily optimized and can be made in aqueous solvents with greater than a 90% yield. Furthermore, Dha and Dhb residues are easily engineered in lantibiotics by site directed mutagenesis or by an organosynthesis method, further facilitating the production of unique analogs with enhanced function.

Conclusion

The paucity of new antibiotics in the drug development pipeline has prompted serious concern from the scientific community that was nicely articulated by the Infectious Disease Society of America (IDSA) in a report entitled 'Bad Bugs, No Drugs'. This report as well as letters to congressional members from IDSA and from the President of the American Society of Microbiology (ASM) has prompted the US Congress to pass the Antibiotic Access and Innovation Amendment in hopes of increasing the amount of antibiotics being developed for therapeutic use. In the cases tested, type A (I) lantibiotics have a well-characterized mechanism of bactericidal activity that is not easily amenable to the development of bacterial resistance. Furthermore, they exhibit the pharmacological characteristics for therapeutic use. Given the current innovations in their manufacture, they are well suited to help fill the antibiotic pipeline.

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