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Invited Review

Combination of antibiotic mechanisms in lantibiotics

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Abstract

Recent studies on the mode of action have revealed exciting features of multiple activities of nisin and related lantibiotics making these peptides interesting model systems for the design of new antibiotics (Molec. Microbiol. 30 (1998) 317; Science 286 (1999) 2361; J. Biol. Chem. 276 (2001) 1772.). In contrast to other groups of antibiotic peptides, the lantibiotics display a substantial degree of specificity for particular components of bacterial membranes. Mersacidin and actagardine were shown to bind with high affinity to the lipid coupled peptidoglycan precursor, the so-called lipid II, which prevents the polymerisation of the cell wall monomers into a functional murein sacculus. The lantibiotics nisin and epidermin also bind tightly to this cell wall precursor; however, for these lantibiotics the binding of lipid II has two consequences. Like with mersacidin blocking of lipid II inhibits peptidoglycan biosynthesis; in addition, lipid II is used as a specific docking molecule for the formation of pores. This combination of lethal effects explains the potency of these peptides, which are active in nanomolar concentration. Other type-A lantibiotics are believed to also use docking molecules for pore formation, although identification of such membrane components has not yet been achieved. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: Nisin; Mersacidin; Lipid II; Target mediated pore formation; Inhibition of cell wall biosynthesis

1. Introduction

Lantibiotics are antimicrobial peptides produced by a wide range of Gram-positive bacteria. They represent a subgroup of bacteriocins, which is characterised by the presence of unique modified amino acids, particularly dehydroamino acids and the thioether amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan). In contrast to ''classical'' peptide antibiotics produced by multienzyme complexes [1,2], lantibiotics are ribosomally synthesised as precursor peptides, which are subsequently converted into the biologically active peptides through post-translational modifications. A number of modified amino acids have been found in lantibiotics including e.g.: *meso*-lanthionine (Lan), *threo*-β-methyllanthionine (MeLan), *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys), *S*-[(*Z*)-2-aminovinyl]-3-methyl-D-cysteine (AviMeCys), 2,3-didehydroalanine (Dha), 2,3-didehydrobutyrine (Dhb) and D-alanine (Fig. 1). The reaction of Dha or Dhb with a cysteine residue results in the formation of the intramolecular ring-structures Lan and MeLan, respectively. The post-translationally modified propeptides are activated by proteases and exported from the producing cells.

Lantibiotics do not form a homogeneous group. Regarding the enzymes taking part in modifications as well as export and processing, two different classes of lantibiotics can be distinguished [3]. Class I lantibiotics are modified by two enzymes, LanB and LanC, catalysing the dehydration of hydroxyamino acids and the formation of the thioether rings, respectively; proteolytic processing and export from the producing cells are performed by dedicated proteases LanP and ABC-transporters LanT. Class II lantibiotics are modified by only one enzyme, LanM, and secreted and activated through hybrid ABC-transporters with an additional proteolytic domain at their N-terminus. According to a proposal of Jung [7], based on the information on structures and modes of action available at that time, the lantibiotics were grouped into the elongated, amphiphilic, screw shaped, membrane-depolarising type-A peptides and the small, globular and enzyme inhibitory type-B lantibiotics. However, in the last decade a significant number of new lantibiotics with intermediate features has been characterised, making a

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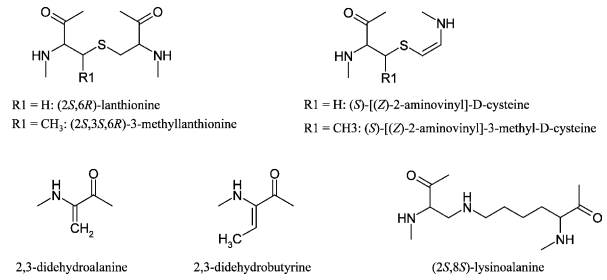

Fig. 1. Structures of some of the modified amino acids found in lantibiotics.

categorisation on the basis of structural and functional features more difficult.

2. Type-A lantibiotics—formation of target independent pores

Type-A lantibiotics are typically active against Gram-positive strains; Gram-negative bacteria are only affected when the outer membrane is disrupted e.g. by ion chelators such as EDTA or citrate [5,6]. The most prominent member of the Type-A group is nisin (Fig. 2). The first report on the mechanism of this prototype type-A lantibiotic dates back to 1960 when Ramseier [7] observed leakage of UV-absorbing intracellular compounds from treated cells and suggested a detergent effect. Subsequent experiments showed that nisin or related lantibiotics kill bacterial cells primarily by interference with energy transduction occurring at the cytoplasmic membrane [8,9]. Addition of nisin and other type-A peptides immediately inhibits biosynthesis processes of macromolecules such as DNA, RNA, proteins and polysaccharides [10]. Furthermore, bacterial cells are unable to actively take-up amino acids and become leaky for inorganic ions and small metabolites [11].

A concept of energisation-dependent activity was deduced from experiments with cytoplasmic vesicles and intact cells as well as with model membranes. Conductance measurements using artificial bilayer membranes (black lipid membranes, BLM) were in good agreement with the results obtained with intact cells and physiological membranes [5,12–15]. There was no macroscopic membrane conductivity below a certain threshold potential; the minimum voltage ranged from -50 to -100 mV for various peptides, and calculated pore diameters were between 1 nm for nisin and about 2 nm for subtilin with a lifetime in the millisecond range [5,13,14].

Initially, it was discussed that a ''barrel-stave'' model may adequately describe the activity of these lantibiotics [5,8,10]. A barrel-stave model, in which α-helical amphipathic peptides initially bind via electrostatical interaction to the outer leaflet in a parallel orientation to the membrane surface, had been elaborated by Boheim [16] and others [17]. In order to avoid the unfavourable position of polar residues to the lipid acyl chains, several monomers of peptides have to assemble forming a bundle of helices. After insertion of the peptides into the membrane the non-polar side-chains of the peptides interact with the hydrophobic lipid core of the membrane and the hydrophilic side-chains point inward, which results in the formation of a water-filled pore. Both the size and the stability of the barrel-stave channel depend on the number of peptides involved in

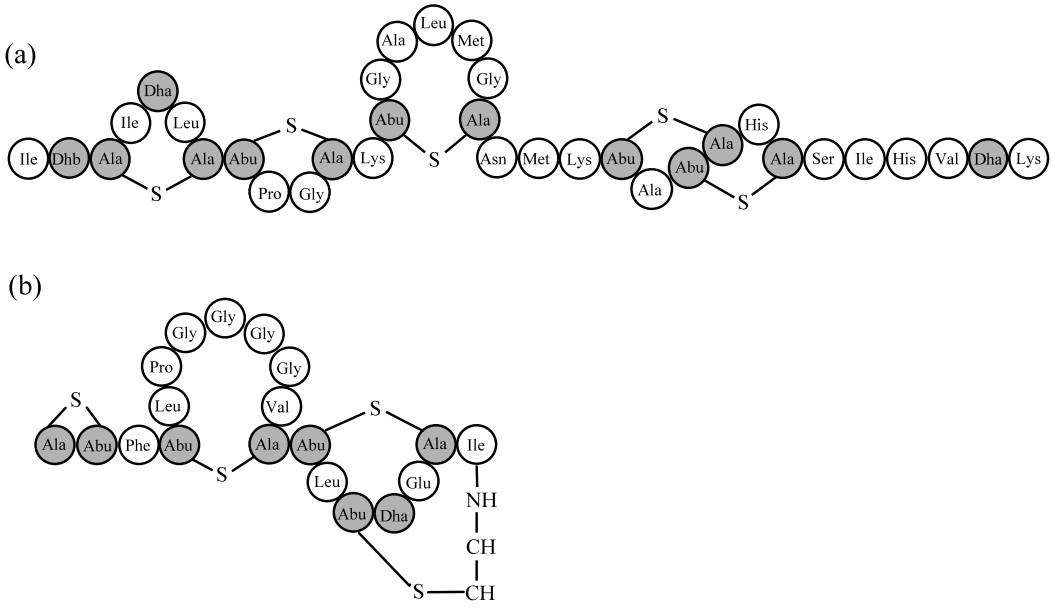

Fig. 2. Primary structure of nisin (a) and mersacidin (b). Dha: didehydroalanine; Dhb: didehydrobutyrin; Abu: α-aminobutyric acid; Ala-S-Ala = lanthionine, Abu-S-Ala: 3-methyllanthionine.

pore formation [5]. Biophysical studies with the defence peptide alamethicin [18] and the neurotoxin pardaxin [19,20] support the barrel-stave mechanism for peptide insertion and organisation in the membrane.

When conformational data derived from NMR studies applying nisin in the presence of membrane-mimicking micelles [21,22] became available, a "wedge model" for pore formation by lantibiotics was proposed [22–24]. This model takes into account that type-A lantibiotics are rather flexible in aqueous solution and defined structural elements have only been identified in small thioether rings [25,26]. Upon contact with the membrane, the peptides adopt an amphiphilic conformation with the charged residues aligned to one face of the molecules and the hydrophobic residues aligned to the other. The cationic peptides interact with the phospholipid head groups by ionic forces causing a locally disturbed bilayer structure, while the hydrophobic residues insert into the membrane [21,22,27].

Studies with nisin demonstrated that the C-terminal region [28,29] as well as the overall negative surface charge of the membrane [27,30–32] were important for binding and pore formation. It is assumed that several molecules have to associate within the membrane in order to form a pore [24], since lantibiotics are too short to transverse the membrane more than once. On the other hand, several transmembrane segments are necessary to form a pore with a diameter of 1 nm or more as deduced from BML studies. The formation of pores leads to dissipation of the membrane potential and promotes a rapid efflux of small metabolites such as amino acids or ATP, which in turn immediately stops all cellular biosynthetic processes [8,11–15]. While the models derived from studies on artificial membrane may describe the behaviour of peptides in pure lipid bilayers, it has to be pointed out that generally micromolar concentration are used to observe effects in such systems. In vivo, however, nisin and related bacteriocins frequently kill bacteria in the nanomolar concentration range, which was taken as an indication for additional activities being involved in killing microbes.

3. Type-A lantibiotics—formation of target mediated pores

Since type-A lantibiotics can act on artificial membranes, binding to specific receptors in the cell membrane is not a prerequisite for activity per se [33]; therefore, a concept of specific targets being involved in the membrane interaction had not been considered. However, for nisin, a finite number of binding sites and specific antagonisation of nisin activity by the inactive N-terminal nisin fragment 1–12 had been observed [34], indicating that a defined binding site may be blocked by the fragment. With that respect, it was important to recall publications by Linnett and Strominger [35,36] who reported that nisin inhibits peptidoglycan biosynthesis, and that it binds to the membrane-bound peptidoglycan precursor undecaprenylpyrophosphoryl-MurNAc(pentapeptide)-GlcNAc, the so-called lipid II.

These reports raised the idea that lipid II may be involved in the formation of pores. This was subsequently shown for nisin and epidermin using lipid II supplemented liposomes [37]. Detailed in vitro studies then demonstrated that lipid II serves as a docking molecule for specific binding to the bacterial membrane [38,39] (Fig. 3). Genetically engineered nisin variants helped to identify the structural requirements for the interaction of the peptide with lipid II [39]. Mutations affecting the conformation of the N-terminal part of nisin comprising rings A through C (e.g. [S3T]nisin), led to reduced binding and increased the peptide concentration necessary for pore formation; i.e. the binding constant for the [S3T]mutant was 0.043×10^7 (M^{-1}) as compared to 2×10^7 (M^{-1}) for the wild type peptide and the minimum concentration for pore formation increased from the 2 to the 50 nM range. Peptides with mutations in the flexible hinge region (e.g. [ΔN20/ΔM21]nisin) were completely inactive in the pore formation assay. When tested in vivo against living bacteria, their activity was only reduced to some extent. The remaining in vivo activity was shown to be due to the unaltered capacity of the mutated peptide to bind to lipid II, and thus to inhibit the incorporation of lipid II into the peptidoglycan network. The N-terminal part of nisin is essential for binding to the membrane bound cell wall precursor lipid II and the resulting inhibition of the peptidoglycan synthesis. In contrast to the nisin induced pore formation without a docking-molecule, a negative surface charge of the membrane is not necessary. However, the positively charged C-terminus of nisin is still important for the initial binding to the anionic cell wall polymer and thus for antimicrobial activity in vivo [40]. The combination of two killing mechanisms, inhibition of the peptidoglycan synthesis and pore formation, in one molecule potentiates in vivo the antibiotic activity and results in nanomolar MIC values, a strategy that may well be worth considering for the construction of novel antibiotics.

4. Additional activities of type-A lantibiotics

In addition to pore formation and inhibition of the cell wall biosynthesis, nisin and the related cationic lantibiotic Pep5 have been shown to induce autolysis of susceptible staphylococcal cells, resulting in massive cell wall degradation, most markedly in the area of the septa between dividing daughter cells. The peptides are able to release two cell wall hydrolysing enzymes, an

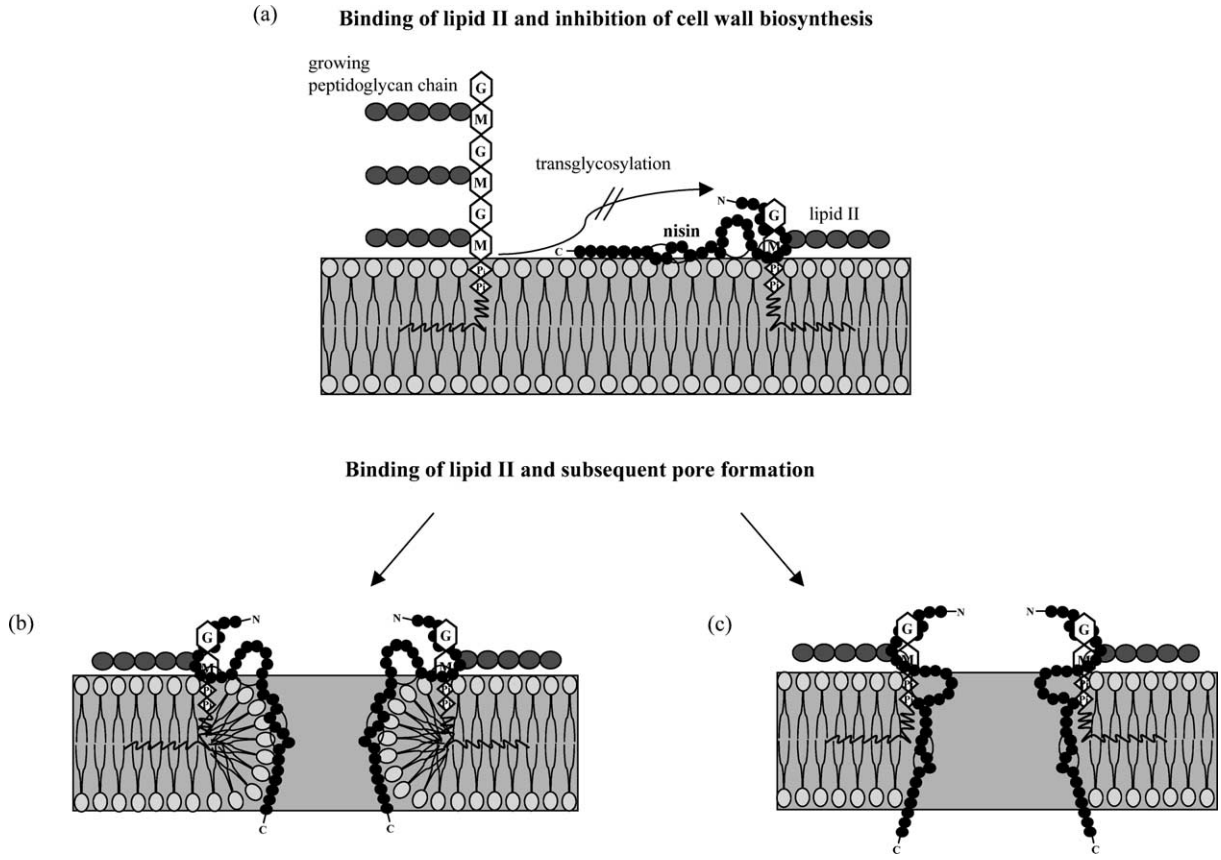

Fig. 3. Models of target-mediated action of nisin. (a) Based on the results of Wiedemann et al. [42] it has been proposed that nisin first binds to the outwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry. The N-terminal segment of nisin is essential for binding whereas the C-terminal part is then assumed to be subsequently translocated across the membrane, in accordance to pore formation in the absence of lipid II [32]. For this step the flexible hinge region between the third and the fourth ring of nisin is important. Several nisin/lipid II complexes are presumed to assemble for a functional pore. Currently, no information is available on the actual pore opening process. In (b) nisin is assumed to stay surface bound when translocating across the membrane, then following the wedge model. In (c) it is assumed that nisin dips into the bilayer, interacting with the acyl chains; such a situation, in essence, follows the pore formation as proposed by the barrel-stave model.

N-acetylmuramoyl-L-alanine amidase and an *N*-acetylglucosaminidase, which are strongly cationic proteins binding to the cell wall via electrostatic interactions with the negatively charged teichoic-, teichuronic-, and lipoteichoic acids; tight binding to these polymers keeps the autolysins inactive [41]. The cationic peptides displace enzymes from the cell wall intrinsic inhibitors by a cation exchange-like process, resulting in apparent enzyme activation and rapid cell lysis [42].

Furthermore, nisin and subtilin inhibit the germination of bacterial spores [43,44]. This activity obviously depends on the presence of Dha residues in position 5 of both peptides. It is presumed that the double bond provides a reactive group for interaction with a spore-associated factor that is essential for outgrowth of spores.

A further special feature of nisin, subtilin and salivaricin A has been shown by Kuipers et al. [45–47] in that these lantibiotics autoregulate their own biosynthesis, a phenomenon called quorum sensing. These peptides function as signal molecules for measuring the cell density of a population. The secreted fully modified peptides induce the transcription of the biosynthesis genes via signal transduction by the two-component regulator system LanKR in a pheromone-like manner. In staphylococci, another quorum sensing phenomenon has been described in which the expression of virulence factors and other extracellular proteins is controlled by the *agr* two-component regulator system [48]. Modified autoinducing peptides produced by other *Staphylococcus aureus* strains inhibit the *agr* expression in other strains via non-productive binding to the sensor kinase. Such peptides could serve as model systems for developing novel antibiotics, which inhibit the synthesis of virulence or colonisation factors.

5. Type-B lantibiotics—mersacidin and actagardine

The type-B lantibiotics mersacidin and actagardine are active against a variety of Gram-positive bacteria, with actagardine being most effective against

streptococci and obligate anaerobes [49,50], while mersacidin (Fig. 2) is almost equally active against staphylococci, streptococci, bacilli, clostridia, corynebacteria, peptostreptococci, and *Propionibacterium acnes* [34,51,52]. Gram-negative bacteria are not susceptible, since peptides cannot pass the outer membrane of bacteria; neither can they penetrate the eucaryotic cell membrane, since *Listeria monocytogenes*, while being susceptible in vitro, is not affected when persisting within eucaryotic cells [53].

With respect to a potential chemotherapeutical application, the type-B lantibiotics mersacidin and actagardine are the most promising antimicrobial agents amongst the lantibiotics. Although both lantibiotics in vitro exhibit only moderate MIC values, mersacidin has attracted recent attention due to its significant in vivo activity. It effectively cured systemic staphylococcal infections in mice (even those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains) [34], as well as subcutaneous staphylococcal abscesses in rats [51]. In both cases the lantibiotic equalled or even exceeded the activity of vancomycin.

Mersacidin and actagardine were shown to interfere with the cell wall biosynthesis by inhibiting the incorporation of glucose and D-alanine into cell wall material of *Staphylococcus simulans* 22, whereas DNA, RNA and protein synthesis proceeded unhindered. Both peptides inhibit peptidoglycan biosynthesis at the level of transglycosylation by forming a complex with the membrane-bound peptidoglycan precursor lipid II [54,55]. The binding of [^{14}C]mersacidin to growing cells as well as to isolated membranes capable of in vitro peptidoglycan synthesis was strictly dependent on the availability of lipid II, and antibiotic inhibitors of lipid II formation strongly interfered with binding of mersacidin. Furthermore, labelled mersacidin tightly associated with micelles formed from purified and labelled lipid II, and the addition of isolated lipid II to the culture broth efficiently antagonised the bactericidal activity of mersacidin. The molecular target site of mersacidin and actagardine on lipid II differs from that of nisin (see above) and of the glycopeptide antibiotic vancomycin, which binds to lipid II via the C-terminal D-Ala- D-Ala of the pentapeptide side-chain; in contrast, mersacidin and actagardine rather interact with the disaccharide–pyrophosphate moiety of lipid II (Fig. 4) [55]. The two lantibiotics contain one ring structure that has been almost completely conserved in both molecules, indicating its importance for activity [34,54,56].

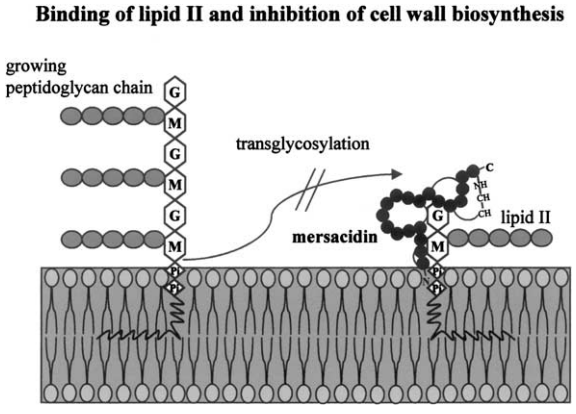

Fig. 4. Model for the inhibition of the peptidoglycan biosynthesis by the lantibiotic mersacidin. The lantibiotic binds to the disaccharide–pyrophosphate moiety of lipid II, thereby blocking the polymerisation of the peptidoglycan.

The cinnamycin-like type-B lantibiotics display antibactericidal activity against a few bacterial strains, in particular specific *Bacillus* strains. Treated cells show increased membrane permeability [57] as well as an impaired ATP-dependent protein translocation [58] and calcium uptake [59]. In addition, duramycin and cinnamycin inhibit a number of metabolic processes in eucaryotic cells by binding to phosphatidylethanolamine [60,61]; e.g. induction of haemolysis of erythrocytes [62] or inhibition of phospholipase A_2, thereby interfering with prostaglandin and leucotriene biosynthesis [63].

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