Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci

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**Objective:** We have sought ways to circumvent resistance, by combining nisin with other antibiotics known to target bacterial cell wall biosynthesis.

**Methods:** Twenty strains each of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) were tested in vitro by standardized methods against nisin alone and combined with bacitracin, ramoplanin and chloramphenicol.

**Results:** Ramoplanin was the most potent compound, and bacitracin had the least activity. Two-way synergy was observed with nisin and ramoplanin. However, chloramphenicol was clearly antagonistic to the activity of nisin.

**Conclusions:** Observations of synergy between nisin and ramoplanin against MRSA and VRE offer a promising approach to the concept of combining nisin with inhibitors of cell wall peptidoglycan. Further investigations are needed in order to develop this approach as a clinical possibility.

**Introduction**

Nisin, an antibacterial substance produced by *Lactococcus lactis* (formerly *Streptococcus lactis*, Lancefield group N), discovered more than 80 years ago, is widely used as a food preservative. It is regarded as a bacteriocin, but is atypical in having a wide spectrum of activity against Gram-positive bacteria. Nisin is a polypeptide containing 34 amino acid residues (mol. wt 3353), including the unusual compounds lantionine and β-methylthioninone: for this reason it is classed as a 'lantibiotic'.

Other peptide antibiotics, such as magainin and cecropins, act by forming pores in bacterial membranes, causing cell death due to loss of essential intracellular substances. Nisin also does this, but, like vancomycin, interacts with lipid intermediates, preventing peptidoglycan synthesis. At present it is not agreed which of these two mechanisms is the more important.

We have investigated the possible therapeutic value of nisin because of its activity against multi-resistant Gram-positive cocci such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Because resistance to nisin has occurred in pneumococci, *Listeria monocytogenes* and pediococci, it seemed logical to test nisin in combination with appropriate antibiotics, which must be active in their own right against MRSA and VRE and preferably have a mechanism of action that predisposes towards synergy. From the reactions shown in Figure 1, we chose several steps in the enzyme sequence, sensitive to ramoplanin (a lipoglycodepsipeptide antibiotic), an inhibitor of the transferase that converts lipid I into lipid II; bacitracin, which inhibits the pyrophosphorylating enzyme that generates the lipid phosphate precursor of lipid I; and chloramphenicol, which causes accumulation of cell wall peptidoglycan (cell wall thickening) as a secondary effect.
Materials and methods

Antibiotics
Nisin (2.5% w/w; Sigma, Poole, Dorset, UK) was suspended at 10 mg/mL in 0.02 M HCl, and the supernatant fraction following centrifugation at 10,000 g for 10 min (containing 250 mg/L pure nisin) was serially diluted in distilled water. Ramoplanin (potency 88.3% from Dr F. Parenti, Biosearch Italia SPa, Geranzano, Italy) and bacitracin (Zn salt, 63 IU/mg; Riker Laboratories, Loughborough, UK) were dissolved in distilled water. Chloramphenicol (Ph Eur grade; Parke-Davis, Eastleigh, UK) was dissolved in ethanol at 5 g/L and a stock solution of 640 mg/L was made in distilled water.

Bacteria
Twenty MRSA and 20 VRE (seven Enterococcus faecalis, 13 Enterococcus faecium) from our culture collection, isolated from patients, were tested. S. aureus Oxford was used as a standard strain.

Microbiological methods
Antibiotic Medium #1 and IsoSensitest agar (ISA) were from Oxoid (Basingstoke, UK).

Strains were cultured overnight in brain–heart infusion (BHI) broth (Oxoid), at 37°C and diluted to give a spot of ~10^4 cfu inoculated using the Denley Multipoint inoculator (Billinghurst, UK).

MICs were determined for antibiotics alone and in various combinations, by the plate dilution method, as described previously. Antibiotics were incorporated into plates containing 15 mL of ISA, in amounts to give concentrations closer than doubling dilutions, in order to observe small amounts of interactions. Interactions were defined, after drawing isobolograms, in terms of summed fractional inhibitory concentrations (ΣFICs), thus: synergy ΣFICs ≤ 0.7; addition 0.7–1.3; antagonism ΣFICs ≥ 1.3. Specific interactions were characterized as ‘one-way’ if the isobologram was asymmetrical, or ‘two-way’ if symmetrical.

Bacterial activity was investigated by incubating each strain (10^6 cfu/mL in BHI) in the presence of nisin: 1 mg/L for the enterococci, 2 mg/L for the MRSA. Sixteen MRSA strains were also challenged with nisin 64 mg/L. Viable counts were performed at hourly intervals.

Results

Inhibitory activities of individual antibiotics
MICs of nisin, chloramphenicol, ramoplanin and bacitracin alone against the MRSA and VRE strains are shown in Table 1. Ramoplanin was the most potent agent; nisin was slightly more active against VRE than MRSA. Bacitracin had the lowest activity.

Antibiotic interactions
MRSA. 1. Nisin + chloramphenicol: for 18 of the 19 strains tested, chloramphenicol antagonized the activity of nisin, but the reverse interaction did not occur; for nine of these 18 strains, the interaction was marked, isobolograms showing ΣFICs ≥ 2. Addition was found for the other strain.
Nisin versus MRSA and VRE

Table 1. Inhibitory activities (MICs) of nisin, chloramphenicol, ramoplanin and bacitracin against 20 strains each of MRSA and VRE

<table>
<thead>
<tr>
<th>Species (no.of isolates tested)</th>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
<th>range</th>
<th>MIC50</th>
<th>MIC90</th>
<th>geometric mean</th>
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<tr>
<td>MRSA* (20)</td>
<td>nisin</td>
<td>1.5–16</td>
<td>6</td>
<td>12</td>
<td>5.3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chloramphenicol</td>
<td>3–12</td>
<td>6</td>
<td>8</td>
<td>6.7</td>
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<td></td>
<td>ramoplanin</td>
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<td>1.5</td>
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<tr>
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<td>bacitracin</td>
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<td>16</td>
<td>16</td>
<td>9.8</td>
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<tr>
<td>VRE (20)</td>
<td>nisin</td>
<td>1.5–16</td>
<td>4</td>
<td>8</td>
<td>4.3b</td>
<td></td>
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<tr>
<td></td>
<td>chloramphenicol</td>
<td>4–32</td>
<td>6</td>
<td>32</td>
<td>9.6c</td>
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<tr>
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<td>ramoplanin</td>
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<td>0.75</td>
<td>1.5</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bacitracin</td>
<td>12–128</td>
<td>32</td>
<td>&gt;128</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*MICs (mg/L) for reference strain, S. aureus Oxford: nisin 4, chloramphenicol 8, ramoplanin 0.5, bacitracin 16.

1. Nisin + chloramphenicol: two-way synergy (ΣFICmin 0.21–0.58) was observed for nine strains, addition for 10.

2. Nisin + ramoplanin: five strains showed addition, 12 showed small amounts of two-way synergy (ΣFICmin 0.38–0.69). Also, for 12 strains the activity of nisin was antagonized by low concentrations of ramoplanin, as found with MRSA strains (see above). Antagonism was also observed for one VRE strain by following growth curves: this strain did not grow in 4 mg/L nisin alone, but did when 0.19 mg/L ramoplanin was added.

3. Nisin + bacitracin: for 16 strains there was one-way antagonism of nisin by bacitracin (ΣFICmax 1.7–3.38); the remaining strains showed addition.

Bactericidal activity of nisin

MRSA. All 20 MRSA strains used for the antibiotic combination studies were incubated with nisin 2 mg/L, and sampled at hourly intervals for 6 h, then after 24 h. Nisin MICs ranged from 2 to 16 mg/L, thus the challenge dose was sub-MIC. After 2 h, viable counts from 16 strains had fallen from 10^6 to <300/mL; 11 strains regrew overnight, but remained fully sensitive.

A similar experiment carried out using a higher concentration of nisin (64 mg/L) suggested that there may be an ‘Eagle effect’: after 2 h, viable counts from all the strains remained >300/mL.

VRE. Using a challenge concentration of 1 mg/L nisin, a 3-log kill was found within 2 h for 14 strains, and within 5 h for 17 strains. Again, when regrowth was found, the organisms remained fully sensitive to nisin.

Detailed time–kill experiments were done on four VRE strains. Nisin was rapidly bactericidal, a 3-log kill being found within 1 h for all strains with MIC concentrations, and killing was also rapid at concentrations <MIC. For example, one-third MIC (1 mg/L in this case) brought about a 4-log kill in 1 h of VRE # 11; regrowth occurred after 24 h, the organisms remaining sensitive. Conversely, ramoplanin was much more slowly bactericidal.

Discussion

The highly selective targeting by certain antibiotics of the enzymes involved in the synthesis and assembly of bacterial cell wall peptidoglycan has provided one of the outstanding features of modern antimicrobial therapy. Development of resistance to some of these antibiotics (e.g. β-lactams and vancomycin) has focused attention on possible alternative approaches to this serious problem. We suggest here the possible therapeutic use of the lantibiotic nisin, long recognized as a food preservative. Others have investigated different polypeptide agents, such as magainins and other lantibiotics. Ideally, the ultimate goal would be to provide a bacteriologically effective combination of agents with nisin. In order to do this, knowledge of the specific mode of action is clearly of great importance, and although this has not been established...
precisely, there are already a number of clues as to what bacterial targets are involved in the mode of action of nisin and other lantibiotics. For example, Giacometti et al. conclude that nisin acts by triggering the hydrolyases, bringing about lysis, while Breukink et al. believe the target for nisin (and other lantibiotics) is the specific synthesis of lipid II. Like Hancock we believe the latter seems the more convincing explanation. However, lysis (as reported for pneumococci) may be a secondary event arising from further disruption of membrane structure (pore formation) following specific inhibition. In due course, it will emerge which is the primary reaction as opposed to the secondary event in the sequence of interaction of nisin with bacteria. This will add to the understanding of the lantibiotic mode of action and suggest further strategies for selecting synergic partners. The interactions of ramoplanin and nisin reported here strongly support the rationale of using this strategy.

More information is needed on the exact molecular quantities of the agents required to induce the specific membrane events and the precise time sequence of the reactions blocked and/or induced by these antibiotics. Important questions thus remain to be answered, for example, are pores in the membrane developed simultaneously with the inhibition of lipid II formation and by the same quantity of nisin, and does nisin attachment sterically hinder or enhance binding of other antibiotics to their target sites? The answers to such questions should throw much light on the sequence and importance of these antibacterial interactions.

Finally, the question of the therapeutic use of nisin alone or in combination justifies further study. Animal experiments have been encouraging, and some clinical trials are reportedly in progress. We are at present investigating formulations of nisin for clinical use.

Acknowledgements

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References


