Nisin/β-lactam adjunct therapy against *Salmonella enterica* serovar Typhimurium: a mechanistic approach

Aman Preet Singh1, Simran Preet2 and Praveen Rishi1*

1Department of Microbiology, Basic Medical Sciences Block, Panjab University, Sector-14, Chandigarh 160014, India; 2Department of Biophysics, Basic Medical Sciences Block, Panjab University, Sector-14, Chandigarh 160014, India

*Corresponding author. Tel: +91-172-2534146; Fax: +91-172-2541770; E-mail: rishipraveen@yahoo.com

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Objectives: Multidrug resistance exhibited by *Salmonella* strains has proved to be a big hurdle in the development of an effective anti-*Salmonella* therapy. In this context, we had previously demonstrated strong synergism of nisin/ceftriaxone and nisin/cefotaxime combinations against *Salmonella enterica* serovar Typhimurium. However, the mechanism remained unexplored. The present study was therefore planned in order to evaluate the underlying mechanisms responsible for the synergistic effect of nisin in combination with these β-lactam antibiotics against serovar Typhimurium.

Methods: A membrane permeabilization assay along with pulse labelling studies were performed to confirm the ability of the combinations to permeabilize the bacterial membrane and to verify their effects on macromolecule synthesis. Additionally, analysis of peroxidative liver damage was performed and levels of nitric oxide, antioxidant enzymes, tumour necrosis factor-α and nuclear factor-κB were also measured.

Results: 1-N-phenylnapthylamine (NPN) uptake assay results confirmed a permeabilization-dependent mechanism, as NPN was taken up by treated cells in a time- and concentration-dependent manner, indicating that the combination influenced membrane permeability. Likewise, dose- and time-dependent inhibition of DNA, RNA and protein synthesis in the presence of both the combinations was observed. Interestingly, synergistic results inferred from in vivo assays confirmed the immuno-modulatory effects of the combinations in the treated mice.

Conclusions: Nisin/ceftriaxone and nisin/cefotaxime combinations exert their antibacterial activity against *Salmonella* by multiple modes of action that involve membrane permeabilization, inhibition of DNA, RNA and protein synthesis and direct immuno-modulatory activity.

Keywords: antimicrobial peptides, in vivo, immuno-modulation, synergy, TNF-α

Introduction

The emergence of resistance to β-lactam and aminoglycoside antibiotics resulted in the discovery of newer classes of synthetic antibiotics, including quinolones and higher-generation cephalosporins.1 However, reports of increasing MIC values and the subsequent increase in incidence of treatment failures due to infection with typhoidal salmonellae exhibiting reduced susceptibility and resistance to these antibiotics suggest that antibiotic therapy will not remain effective for much longer.2–4 This worldwide escalation of bacterial resistance to conventional antibiotics is a serious concern for modern medicine. In view of this development, the scientific community is forced to find some alternative, either by decreasing the amount of antibiotics administered or by ending their use completely.

In this context, antimicrobial peptides (AMPs) of prokaryotic and eukaryotic origin have recently gained interest owing to their versatile applicability for use against infectious diseases.5–7 Besides their antimicrobial activity, AMPs are also known to possess chemotactic activities and are therefore recognized as linkers of natural and acquired immune responses.8,9 Lately, co-therapy using two or more antimicrobial agents has been exploited; in particular, combinations of bacteriocins and antibiotics have been successfully employed to overcome antibiotic resistance in selected cases.10,11 Various studies have been carried out to elucidate the mechanisms of these clinically relevant synergistic interactions, which have shown surprising variability in the ways that pathogens are killed, including new intracellular targets, unsuspected modes of membrane interaction and blockade of soluble virulence factors independently of direct interaction with bacteria or modulation of the immune system.12,13

In this context, one promising alternative is the use of nisin, a ribosomally synthesized and post-translationally modified...
bacteriocin. In our earlier study, we demonstrated that the use of nisin/antibiotic combinations against Salmonella may be perceived as a promising solution to the growing problem of resistance to conventional antibiotics, particularly by facilitating the entry of drugs into cells. In this study, nisin/ceftriaxone and nisin/cefotaxime combinations demonstrated excellent in vitro and in vivo synergism against the serovar Typhimurium. Therefore, combination with a suitable antibiotic might be used to increase in vivo activity in terms of direct killing, as well as more pronounced immuno-modulatory activity. However, the mechanism(s) of this synergism remains unexplored. In the light of these facts, the present study aimed at exploring the possible in vitro (in terms of membrane permeabilization and macromolecular synthesis) and in vivo mechanism(s) responsible for the synergistic effect of nisin in combination with conventional antibiotics against Salmonella enterica serovar Typhimurium.

Materials and methods

Ethics statement

The experimental protocols were approved by the Institutional Animal Ethics Committee (approval ID IAEC/156, dated 25.08.2011) of the Panjab University, Chandigarh, India (registration number 45/1999/CPCSEA) and performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation. All efforts were made to minimize the suffering of animals.

Animals

Female BALB/c mice (18–22 g, 4–5 weeks old) were procured from Central Animal House, Panjab University, Chandigarh, India. The animals were housed under standard conditions with free access to food and water.

Bacterial strain and growth medium

NCTC74, a standard strain of Salmonella enterica serovar Typhimurium originally provided by the Central Research Institute, Kasauli, India, was used in the present study. This strain has been maintained in our laboratory for the last several years and has also been used in recent studies. The strain was maintained on nutrient agar slants and preserved at −80°C by making 20% glycerol stocks. Every time the strain was used in an experiment, its purity was confirmed biochemically and serologically.

For preparation of bacterial cell suspensions, bacterial cells grown over-night (at 37°C, 150 rpm) in nutrient broth (5.0 g/L peptone, 5.0 g/L NaCl, 1.5 g/L beef extract, 1.5 g/L yeast extract, pH 7.4 ± 0.2) were harvested by centrifugation (3738 g, 15 min), washed once with 10 mM sodium PBS (pH 7.2), and resuspended in PBS to a final concentration of ~10^8 cfu/mL.

Agents

Nisin (2.5% w/w), EDTA, ceftriaxone and cefotaxime powder were obtained from Sigma-Aldrich (St Louis, MO, USA). Ceftriaxone, cefotaxime and EDTA were dissolved in distilled water and nisin was dissolved in 0.2 N HCl. Stock solutions of 500 mM for EDTA and 1000 mg/L for other agents were prepared and used within 1 week. When calculating nisin concentrations, the percentage of active ingredient was used.

Determination of MICs and fractional inhibitory concentrations (FICs)

The MICs and FICs of all the test agents employed in the present study were evaluated as described by us previously. The MIC was defined as the lowest concentration of each antibiotic not producing any visible microbial growth. The FICs were calculated after dividing the MICs of the test agents in combination by the MICs of test agents alone, separately. The FIC index, obtained by adding both FICs, was interpreted as indicating a synergistic effect when it was ≤0.5, as additive or indifferent when it was >0.5 and ≤2.0, and as antagonistic when it was >2.0.

Membrane permeabilization assay

The ability of nisin, ceftriaxone and cefotaxime to permeabilize the membrane of serovar Typhimurium was evaluated by a 1-N-phenylnapthylamine (NPN) uptake assay as described by us previously. To evaluate the effect at different peptide to lipid ratios, subinhibitory and higher concentrations of tested agents were used. Briefly, 50 μL of suspension containing mid-log-phase bacterial cells (1×10^7 cfu/mL) at desired concentrations was suspended in 100 μL of 5 mM HEPES buffer (pH 7.4) containing 10 μM NPN in 1.5 mL tubes. After 5 min of incubation, various concentrations of test agents (at their respective MICs and FICs; shown in Figure 1) were added, and the increase in fluorescence of NPN was monitored at excitation and emission wavelengths of 340 and 415 nm, respectively, with a slit width of 5 nm. EDTA (a known membrane

Figure 1. Concentrations of tested agents used in the membrane permeabilization assay. CRO, ceftriaxone; CTX, cefotaxime.
permeabilizer; 10 mM) was added to the control tubes. The emission and excitation wavelengths were determined after analysing the fluorescence spectrum of NPN in the presence of bacterial cells (without any membrane permeabilizer) at different excitation wavelengths using an LS55-Perkin Elmer luminescence spectrophotometer as described by us previously. To measure the time-dependent increase in permeabilization of various tested agents in bacterial cells, relative fluorescence units (fluorescence value of the cell suspension with the test substance and NPN minus the corresponding value of the cell suspension and NPN without the test substance) were also determined at different time intervals.

Effect on macromolecular synthesis (pulse labelling studies)

The effect of nisin/ceftriaxone and nisin/cefotaxime combinations on intracellular metabolic pathways was also studied, using a macromolecular synthesis assay that monitored inhibition of synthesis of key macromolecules such as DNA, RNA and proteins by using [methyl-3H]thymidine, [methyl-3H]uridine and L-[methyl-3H]leucine, respectively, as described by us previously. Investigations were carried out over a range of subinhibitory concentrations, inhibitory concentrations and twice the inhibitory concentrations of the tested combinations, i.e., 0.5×FIC, FIC and 2×FIC, respectively, against S. enterica serovar Typhimurium. The radioactivity incorporated in the cells was calculated using a standard curve plotted between cpm and radioactivity (mCi) for all three radiolabelled precursors (at various concentrations). The calculated radioactivity was then converted to molar concentrations of each of the precursors by using the following formula:

\[
\text{moles of precursor incorporated} = \frac{\text{calculated radioactivity}}{\text{specific activity (for each precursor)}}
\]

In vivo studies: experimental design

Mice were infected with 0.25 mL of 10^7 cfu/mL of serovar Typhimurium orally. Seven days after the challenge, establishment of Salmonella infection was confirmed by bacterial translocation into the intestines, livers and spleens of the infected mice. Seven days post-infection, mice were divided into six groups, each comprising six to eight mice. A group of six mice was set aside to serve as the uninfected control. Following treatment, the following groups were established: (i) control group: mice in this group were injected with 0.1 mL sterile saline subcutaneously (sc) and served as the control (infected) group; (ii) infected and nisin treated: mice in this group were administered 50 mg/kg body weight of nisin sc; (iii) infected and ceftriaxone treated: mice in this group were administered 50 mg/kg body weight of ceftriaxone sc; (iv) infected and cefotaxime treated: mice in this group were administered 100 mg/kg body weight of cefotaxime sc; (v) infected and nisin/ceftriaxone treated: mice in this group were co-administered nisin (50 mg/kg body weight sc) and ceftriaxone (50 mg/kg body weight sc); and (vi) infected and nisin/cefotaxime treated: mice in this group were co-administered nisin (50 mg/kg body weight sc) and cefotaxime (100 mg/kg body weight sc).

All the test agents were injected in four doses sc with 12 h intervals, individually and in combination. The doses used in the present study were selected on the basis of preliminary studies done to determine the reduction in bacterial load in different target organs.

Post-mitochondrial supernatant preparation

At 48 h post-therapy, mice from all groups were sacrificed and their livers were removed aseptically and rinsed in 0.05 M PBS (pH 7.4). A 10% (w/v) homogenate of each liver was prepared in PBS using a Potter–Elvehjem homogenizer. An aliquot of the liver homogenate was used for the estimation of lipid peroxidation and tumour necrosis factor-α (TNF-α). However, for the estimation of nitrite, superoxide dismutase (SOD) and catalase activities, a post-mitochondrial preparation was prepared. For this purpose, the remaining tissue homogenates were centrifuged at 8900 g for 20 min at 4°C in a refrigerated centrifuge. The supernatants thus obtained were called post-mitochondrial supernatants. They were used to avoid interference from reactive oxygen and reactive nitrogen species, which are otherwise generated during the essential cellular metabolic processes at mitochondrial sites.

Extent of peroxidative liver damage

Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids (PUFAs). The peroxidation of PUFAs is a feature of many types of cell injury in which free radical intermediates are produced in excess. Lipid peroxidation can cause changes in membrane fluidity and permeability and can increase the rate of protein degradation, which eventually leads to cell lysis. Measurement of lipid peroxidation products, such as malondialdehyde (MDA), is an indication of the extent of damage. Quantitative measurement of lipid peroxidation in liver was performed according to the method of Wills as described previously. The results were expressed as nmol of MDA/mg of protein, using the molar extinction coefficient of chromophore (1.56×10^5 M^-1 cm^-1). The protein content of tissue homogenates was calculated as described previously.

Estimation of enzymatic antioxidant activity

Development of tissue injury and the outcome of the disease depend on the balance between the generation of toxic radicals and tissue antioxidant status. Mammalian cells are equipped with antioxidant systems to combat free radical-mediated damage. In general, antioxidants consist of non-enzymatic substances and enzymes such as SOD and catalase. Therefore, changes in the functional status of antioxidant enzymes in the livers were also estimated in the present study. SOD activity was assayed according to the method of Kono and was expressed as units of SOD/mg of protein, where a unit of activity was defined as the amount of SOD required to inhibit the rate of reduction of nitro blue tetrazolium (NBT) by 50%. The catalase activity was assayed by the method of Luck and was expressed as mmol of H_2O_2 decomposed/min/mg of protein using the molar extinction coefficient of the chromophore (0.0394 mM^-1 cm^-1).

Estimation of nitrite levels

Nitric oxide (NO) is an important signalling molecule that is produced in large quantities during host defence and immunological reactions. However, excessive amounts of NO are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation. NO is a fairly short-lived molecule (with a half-life of a few seconds) produced from enzymes known as NO synthases (NOSs). Estimation of nitrite levels is an indirect measure of NO content because reactive nitrogen intermediates, such as nitrates and nitrates, are known to be the end products of oxidative metabolism of labile NO and their quantification is regarded as an indicator of NO generation. The amount of NO was determined by the Griess reaction, as described previously. The assay is based on the propensity of NO to be oxidized to nitrate and nitrite under physiological conditions. For this, 100 μL aliquots of post-mitochondrial supernatant were mixed with 100 μL of Griess reagent (0.1% naphthylethylene diamine dihydrochloric acid and 1% sulphanilamide in 5% phosphoric acid) and incubated at room temperature (28–30°C) for 10 min. Absorbance was measured at 546 nm. Nitrite levels in all the samples were quantified according to the standard graph for sodium nitrite.
Liver TNF-α assay

In order to substantiate the role of TNF-α in altering nitrite and antioxidant levels, an ELISA assay for TNF-α was performed in the liver homogenates using a commercially available cytokine assay kit (RayBiotech, Norcross, GA, USA) according to the manufacturer’s instructions. Briefly, standards for TNF-α were dispensed in 96-well microtitre plates pre-coated with monoclonal antibody specific for mouse TNF-α. To each of the designated wells, 25 μL of each test sample and 75 μL of assay diluent were added, the plates were sealed with acetic plate sealer and they were then incubated at room temperature for 2.5 h. Plates were then washed five times with the wash buffer and 100 μL of biotin antibody was dispensed into each well. Plates were again sealed and incubated at room temperature for 1 h, after which they were washed five times with the wash buffer and then 100 μL of streptavidin solution was dispensed into each well. Plates were again sealed and incubated at room temperature for 45 min. Plates were then washed five times with the wash buffer and 100 μL of substrate solution was dispensed into each well. Plates were finally incubated at room temperature (in darkness) for 30 min. Stop solution (50 μL) was added to each well and absorbance was measured at 450 nm. The results were expressed as pg/mL of TNF-α released.

Assay for nuclear factor-κB (NF-κB) p50 subunit

Expression of TNF-α is directly related to NF-κB levels because, upon activation, NF-κB results in the expression of inflammatory mediators, including cytokines (particularly TNF-α and IL-6), chemokines, lipid mediators, enzymes and adhesion molecules. Likewise, blocking of NF-κB activation may further down-regulate TNF-α, as has been reported previously. Therefore, an assay for the NF-κB p50 subunit in the nuclear extracts was performed in nisin- and antibiotic-treated groups (alone and in combination) using a commercially available kit (Transcription Factor Assay Kit, Upstate Biotechnology, NY, USA) according to the manufacturer’s instructions. This assay combines the principle of the electrophoretic mobility shift assay (EMSA) with the 96-well-based enzyme linked immunosorbent assay (ELISA). Briefly, nuclear extracts from all the tested groups were prepared using Chemicon’s Nuclear Extraction Kit. During the assay, the capture probe, a double-stranded biotinylated oligonucleotide containing the consensus sequence for NF-κB, was mixed with the nuclear extract in the transcription factor assay buffer provided, directly in the streptavidin-coated plate, and incubated in the plate well for 2 h at room temperature. Plates were then washed to remove the unbound material. The bound NF-κB transcription factor subunit p50 was detected with a specific primary antibody, mouse anti-NF-κB p50. Horseradish peroxidase-conjugated secondary antibody was then used for detection using 3,3′,5′-tetramethylbenzidine (TMB/E) as the substrate and absorbance was read at 450 nm. Positive and negative controls were also run simultaneously.

Statistical analysis

Data are expressed as mean ± SD of three to five independent experiments. Statistical analysis was done by Student’s unpaired t-test and one-way analysis of variance, followed by pairwise comparison procedures (Tukey test), using Jandel SigmaStat statistical software, version 2.0. In all cases, statistical significance was defined as a P value of <0.05.

Results

Membrane permeabilization assay

The series of emission spectra obtained with different excitation wavelengths (slit width 5 nm) for NPN in presence of S. enterica serovar Typhimurium exhibited an absorption maximum at ~415 nm. The most effective excitation wavelength was found to be 340 nm; an almost similar response was obtained by exciting at 330 or 350 nm. In the absence of serovar Typhimurium, NPN in HEPES buffer yielded weak fluorescence that peaked at 457 nm (excitation at 340 nm, data not shown).

Nisin alone did not show any significant effect upon addition to the cell suspension in the presence of NPN, while a significant increase in fluorescence intensity was observed when ceftriaxone and cefotaxime alone were added to the cell suspension compared with the intensity observed with NPN alone. However, both nisin/ceftriaxone and nisin/cefotaxime combinations were found to permeabilize the bacterial outer membrane to NPN more prominently in a time-dependent manner. Incubation of the cells with NPN in the presence of either of the two tested combinations resulted in maximum blue shift in emission peak with increasing fluorescence intensity compared with the intensity of the peak observed when the cells were incubated with NPN in the absence of the combinations (Figure S1, available as Supplementary data at JAC Online). These results suggested that both nisin/ceftriaxone and nisin/cefotaxime have the ability to permeabilize the outer membrane of S. enterica serovar Typhimurium. Furthermore, relative fluorescence units were also found to be significantly increased (P<0.05) in a dose- and concentration-dependent manner in the presence of either combination, indicating the increasing permeabilization of antimicrobial agents with time (compared with controls) (Figure 2).

Effect on macromolecule synthesis (pulse labelling studies)

Dose- and time-dependent inhibition of DNA, RNA and protein synthesis in the presence of either of the tested combinations was observed. In control cells, which were not exposed to the tested combinations, DNA synthesis was found to be increased after 60 min of exposure. Inhibition of incorporation of thymidine after 60 min was 51.85% (P<0.05), 82.41% (P<0.05) and 94.44% (P<0.05) in the presence of 2 mg/L nisin + 0.38 mg/L ceftriaxone (0.5×FIC), 4 mg/L nisin + 0.75 mg/L cefotaxime (FIC) and 8 mg/L nisin + 1.5 mg/L cefotaxime (2×FIC), respectively, compared with control cells (grown in the absence of the combinations) (Figure 3a). Likewise, 49.07% (P<0.05), 81.94% (P<0.05) and 92.41% (P<0.05) inhibition of thymidine incorporation was observed after 60 min when serovar Typhimurium was incubated in the presence of 2.5 mg/L nisin + 0.5 mg/L cefotaxime (0.5×FIC), 5 mg/L nisin + 1 mg/L cefotaxime (FIC) and 10 mg/L nisin + 2 mg/L cefotaxime (2×FIC), respectively (Figure 4a).

Similarly, nisin/ceftriaxone and nisin/cefotaxime also exerted an inhibitory effect on RNA synthesis in serovar Typhimurium as 35.09% (P<0.05), 78.07% (P<0.05) and 89.47% (P<0.05) inhibition in the presence of nisin/ceftriaxone (Figure 3b) and 38.6% (P<0.05), 80.35% (P<0.05) and 87.72% (P<0.05) inhibition (in the presence of nisin/cefotaxime) of incorporation of uridine was observed when the cells were grown in the presence of 0.5×FIC, FIC and 2×FIC of the combinations, respectively, for 60 min (Figure 4b).

The tested combinations also exhibited a profound effect on protein synthesis by serovar Typhimurium as the percentage inhibition of incorporation of leucine after 60 min was found to be 37.5% (P<0.05), 83.4% (P<0.05), 93.55% (P<0.05) in the
presence of nisin/ceftriaxone (at 0.5 × FIC, FIC and 2 × FIC, respectively) (Figure 3c) and 35.55% (P, 0.05), 82.81% (P, 0.05) and 91.8% (P, 0.05) in the presence of nisin/cefotaxime (at 0.5 × FIC, FIC and 2 × FIC, respectively) compared with leucine incorporation in control cells (Figure 4c). It can be concluded from these results that the nisin/ceftriaxone and nisin/cefotaxime combinations exert their most significant effect on DNA synthesis with lesser effects on protein and RNA synthesis.

**Extent of peroxidative liver damage**

Infection with serovar Typhimurium induced significant (P<0.05) lipid peroxidation in the infected mice, as indicated by increased MDA levels compared with uninfected mice (202.73 ± 27.02 nmol/mg of protein in infected controls versus 95.04 ± 10.47 nmol/mg of protein in uninfected controls). However, nisin alone did not show a significant effect, while significant decreases in the levels of MDA were observed in mice treated with ceftriaxone and cefotaxime alone compared with the untreated infected group. On the other hand, adjunct therapy with nisin/ceftriaxone (113.13 ± 5.66 nmol/mg of protein) and nisin/cefotaxime (117.89 ± 6.26 nmol/mg of protein) gave much larger reductions, observed as decreased MDA levels (Table 1).

**Estimation of enzymatic antioxidant activity**

Infection with serovar Typhimurium induced a significant decrease (P<0.05) in the activity of enzymatic antioxidants (SOD and catalase) compared with the uninfected control group (1.94 ± 0.24 U/mg of protein in SOD activity and 46.04 ± 2.87 mmol of catalase/mg of protein). The group treated with nisin alone did not show any significant change in SOD and catalase activity compared with the untreated infected group. However, mice treated with the combinations showed a spectacular restoration of SOD and catalase activity, which attained values close to those in uninfected controls (Table 1).

**Estimation of nitrite levels**

The group treated with nisin alone (14.13 ± 2.38 μmol/mg of protein) did not show any significant change in nitrite levels compared with the untreated infected group (14.8 ± 3.05 μmol/mg of protein), while a significant decrease was observed in the
groups treated with ceftriaxone alone (7.86 ± 1.16 μmol/mg of protein) and cefotaxime alone (8.36 ± 0.66 μmol/mg of protein) compared with the infected control group. Interestingly, groups co-administered nisin and antibiotics showed maximum decreases in nitrite levels, close to the normal levels observed in the uninfected group (5.32 ± 0.31 μmol/mg of protein in the nisin/ceftriaxone-treated group and 5.72 ± 1.13 μmol/mg of protein in the nisin/cefotaxime-treated group versus 3.63 ± 0.54 μmol/mg of protein in the uninfected group) (Table 1).

Liver TNF-α assay

Infection with serovar Typhimurium induced significant TNF-α generation (P < 0.05) (277.51 ± 22.5 pg/mL) compared with the uninfected control group (68.26 ± 8.25 pg/mL). Levels of TNF-α observed in the nisin-treated group (264.43 ± 20.62 pg/mL) were similar to the levels observed in the untreated infected group, while groups that were treated with ceftiraxone alone (158.53 ± 10.05 pg/mL) and cefotaxime alone (160.77 ± 10.53 pg/mL) showed significant decreases. However, groups that were treated with combinations of nisin/ceftiraxone and nisin/cefotaxime showed more prominent effects (106.86 ± 7.18 pg/mL in the nisin/ceftiraxone-treated group and 111.64 ± 9.19 pg/mL in the nisin/cefotaxime-treated group) (Figure 5).

Assay for NF-κB p50 subunit

Levels of the NF-κB p50 subunit were significantly (P < 0.01) elevated in the group infected with serovar Typhimurium compared with the uninfected control group (Figure 6). However, no significant change in level was observed in the group treated with nisin alone, while the groups treated with ceftiraxone alone and cefotaxime alone showed significantly attenuated activation of NF-κB. Conversely, levels of the NF-κB p50 subunit were further reduced significantly (P < 0.001) in groups treated with nisin/ceftiraxone or nisin/cefotaxime (Figure 6).

Discussion

The synergistic activity of AMPs with antibiotics has been explained by many hypotheses but little evidence has been obtained, mostly because of the scarcity of conclusive data on the molecular mechanism of action for most agents. However, the frantic search for effective therapies in infections...
with multidrug-resistant bacteria, combined with the characterization of the molecular mechanism of action of some AMP classes, is contributing to bridging the gap between basic research and clinical applications in translational research on AMPs. Recently we demonstrated the in vivo therapeutic potential of nisin/β-lactam combinations against serovar Typhimurium. In the present study, we aimed to explore the basic mechanism that might be responsible for this synergistic effect.
The most common explanation for synergism is permeabilization of the bacterial membrane, which would facilitate the penetration of the antibiotic into the cell.\textsuperscript{31,32} Our NPN uptake assay results confirmed this permeabilization-dependent mechanism for the tested nisin/antibiotic combinations against serovar Typhimurium. The hydrophobic fluorescent probe NPN was used as an indicator of membrane integrity. It has a low fluorescence quantum yield in aqueous solution but fluoresces strongly in the hydrophobic environment of a biological membrane. It provides a sensitive probe for outer membrane barrier function because it is...

\begin{figure}[h]
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\caption{Modulatory effects of nisin, ceftriaxone and cefotaxime alone and in combination on hepatic TNF-\(\alpha\) levels in mice infected with serovar Typhimurium. Values are expressed as mean±SD of three independent observations. *\(P<0.05\) versus uninfected mice (uninfected control). \#\(P<0.05\) versus log\(_{10}\) cfu of serovar Typhimurium in untreated mice (infected control). €\(P<0.05\) versus mice treated with nisin alone. CRO, ceftriaxone; CTX, cefotaxime. Numbers in the bar labels are drug doses (mg/kg).}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Effects of nisin, ceftriaxone and cefotaxime alone and in combination on Salmonella-induced activation of NF-\(\kappa\)B in liver. Values are expressed as mean±SD of five different observations. *\(P<0.05\) versus uninfected mice (uninfected control). \#\(P<0.05\) versus untreated mice (infected control). *\(P<0.05\) versus mice treated with nisin alone. \#\(P<0.05\) versus mice treated with ceftriaxone alone. †\(P<0.05\) versus mice treated with cefotaxime alone. CRO, ceftriaxone; CTX, cefotaxime. Numbers in the bar labels are drug doses (mg/kg).}
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Synergistic mechanism of nisin/β-lactam combination

...excluded from intact microbial cells but can enter at points where membrane integrity is compromised. Normally, the intact outer membrane excludes hydrophobic molecules, but through the action of the tested combinations in destabilization of the bacterial membrane, the phospholipids become accessible and NPN enters into the damaged phospholipid layer, where it emits stronger fluorescence.\(^{33}\) Nisin alone did not show any permeabilization, but ceftriaxone and cefotaxime alone were observed to permeabilize the bacterial membrane. However, a more pronounced increase was observed in the presence of either of the tested combinations. Owing to the properties of β-lactam antibiotics as metal chelators, it may be possible that these antibiotics induce morphological changes in target cells, which might help increase permeabilization of nisin across the bacterial outer membrane, enabling it to work against otherwise nisin-resistant Salmonella.\(^ {14,34,35}\) Further, nisin may also render the drug efflux pumps ineffective, thereby increasing cellular drug accumulation by altering cell permeability, leading to collapse of the membrane potential and causing rapid efflux of materials present in the cytosolic milieu.\(^ {36}\) These two mechanisms might have acted mutually, leading to an enhanced anti-Salmonella effect, as observed in the NPN assay in the present study. The marked blue shift accompanied by an increase in fluorescence intensity observed in the emission spectrum of NPN in the presence of nisin/ceftriaxone and nisin/cefotaxime indicated the movement of NPN into a more hydrophobic environment. Further, NPN was taken up by damaged cells in a time- and concentration-dependent fashion, indicating that the combinations influenced membrane permeability. These observations are in concordance with our earlier scanning electron microscopy findings suggesting disruption of the bacterial membrane.\(^ {14}\)

In addition to membrane disruption, another explanation for synergy is an effect on intracellular targets such as nucleic acids and proteins.\(^ {37}\) To validate this, a macromolecular synthesis assay was performed. Dose- and time-dependent inhibition of DNA, RNA, and protein synthesis was observed. Interestingly, the nisin/ceftriaxone and nisin/cefotaxime combinations were more effective in inhibiting DNA synthesis than in inhibiting protein and RNA synthesis. It might also be possible that membrane permeabilization affects macromolecular synthesis due to leakage of cell contents and ions that are essential for the activity of intracellular enzymes, thereby interfering with essential metabolic processes inside the target cells.\(^ {16}\)

AMP/antibiotic combinations often have immuno-modulatory properties, which can provide a significant or even prominent means of clearing infections.\(^ {12,25}\) In this context, the immuno-modulatory efficacy of nisin has been well documented and has been reported to alter both the cell-mediated and the humoral immune response.\(^ {40-45}\) Since the liver is implicated in biological processes, its damage has severe consequences for metabolism, the immune response, detoxification and antimicrobial defences. Therefore, in order to confirm the in vivo immuno-modulatory properties of the tested combinations, levels of oxidants and antioxidants, TNF-α and the NF-κB p50 subunit were also assessed in the livers of treated and non-treated mice.

There is evidence that Salmonella infection results in excessive production of reactive oxygen species, leading to lipid peroxidation and finally to tissue damage.\(^ {15}\) In the present study, nisin/β-lactam adjunct therapy led to a significant decrease in the extent of lipid peroxidation (in terms of MDA level) and restored SOD and catalase activity in the treated mice. These combinations might have exerted this synergistic effect by scavenging free radicals (efficient detoxification of H₂O₂) and by up-regulating antioxidant activity, thus countering oxidative stress and thereby providing protection against oxidative damage.

The drastically elevated levels of nitrite observed in the Salmonella-infected groups might have been associated with TNF-α, as it is known for its potent stimulatory activity of inducible NOS, which increases NO levels.\(^ {28,44}\) Interestingly, we observed a significant decrease in nitrite levels and restoration of the TNF-α level in all groups treated with both tested combinations. In agreement with our findings, it has been reported previously that β-lactams can attenuate TNF-α levels,\(^ {45,46}\) an effect that might have led to the NO levels seen following treatment in the present study. Moreover, cellular sensitivity and resistance to TNF-α is correlated with decreased and increased levels of SOD, respectively.\(^ {47,48}\) Our TNF-α assay results confirmed these assumptions, as increased levels of SOD, and decreased levels of TNF-α were observed in the presence of nisin/ceftriaxone and nisin/cefotaxime combinations. Owing to the properties of the tested combinations, levels of oxidants, antioxidants, and TNF-α might have exerted this synergistic effect by scavenging free radicals and providing protection against oxidative damage.

In conclusion, we report that nisin/β-lactam combinations exert their anti-Salmonella activity by displaying multiple modes of action, such as membrane permeabilization and inhibition of macromolecular synthesis, as well as by immuno-modulation. These observations seem to be significant value as the combinations provide the basis for developing therapeutic formulations that may serve as an alternative strategy to combat Salmonella infections.

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**Transparency declarations**
None to declare.
Supplementary data
Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
Synergistic mechanism of nisin/β-lactam combination