Intracellular activity of the peptide antibiotic NZ2114: studies with Staphylococcus aureus and human THP-1 monocytes, and comparison with daptomycin and vancomycin

Karoline Sidelmann Brinch¹*, Paul M. Tulkens², Francoise Van Bambeke², Niels Frimodt-Møller³, Niels Høiby⁴ and Hans-Henrik Kristensen¹

¹Novozymes A/S, Pharma Discovery, Krogshøjvej 36, DK-2880 Bagsværd, Denmark; ²Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, UCL 7370 avenue E. Mounier 73, B-1200 Brussels, Belgium; ³National Center for Antimicrobials & Infection Control, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark; ⁴Department of Clinical Microbiology, Rigshospitalet, University of Copenhagen, Juliane Maries Vej 22, DK-2100 Copenhagen Ø, Denmark

*Corresponding author. Tel: +45-4446-4787; Fax: +45-4446-3233; E-mail: kbri@novozymes.com

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Objectives: Staphylococcus aureus survives inside eukaryotic cells. Our objective was to assess the activity of NZ2114, a novel peptidic antibiotic, against intracellular S. aureus in comparison with established antistaphylococcal agents acting on the bacterial envelope with a distinct mechanism.

Methods: The extracellular (broth) and intracellular (THP-1 monocytes) activities of NZ2114 were compared with those of vancomycin and daptomycin against methicillin-susceptible S. aureus (MSSA), methicillin-resistant S. aureus (MRSA) and vancomycin-resistant S. aureus (VRSA).

Results: All three compounds showed an extracellular bactericidal effect (≥3 log₁₀ kill) against MSSA and MRSA. Daptomycin and NZ2114 also exhibited bactericidal activity against VRSA. The extracellular killing was concentration dependent for all three compounds within the range of drug concentrations tested. The intracellular experiments demonstrated a maximal intracellular effect of NZ2114 after 24 h as a 5 log₁₀ cfu reduction against MSSA (ATCC 25923), while the activity was a 0.9 log₁₀ cfu reduction against MRSA and a 0.2 log₁₀ cfu reduction against VRSA. For comparison, the intracellular activity of daptomycin was a 1.0 log₁₀ cfu reduction against MSSA, a 0.8 log₁₀ cfu reduction against MRSA and a 0.3 log₁₀ cfu reduction against VRSA. Vancomycin showed activity against both MSSA and MRSA (0.6 log₁₀ cfu reduction), whereas VRSA was resistant to vancomycin.

Conclusions: NZ2114 displayed similar extracellular and intracellular activities as daptomycin, and was more effective than vancomycin against the intracellular forms of susceptible bacteria. However, the study also showed that the intracellular activities of NZ2114 and daptomycin are weaker than their extracellular activities.

Keywords: antimicrobial peptides, killing kinetics, plectasin

Introduction

Staphylococcus aureus causes a wide spectrum of mild to severe infections in both humans and animals.¹ Several factors contribute to the persistence and recurrence of these infections, but an important feature is the ability of the bacteria to invade and survive inside phagocytes and other cells.² Recent studies showed no direct correlation between the accumulation of antibiotics in host cells and their activity against intracellular S. aureus, and antibiotics commonly recommended for infections caused by resistant strains, such as vancomycin and daptomycin, exhibit poor intracellular activity.³ This supports the need to assess each drug individually for intracellular antistaphylococcal activity, especially when dealing with new compounds.

NZ2114 is a variant of plectasin, a defensin found in the pezizean fungus, Pseudoplectania nigrella. This peptide has shown a potent antimicrobial effect against various Gram-positive bacteria, including resistant strains of S. aureus,⁴ as its mode of action involving Lipid II and its precursors is different from that of currently used antistaphylococcal compounds.⁵

Our aim was to assess NZ2114 for antistaphylococcal activity in the THP-1 monocyte model in comparison with two well-established and clinically used antistaphylococcal compounds...
also acting on the bacterial envelope, daptomycin and vancomycin, using both susceptible and resistant isolates.

**Materials and methods**

**Bacterial strains, susceptibility testing, sources of antibiotics and cells**

Methicillin-susceptible *S. aureus* (MSSA) ATCC 25923, methicillin-resistant *S. aureus* (MRSA) #428 (clinical isolate from axil: Statens Serum Institut) and vancomycin-resistant *S. aureus* (VRSA) [VRSA2, Pennsylvania H1P11983 obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) programme (operated by Eurofins Medinet, Inc., Herndon, VA, USA; supported under NIAID/NIH contract #HHSN272007000055C)] were used in the studies. MICs were determined in Mueller–Hinton broth (with 50 mg/mL CaCl₂ for daptomycin) at pH 5.4 and 7.4. NZ2114 (mol. wt: 4417 Da) was provided by Novozymes ( Bagsværd, Denmark) and formulated in KING buffer pH 5.0 (Fresenius Kabi, Copenhagen, Denmark). Daptomycin was from Cubicin® (Novartis International AG, Basel, Switzerland) and vancomycin was from Sigma–Aldrich (St Louis, MO, USA). Myelomonocytic cells THP-1 cells (ATCC TIB-202) were obtained from the ATCC (Manassas, VA, USA).

**Extracellular and intracellular dose–kill curve studies**

These studies were performed as previously described.³ For extracellular activity, bacteria were used at a density of 10⁶ cfu/mL and the number of viable bacteria was determined after 24 h of incubation with antibiotics. For intracellular activity, opsonized bacteria (5×10⁵ cfu/mL) were added to THP-1 monocyte cultures at a bacterium-to-monocyte ratio of 4:1. After 1 h, non-phagocytosed bacteria were removed by exposure to 50 mg/L gentamicin for 45 min. Monocytes were then resuspended in standard culture medium and a first sample taken for determination of the initial cfu content. A second sample was taken after 24 h incubation in the presence of antibiotics at 37°C in a 5% CO₂ atmosphere. In both cases, cfu were measured by automated colony counting and the results expressed as cfu per mL (extracellular bacteria) or mg cell protein (intracellular bacteria).

**Cell cytotoxicity**

The effect on viability of NZ2114, vancomycin and daptomycin on THP-1 monocytes was assessed by Trypan Blue exclusion test, with cells exposed to the compounds at up to 256 mg/L for 24 h. Standard culture medium and 70% ethanol served as negative and positive controls, respectively.

**Curve-fitting and statistical analyses**

For the analysis of dose–effect relationships, the Hill equation (slope = 1) was used to calculate the relative maximal efficacy (Eₘₐₓ), the static concentration (Cₜₐₜ), and the goodness of fit (R²), as determined by non-linear regression using GraphPad Prism® 5.0 (GraphPad Prism Software, San Diego, CA, USA). Multiple comparisons between Eₘₐₓ values for all three compounds were performed by one-way analysis of variance with the Tukey post-hoc test (P<0.05). Comparisons of corresponding Eₘₐₓ values of extracellular and intracellular activities for each compound were performed using the unpaired, two-tailed t-test (P<0.05). Analysis of covariance (Tukey’s) was performed for extracellular versus intracellular concentration.

**Results**

**Susceptibility studies**

MICs of NZ2114, daptomycin and vancomycin at pH 7.4 were: 4, 1 and 2 mg/L for ATCC 25923; 2, 1 and 1 mg/L for MRSA #428; and 4, 1 and >128 mg/L for VRSA2. The activities of both NZ2114 and daptomycin were impaired by the acidic pH, with increases in MIC up to 16-fold compared with at pH 7.4. In contrast, the activity of vancomycin was unaffected by this pH change.

**Extracellular concentration–effect studies**

The extracellular killing effects of NZ2114, daptomycin and vancomycin at concentrations ranging from 0.001- to 128-fold the MIC over a 24 h period on susceptible strains are shown in Figure 1 and Table 1. All three compounds exhibited a bactericidal effect (Eₘₐₓ > 3 log₁₀ decrease in cfu compared with the initial inoculum).

**Cell toxicity**

The viability of THP-1 monocytes was fully maintained in THP-1 cells exposed to NZ2114, daptomycin and vancomycin at concentrations of up to 256 mg/L (≤1% of dead cells; no difference from control medium, >99% stained cells with 70% ethanol).

**Intracellular concentration–effect studies**

Figure 1 and Table 1 show the intracellular activities of NZ2114, daptomycin and vancomycin against *S. aureus* phagocytosed by THP-1 monocytes when tested over a wide range of concentrations (0.01- to 128-fold the MIC) for 24 h. The maximal relative efficacy (Eₘₐₓ) of all compounds was considerably reduced intracellularly when compared with the extracellular values.

Against *S. aureus* ATCC 25923, NZ2114 retained an Eₘₐₓ of −1.5 log₁₀ cfu, a level significantly better than observed with vancomycin (−0.6 log₁₀ kill) and daptomycin (−1.0 log₁₀ cfu). Notably, the static concentration (Cₜₐₜ) of NZ2114 and of daptomycin for these strains was close to their MIC in broth. This is in contrast to vancomycin, for which the static concentration (Cₜₐₜ) was approximately three times its MIC in broth. Against the MRSA #428 strain, all compounds had an Eₘₐₓ of less than −1 log₁₀ cfu, with a trend towards better activity with NZ2114 and daptomycin. The static concentration (Cₜₐₜ) of vancomycin was considerably higher than its MIC (~7-fold), in contrast to what was observed for NZ2114 or daptomycin.

The activity of NZ2114 and daptomycin against VRSA2 was only bacteriostatic, with the corresponding static concentration (Cₜₐₜ) being close to the MIC in broth.

**Discussion**

Antibacterial peptide antibiotics are a novel class of drugs active against resistant strains, with NZ2114 representing a potential candidate for development based on its pharmacodynamic profile in a murine model.⁶ The present study showed that NZ2114: (i) displayed similar extracellular and intracellular activities as daptomycin, which is long known to be a highly bactericidal anti-MRSA agent⁷ and (ii) was more effective than...
vancomycin against the intracellular forms of susceptible bacteria. The mechanisms causing such a reduction of intracellular activity, in comparison with what is seen in broth, remain largely hypothetical. For NZ2114, however, this could be caused by the acidic environment of the phagolysosomes where intracellular S. aureus multiply in THP-1 cells. We saw, indeed, that the MIC

**Figure 1.** Activity of NZ2114, daptomycin and vancomycin against S. aureus [(a) MSSA, (b) MRSA and (c) VRSA] extracellularly in broth (filled inverted triangles) and intracellularly in THP-1 monocytes (open circles). The ordinate shows the change in cfu/mg of protein (intracellular) or cfu/mL (extracellular) after 24 h of incubation compared with the initial inoculum. The broken line at $y=0$ corresponds to the bacteriostatic activity. The abscissa shows the extracellular concentrations of plectasin applied, with the broken lines corresponding to the MIC values.
of NZ2114 was markedly increased when the pH was lowered from 7.4 to 5.4. Yet, the intracellular activities of NZ2114 and of daptomycin remain weaker than their extracellular activities, which has been observed for most antistaphylococcal drugs so far. However, the level of maximal relative activity of NZ2114 against MSSA and MRSA compares to that of daptomycin, plectasin and antistaphylococcal β-lactams (including ceftobiprole).

The model used has several limitations. First, there was no correlation between pharmacodynamic and pharmacokinetic parameters, since we did not assay for the intracellular drug content. Second, protein binding was not taken into account and correlation between pharmacodynamic and pharmacokinetic parameters, since we did not assay for the intracellular drug content. The model used has several limitations. First, there was no correlation between pharmacodynamic and pharmacokinetic parameters, since we did not assay for the intracellular drug content. Second, protein binding was not taken into account and correlation between pharmacodynamic and pharmacokinetic parameters, since we did not assay for the intracellular drug content.

Statistical analyses: (i) comparison per row, corresponding $E_{\text{max}}$ values of extracellular and intracellular activities (all compounds had a significant difference ($P<0.0001$) between intracellular and extracellular $E_{\text{max}}$ values); (ii) comparison per column, multiple comparisons between intracellular $E_{\text{max}}$ values for all compounds [ATCC, NZ2114 had a significantly lower $E_{\text{max}}$ value than both daptomycin and vancomycin ($P<0.05$ and $P<0.01$, respectively); MRSA, NZ2114 had a significantly lower $E_{\text{max}}$ value than vancomycin ($P<0.01$); and VRSA, no difference in $E_{\text{max}}$ value between NZ2114 and daptomycin ($P>0.05$)].

Table 1. Maximal relative efficacy ($E_{\text{max}}$) and static concentration ($C_s$) of NZ2114, daptomycin and vancomycin, as determined from analysis of the data presented in Figure 1

<table>
<thead>
<tr>
<th>Strain and antibiotic</th>
<th>Extracellular</th>
<th>Intracellular</th>
<th>$E_{\text{max}}$ (95% CI), log cfu</th>
<th>$C_s \times \text{MIC}$</th>
<th>$R^2$</th>
<th>$E_{\text{max}}$ (95% CI), log cfu</th>
<th>$C_s \times \text{MIC}$</th>
<th>$R^2$</th>
<th>$P$ value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 25923</td>
<td></td>
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</tr>
<tr>
<td>NZ2114</td>
<td>$-4.07 (-4.52$ to $-3.62)$</td>
<td>1.3</td>
<td>0.967</td>
<td>$-1.51 (-1.80$ to $-1.22)$</td>
<td>0.8</td>
<td>0.901</td>
<td>$&lt;0.001$</td>
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<tr>
<td>daptomycin</td>
<td>$-4.06 (-4.42$ to $-3.69)$</td>
<td>0.3</td>
<td>0.927</td>
<td>$-1.00 (-1.25$ to $-0.76)$</td>
<td>0.6</td>
<td>0.927</td>
<td>$&lt;0.001$</td>
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<tr>
<td>vancomycin</td>
<td>$-3.46 (-4.67$ to $-4.05)$</td>
<td>0.3</td>
<td>0.968</td>
<td>$-0.64 (-0.99$ to $-0.29)$</td>
<td>2.9</td>
<td>0.823</td>
<td>$&lt;0.001$</td>
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<td>S. aureus MRSA #428 (axil, clinical isolate)</td>
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<tr>
<td>NZ2114</td>
<td>$-4.58 (-4.94$ to $-4.22)$</td>
<td>0.3</td>
<td>0.962</td>
<td>$-0.93 (-1.27$ to $-0.58)$</td>
<td>1.1</td>
<td>0.868</td>
<td>$&lt;0.001$</td>
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<tr>
<td>daptomycin</td>
<td>$-4.61 (-5.01$ to $-4.22)$</td>
<td>0.2</td>
<td>0.946</td>
<td>$-0.85 (-1.13$ to $-0.57)$</td>
<td>1.8</td>
<td>0.888</td>
<td>$&lt;0.001$</td>
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<tr>
<td>vancomycin</td>
<td>$-3.37 (-3.87$ to $-2.80)$</td>
<td>0.7</td>
<td>0.907</td>
<td>$-0.66 (-0.99$ to $-0.32)$</td>
<td>6.2</td>
<td>0.895</td>
<td>$&lt;0.001$</td>
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<td>S. aureus VRSA2 (Pennsylvania HIP11983)</td>
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<tr>
<td>NZ2114</td>
<td>$-3.79 (-4.16$ to $-3.41)$</td>
<td>1.2</td>
<td>0.967</td>
<td>$-0.22 (-0.33$ to $-0.11)$</td>
<td>2.3</td>
<td>0.907</td>
<td>$&lt;0.001$</td>
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<tr>
<td>daptomycin</td>
<td>$-3.69 (-4.22$ to $-3.16)$</td>
<td>0.9</td>
<td>0.937</td>
<td>$-0.29 (-0.44$ to $-0.16)$</td>
<td>1.3</td>
<td>0.851</td>
<td>$&lt;0.001$</td>
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</table>

$E_{\text{max}}$ decrease in log cfu after 24 h compared with original inoculum (t=0 h) and extrapolated for an infinitely large antibiotic concentration; $C_s$ concentration (×MIC) resulting in no apparent growth of bacteria; $R^2$, confidence interval.

$^a$P values determined by analysis of covariance for extracellular versus intracellular concentrations between all compounds.

$^s$, $E$, $R$, $C$ values determined by analysis of covariance for extracellular versus intracellular concentrations between all compounds.

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We thank A. Sandberg (Statens Serum Institut), for fruitful discussions, and M.-C. Cambier and M. Vergauwen (Unité de Pharmacologie Cellulaire et Moléculaire, Brussels), for dedicated technical assistance.

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Transparency declarations
K. S. B. and H.-H. K. are employees of Novozymes A/S, the company responsible for the discovery and development of plectasin and NZ2114. N. F.-M. and N. H. are members of the Novozymes advisory board associated with the plectasin project. K. S. B. and H.-H. K. are owners of stock options in Novozymes. The other authors have no known conflicts of interest.
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