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

Karoline Sidelmann Brinch[1]*, Paul M. Tulkens[2], Francoise Van Bambeke[2], Niels Frimodt-Møller[3], Niels Høiby[4] and Hans-Henrik Kristensen[1]

[1]Novozymes A/S, Pharma Discovery, Krogshøjvej 36, DK-2880 Bagsværd, Denmark; [2]Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, UCL 7370 avenue E. Mounier 73, B-1200 Brussels, Belgium; [3]National Center for Antimicrobials & Infection Control, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark; [4]Department 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 $\log_{10}$ 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 $\log_{10}$ cfu reduction against MSSA (ATCC 25923), while the activity was a 0.9 $\log_{10}$ cfu reduction against MRSA and a 0.2 $\log_{10}$ cfu reduction against VRSA. For comparison, the intracellular activity of daptomycin was a 1.0 $\log_{10}$ cfu reduction against MSSA, a 0.8 $\log_{10}$ cfu reduction against MRSA and a 0.3 $\log_{10}$ cfu reduction against VRSA. Vancomycin showed activity against both MSSA and MRSA (0.6 $\log_{10}$ 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 pezizalean 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 HIP11983 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 #HHSN272200700055C)] were used in the studies. MICs were determined in Mueller–Hinton broth (with 50 μg/mL $CaCl_2$ 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 10^6 cfu/mL and the number of viable bacteria was determined after 24 h of incubation with antibiotics. For intracellular activity, opsonized bacteria (5×10^5 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 (37°C in a 5% CO_2 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 (E_{max}), the static concentration (C_s) and the goodness of fit (R^2), as determined by non-linear regression using GraphPad Prism® 5.0 (GraphPad Prism Software, San Diego, CA, USA). Multiple comparisons between E_{max} 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 E_{max} 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 ($E_{max}>3$ $\log_{10}$ 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 (E_{max}) of all compounds was considerably reduced intracellularly when compared with the extracellular values.

Against *S. aureus* ATCC 25923, NZ2114 retained an E_{max} of -1.5 $\log_{10}$ cfu, a level significantly better than observed with vancomycin (-0.6 $\log_{10}$ kill) and daptomycin (-1.0 $\log_{10}$ cfu). Notably, the static concentration (C_s) 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 (C_s) was approximately three times its MIC in broth. Against the MRSA #428 strain, all compounds had an E_{max} of less than -1 $\log_{10}$ cfu, with a trend towards better activity with NZ2114 and daptomycin. The static concentration (C_s) 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 (C_s) 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 agent;[7] and (ii) was more effective than

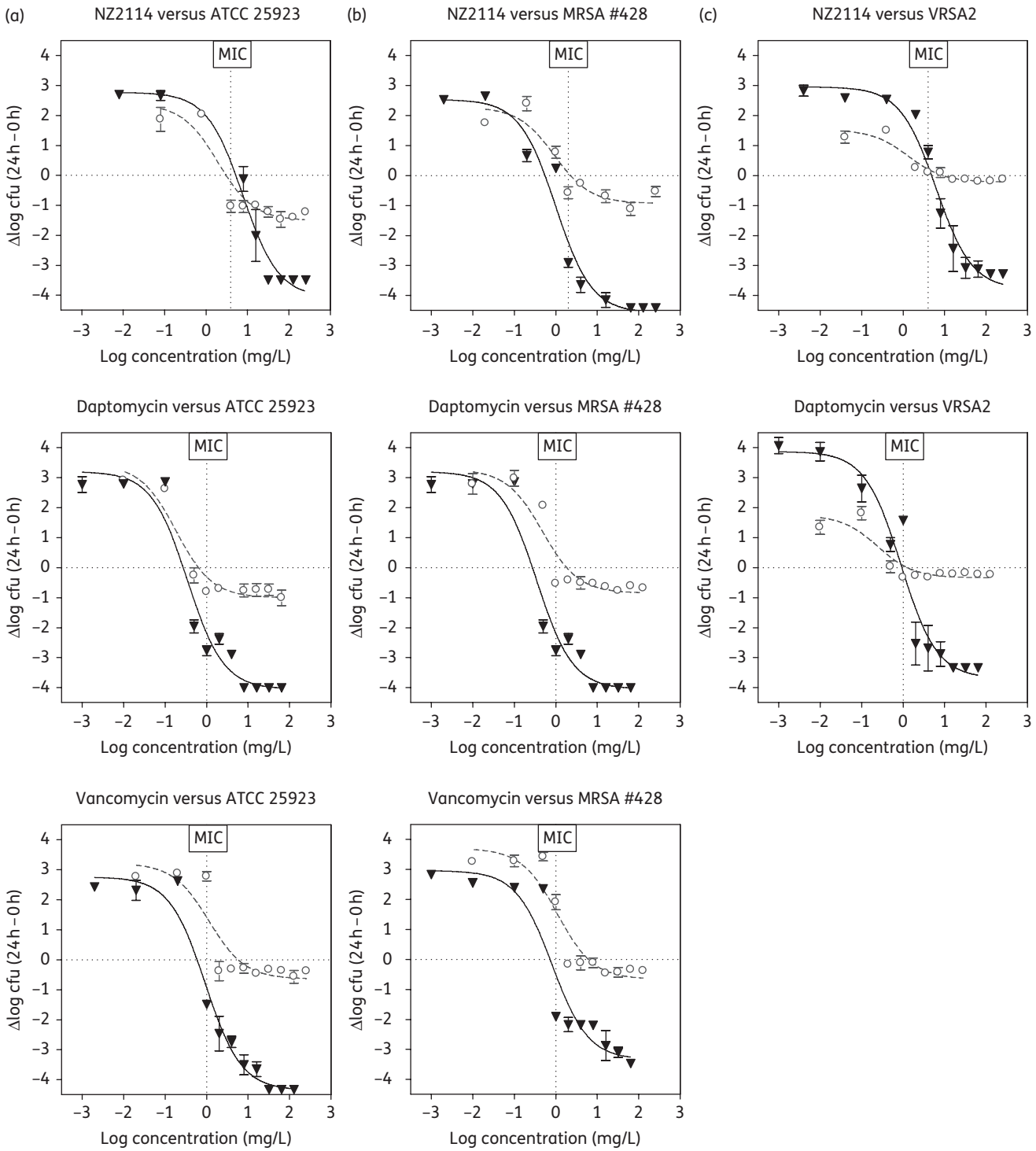

Figure 1. Activity of NZ2114, daptomycin and vancomycin against *S. aureus* [(a) MSSA, (b) MRSA and (c) VRSA] extracellularly in broth (filled inverted triangles) and intracellularly in THP-1 monocytes (open circles). The ordinate shows the change in cfu/mg of protein (intracellular) or cfu/mL (extracellular) 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.

vancomycin against the intracellular forms of susceptible bacteria.[8] 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

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

	Extracellular			Intracellular			
Strain and antibiotic	E_{max} (95% CI), log cfu	C_s, ×MIC	R^2	E_{max} (95% CI), log cfu	C_s, ×MIC	R^2	*P* value[a]
S. aureus ATCC 25923							
NZ2114	−4.07 (−4.52 to −3.62)	1.3	0.967	−1.51 (−1.80 to −1.22)	0.8	0.901	<0.001
daptomycin	−4.06 (−4.42 to −3.69)	0.3	0.927	−1.00 (−1.25 to −0.76)	0.6	0.927	<0.001
vancomycin	−4.36 (−4.67 to −4.05)	0.3	0.968	−0.64 (−0.99 to −0.29)	2.9	0.823	<0.001
S. aureus MRSA #428 (axil, clinical isolate)							
NZ2114	−4.58 (−4.94 to −4.22)	0.3	0.962	−0.93 (−1.27 to −0.58)	1.1	0.868	<0.001
daptomycin	−4.61 (−5.01 to −4.22)	0.2	0.946	−0.85 (−1.13 to −0.57)	1.8	0.888	<0.001
vancomycin	−3.37 (−3.87 to −2.80)	0.7	0.907	−0.66 (−0.99 to −0.32)	6.2	0.895	<0.001
S. aureus VRSA2 (Pennsylvania HIP11983)							
NZ2114	−3.79 (−4.16 to −3.41)	1.2	0.967	−0.22 (−0.33 to −0.11)	2.3	0.907	<0.001
daptomycin	−3.69 (−4.22 to −3.16)	0.9	0.937	−0.29 (−0.44 to −0.16)	1.3	0.851	<0.001

E_{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 (in ×MIC) resulting in no apparent growth of bacteria; CI, confidence interval.
[a]*P* values determined by analysis of covariance for extracellular versus intracellular concentrations between all compounds.
Statistical analyses: (i) comparison per row, corresponding E_{max} values of extracellular and intracellular activities [all compounds had a significant difference ($P<0.0001$) between intracellular and extracellular E_{max} values]; (ii) comparison per column, multiple comparisons between intracellular E_{max} values for all compounds [ATCC, NZ2114 had a significantly lower E_{max} value than both daptomycin and vancomycin ($P<0.05$ and $P<0.01$, respectively); MRSA, NZ2114 had a significantly lower E_{max} value than vancomycin ($P<0.01$); and VRSA, no difference in E_{max} value between NZ2114 and daptomycin ($P>0.05$)].

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[9] and antistaphylococcal β-lactams (including ceftobiprole).[10]

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 because the cellular model does not allow the serum content of the culture medium to significantly vary. Third, cells were exposed to constant concentrations of antibiotics, which is at variance with what will most likely take place *in vivo* if using discontinuous drug administration schedules. Yet, the neutropenic murine thigh infection model applied to NZ2114 recently[6] taught us that a static effect and a 1 $\log_{10}$ cfu decrease from an initial inoculum can be obtained with drug exposure levels corresponding to free AUC_{24}/MIC ratios of 28.5 and 45, respectively. Although this model is very remote from ours and deals primarily with extracellular bacteria, it is interesting to note that we observe: (i) a static effect for intracellular MSSA and MRSA with both NZ2114 and daptomycin when exposing cells for 24 h at extracellular concentrations corresponding roughly to their MIC (generating the equivalent of an AUC_{24}/MIC ratio of 24); and (ii) a 1 $\log_{10}$ cfu decrease for extracellular concentrations ~10-fold higher. Thus, the two models eventually provide reasonably convergent results. Moreover, we also know from our studies with plectasin, in which the cell model used here could be compared with an *in vivo* peritonitis model, that there is a fair degree of similarity between the conclusions that can be drawn from the two sets of results.[9]

The data presented here allow for a direct comparison of drugs with regard to activity. In this context, NZ2114 clearly appears superior to vancomycin and similar to daptomycin, against both vancomycin-susceptible and vancomycin-resistant staphylococci. Together with the results of animal studies,[6] this may help in its rational development to fight against a bacterium that is now a major scourge in hospital and community set-ups.

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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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