Efficacy of NZ2114, a Novel Plectasin-Derived Cationic Antimicrobial Peptide Antibiotic, in Experimental Endocarditis due to Methicillin-Resistant Staphylococcus aureus

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Running title: MRSA endocarditis model and antimicrobial peptides

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ABSTRACT

Cationic antimicrobial peptides (CAPs) play important roles in host immune defenses. Plectasin is a defensin-like CAP isolated from the saprophytic fungus, *Pseudoplectania nigrella*. NZ2114 is a novel variant of plectasin with potent activity against Gram-positive bacteria. In this study, we investigated: \textbf{i)} the \textit{in vivo} pharmacokinetic and pharmacodynamic (PK/PD) characteristics of NZ2114; and \textbf{ii)} the \textit{in vivo} efficacy of NZ2114 in comparison with two conventional antibiotics, vancomycin or daptomycin, in a \textbf{rabbit experimental endocarditis model} (IE) due to a methicillin-resistant *Staphylococcus aureus* (MRSA) strain (ATCC33591). All NZ2114 regimens (5, 10 and 20 mg/kg, iv, \textit{twice daily for three days}), significantly decreased MRSA densities in cardiac vegetations, kidneys and spleen vs. untreated controls, except in one scenario (5 mg/kg vs. splenic MRSA counts). The efficacy of NZ2114 was clearly dose-dependent in all target tissues. At 20 mg/kg, NZ2114 showed a significantly greater efficacy vs. vancomycin (*P* < 0.001), and similar efficacy to daptomycin. Of importance, only NZ2114 (at 10 and 20 mg/kg regimen) prevented post-therapy relapse in \textbf{cardiac vegetations, kidneys and spleen}, while bacterial counts in \textbf{these target tissues} continued to increase in vancomycin- and daptomycin-treated animals. These \textit{in vivo} efficacies were equivalent and significantly correlated with three PK indices investigated: $fC_{\text{max}}$/MIC, $fAUC$/MIC and $f\%T>MIC$, as analyzed by a sigmoid $E_{\text{max}}$ model ($R^2 > 0.69$). The superior efficacy of NZ2114 in this MRSA IE model suggest the potential for further development of this compound for treating serious MRSA infections.
INTRODUCTION

Endovascular infections are very prevalent, life-threatening infectious syndromes (13). *Staphylococcus aureus* is the most common cause of such infections worldwide, with an unacceptably high morbidity and mortality, especially when caused by antimicrobial-resistant strains (e.g., methicillin-resistant [MRSA] and vancomycin-intermediate resistant *S. aureus* [VISA]) (3, 8, 15, 17, 18, 26). Therefore, there is an urgent need to develop new antimicrobial agents for the prevention and treatment of these syndromes.

Plectasin is a defensin-like, cationic antimicrobial peptide (CAP) isolated from the saprophytic ascomycete, *Pseudoplectania nigrella*. This CAP contains 40 amino acids and has potent activity against Gram-positive bacteria *in vitro* and *in vivo* (19, 21). In addition, plectasin shares primary structure features with defensin-like peptides from spiders, scorpions, dragonflies and mussels, and folds into a cystine-stabilized alpha-beta conformation (CS-αβ). Most CAPs have been thought to target the bacterial cell membrane. However, plectasin has a novel mode of antimicrobial action, specifically binding to the key bacterial cell wall precursor, lipid II (21), and thereby interfering with bacterial cell wall biosynthesis. NZ2114 is a new variant of plectasin with improved *in vitro* activity against staphylococci and streptococci, including those resistant to clinically available antibiotics (1, 4, 5, 19-21). Recent studies suggested that NZ2114 has potent activities both *in vitro* and in a number of animal models, including rabbit meningitis, murine peritonitis and thigh infections, and against various strains of *Staphylococcus aureus* and of *Streptococcus pneumoniae* (1, 5, 20). The purpose of the current study was to: i) compare the *in vivo* antimicrobial efficacy of NZ2114 with two standard anti-MRSA antibiotics, vancomycin...
and daptomycin, in a rabbit experimental MRSA endocarditis (IE) model; and ii) characterize the 
*in vivo* pharmacokinetic and pharmacodynamic (PK/PD) profiles of NZ2114 in this model.

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MATERIALS AND METHODS

Bacteria, media, and antibiotic. A vancomycin-susceptible, non-heteroresistant, hospital-associated MRSA strain (ATCC33591) was employed in this study (16, 22). Mueller-Hinton broth (MHB, Difco Laboratories, Detroit, MI) and Mueller-Hinton agar (MHA, Difco Laboratories, Detroit, MI) were used to culture the bacterial strain. NZ2114 was supplied by Sanofi-Aventis (Toulouse, France). Vancomycin and daptomycin were purchased from APP Pharmaceuticals (Schaumburg, IL 60173), and Cubist Pharmaceuticals (Lexington, MA 02421), respectively.

In vitro susceptibility studies. The minimal inhibitory concentrations (MICs) of NZ2114, vancomycin and daptomycin against the MRSA ATCC33591 (5x10^5 CFU/ml) were determined by the standard Clinical and Laboratory Standards Institute microdilution methods (11). All MIC assays were performed in duplicate on three occasions.

In vitro time-kill curves of NZ2114. Time-kill curves of NZ2114 (range 0.5 - 5x MIC) were performed in glass flasks containing a final inoculum of 5 x 10^5 CFU/ml of the study MRSA strain at 37°C with shaking for 24 hr. At 0, 2, 4, 6 and 24 hr of incubation, 0.1 ml aliquots were taken from each group, serially diluted in sterile phosphate-buffered saline, plated onto MHA plates and incubated at 37°C for 24 h for viable count enumeration. Each time-kill experiment was carried out in at least duplicate on separated days.
Experimental IE model. A well-characterized rabbit model of catheter-induced infective endocarditis (IE) was used to study both the pharmacokinetic and pharmacodynamic (PK/PD) profiles of NZ2114, and to compare its anti-MRSA efficacy with vancomycin and daptomycin. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care criteria. All animal studies were approved by the Animal Research Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Briefly, anesthetized rabbits (New Zealand white; Harland Laboratory Inc, California) underwent transcarotid-transaortic valve catheterization. IE was produced by the iv injection of MRSA ATCC33591 (~1 x 10^6 CFU; ID₉₅ inoculum for this strain in the IE model as determined from pilot studies) at 24 h after catheterization.

Antibiotic treatments. At 24 hrs after infection, animals were randomized to receive: i) no therapy (controls); ii) NZ2114 (5, 10 or 20 mg/kg, iv, bid; dose-range established based on pilot PK/PD studies and MRSA MICs); iii) vancomycin (15 mg/kg, iv, bid); dose-regimen based on prior efficacy studies in experimental MRSA IE (12)); or iv) daptomycin (12 mg/kg, iv, once daily; dose-regimen mimics human-like PK at 6 mg/kg/d iv, once daily (7)). Each treatment regimen was administered for 3 days. Untreated controls were sacrificed at 24 hr post-infection to establish the target tissue MRSA densities at the outset of antibiotic therapies. One-half of the antibiotic-treated animals were sacrificed at 24 hr after the last antibiotic dose (to minimize tissue antibiotic carryover effects) for evaluating the treatment efficacies. The remaining antibiotic-treated animals were maintained drug-free for three additional days for analysis of microbiologic relapse as previously described (25). At sacrifices, target tissues were aseptically removed and quantitatively cultured as previously described (24). Culture results were expressed
as mean $\log_{10}$ CFU per gram of tissue ($\pm$ SD). The lower limit of microbiologic detection of the organism density in the above target tissues is $\leq 1 \log_{10}$ CFU per gram of tissue. This value was assigned to all culture-negative ('sterile') target tissues for the purposes of calculating the mean $\log_{10}$ CFU per gram of tissue ($\pm$ SD) for eventual statistical comparisons.

**PK/PD studies.** The NZ2114 pharmacokinetics were determined after single iv bolus injections of 5, 10 or 20 mg/kg at 24 hr post-infection in animals with established MRSA IE (three rabbits per group). Blood samples were collected at 5 minutes, as well as 0.5, 1, 2, 4, 6, 8, 12 and 24 h post- NZ2114 administration. After centrifugation of the heparinized blood samples, the plasma was immediately separated and frozen at -20°C until analysis. NZ2114 plasma concentrations were determined by using a qualified liquid chromatography-mass spectrometry (LC/MS-MS) assay (Applied Biosystems API4000) with a lower limit of detection of 50 ng/ml (10). The PK parameters were calculated from the individual free plasma concentrations using the WinNonlin Professional 5.2 software package, based on the non-compartmental model #201.

Because the PK of vancomycin and daptomycin at the dosages used in this study have been previously determined in the IE model (2, 7, 12, 24), we did not repeat them in this study. Briefly, using vancomycin at 15 mg/kg, iv., mean serum levels are routinely greater than 50 µg/ml at 1-2 h post-dose (> 100 times of the MIC of MRSA ATCC33591), and 2-4 µg/ml at 6 h post-dose, with no detectable trough levels at 12 h post-dose (12). Daptomycin at a dose of 12 mg/kg in rabbits mimics the PK-PD profile observed in humans administered a dose of 6 mg/kg, the currently recommended dose for *S. aureus* bacteremia and right-sided IE (7).
PK/PD index data analysis. The relationship between each PK/PD index, $f\%T>MIC$, free $C_{\text{max}}$/MIC ($fC_{\text{max}}$/MIC), free $\text{AUC}$/MIC ($f\text{AUC}$/MIC), and the efficacy were best fitted using a sigmoid $E_{\text{max}}$ model. The PK/PD correlations were calculated from the individual efficacy data using the WinNonlin Professional 5.2 software, with pharmacodynamic model #107.

Statistical analysis. The in vivo efficacy data in terms of reductions in target organ bacterial counts were analyzed by a one-way ANOVA followed by Dunnett’s adjustment for multiplicity, whereas non-inferiority analyses were based on the comparison of upper 90% confidence limit to a predefined limit data. For the analysis of the proportion of sterile tissue cultures, the Fisher’s exact test was used. $P$ values of $<0.05$ were considered statistically significant. All statistical analyses were conducted using Software SAS® v9.2.
RESULTS

In vitro susceptibility studies. The MICs of NZ2114, vancomycin and daptomycin versus the MRSA ATCC33591 study strain were 0.5, 2.0 and 0.125 µg/ml, respectively.

In vitro time-kill curves of NZ2114. In the absence of NZ2114, the bacterial counts reached their maximal at 24 hr after inoculation (Figure 1). In the presence of 0.5x MIC of NZ2114, the bacterial counts grew more slowly than with the controls. NZ2114 at 1x and 5x MIC reduced bacterial densities by up to 4 log_{10} CFU/ml at 6 hr incubation. However, NZ2114 at 1x MIC failed to inhibit bacterial re-growth at the 24 hr time point (Figure 1).

The efficacy of NZ2114, vancomycin and daptomycin in the IE model. MRSA densities in the different therapy groups are shown in Figure 2. A dose-dependent efficacy of NZ2114 was clearly observed in all target tissues at end-of-treatment as compared with untreated controls. NZ2114, at 5, 10 and 20 mg/kg dosing regimens, significantly reduced mean MRSA vegetation counts by ~2, 3 and 6 log_{10} CFU/g, respectively, as compared to untreated controls. At 10 and 20 mg/kg, the effect was also significant for kidneys and spleen MRSA counts. In addition, NZ2114 at 20 mg/kg, had a significantly greater efficacy in all target tissues as compared to vancomycin (P < 0.05). NZ2114 at 20mg/kg demonstrated an equivalent efficacy to daptomycin at 12mg/kg (Figure 2).

Relapse analysis indicated that for vancomycin and daptomycin, the bacterial load in all three target tissues was higher than the end-of-treatment counts. In contrast, only NZ2114 regimens...
(10 and 20 mg/kg) prevented microbiologic relapse in this experimental IE model (Figure 3). Moreover, NZ2114 at 20 mg/kg yielded the highest percent of sterile target tissue cultures in both treatment and relapse groups (Table 1).

**PK.** The free plasma time-concentration profiles and PK parameters of NZ2114 administered iv at 5, 10 and 20 mg/kg in experimental MRSA IE are shown in Figure 4. Peak NZ2114 plasma levels were observed by 5 min post iv administration (first sampling time). The free NZ2114 half-life ($T_{1/2}$) following single doses of 5, 10 or 20 mg/kg was approximately 2.3 h in the IE model. The free $AUC_{0-12h}$ and peak values for the escalating single doses ranged from 3.09 to 10.3 mg.h/l and 1.78 to 8.44 mg/l, respectively. Over the range of the doses tested, plasma exposures increased with dose, with a mean $fAUC_{0-12h}$ for 10/5 mg/kg and 20/5 mg/kg ratios of 1.4 and 3.3, respectively. The protein binding of NZ2114 in rabbit serum was 91.6-94.5%, which is consistent with the parent plectasin compound in human and murine serum (5).

**NZ2114 PK/PD index determination.** The relationships between each free PK/PD index, $f\%T>MIC$, $fC_{max}/MIC$ and $fAUC_{0-12h}/MIC$ vs. the efficacy of NZ2114 against MRSA are shown in Figure 5. Therapeutic outcomes correlated significantly with the $f\%T>MIC$, $fC_{max}/MIC$ and $fAUC_{0-12h}/MIC$ indices ($R^2=0.69$).
DISCUSSION

The growing problem of resistance to conventional antibiotics, and the need for new antibiotics has stimulated great interest in development of antimicrobial peptides, especially congeners of native host defense peptides, as novel human therapeutics. This impetus has been driven in-part by the demonstration that the emergence of resistance in vitro to antimicrobial peptides is less common and rapid as compared to conventional antibiotics (14, 27). However, significant issues in development of antimicrobial peptides, including potential host toxicities and durability in vivo, and formulation strategies, have hampered their development as therapeutics to date.

Previous studies have shown that plectasin, a defensin-like CAP, and its derivative NZ2114, have potent in vivo activity and low systemic toxicities in several animal models, including meningitis, murine peritonitis-sepsis, pneumonia, and thigh infections (1, 19). However, such studies have not evaluated NZ2114 in a multi-organ model of endovascular infection due to a drug-resistant Gram-positive pathogen. Thus, our present study evaluated the efficacy of NZ2114 vs. conventional therapy in experimental MRSA IE.

Several interesting findings emerged from the present study. Firstly, in general, all regimens, especially NZ2114 and daptomycin were significantly effective in reducing MRSA densities in all three target tissues as compared to untreated controls during therapy. Secondly, the efficacy of NZ2114 against MRSA IE was clearly dose-dependent in terms of reducing target tissue MRSA burdens and in organ sterilizations. Thirdly, NZ2114 (20 mg/kg) was significantly more effective than vancomycin in reducing MRSA counts in target tissues after three days of
treatment, and comparable to daptomycin. Finally, NZ2114 and daptomycin (but not vancomycin) had equivalent efficacy in preventing post-therapy target tissue microbiologic relapses. Notably, after discontinuation of therapy, only NZ2114 at 10 and 20 mg/kg regimens completely prevented post-therapy bacteriologic relapse in target tissues during a three days drug-free period. Of interest, following NZ2114 therapy, approximately 2 logs$_{10}$ CFU/g reductions in vegetation MRSA counts were observed, despite NZ2114 serum concentrations falling below the MIC of the infecting MRSA strain. The mechanism(s) of NZ2114-mediated relapse prevention may be multifactorial. For example, Andes et al have documented a prolonged post-antibiotic effect (3-15 hr) for NZ2114 against \textit{S. aureus} (1). Such prolonged post-antibiotic effects may well provide an advantage for infrequent dosing strategies for this compound, despite its relatively short $T_{1/2}$. In addition, Brinch et al recently showed that NZ2114 and daptomycin (but not vancomycin) exhibited similar extracellular and intracellular anti-MRSA activities against vancomycin-susceptible strains (6). Given the capacity of staphylococci to penetrate and persist within endovascular cells as a means of establishing a reservoir for persistent infection (9, 23), the excellent intracellular bactericidal activities of NZ2114 and daptomycin might yield both favorable therapeutic and relapse-prevention outcomes in IE.

In conclusion, the present findings demonstrate that NZ2114 has dose-dependent efficacy \textit{in vivo} against MRSA in this experimental IE model. In addition, the treatment efficacy of NZ2114 was significantly greater than vancomycin, and equivalent to daptomycin, in this IE model. Together, these results suggest that NZ2114 could be a promising template for further development as a novel anti-MRSA therapeutics.
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ACKNOWLEDGMENTS
REFERENCES


Table 1. Percent of sterile tissue cultures after three days of treatment, and at post-therapy relapse in experimental MRSA IE.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>% sterile cultures (treatment/relapse)</th>
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<tbody>
<tr>
<td></td>
<td>(# animals; treatment/relapse)</td>
</tr>
<tr>
<td></td>
<td>Vegetations</td>
</tr>
<tr>
<td>NZ2114 (5 mg/kg, n=8/7)</td>
<td>0/0</td>
</tr>
<tr>
<td>NZ2114 (10 mg/kg, n=8/8)</td>
<td>0/0</td>
</tr>
<tr>
<td>NZ2114 (20 mg/kg, n=8/8)</td>
<td>50*/50*</td>
</tr>
<tr>
<td>Vancomycin (15 mg/kg, n=6/6)</td>
<td>0/0</td>
</tr>
<tr>
<td>Daptomycin (12 mg/kg, n=6/6)</td>
<td>33/33</td>
</tr>
</tbody>
</table>

* *P < 0.05 vs. NZ2114 5 mg/kg and 10 mg/kg, and vancomycin treatment and relapse groups.

** *P < 0.05 vs. NZ2114 5 mg/kg and vancomycin relapse groups.
Figure 1. *In vitro* time-kill curve of NZ2114. Concentrations = 0, 0.5x, 1x, and 5x MIC against MRSA ATCC33591.

Control ◆; NZ2114 0.5xMIC ■; NZ2114 1xMIC ▲; NZ2114 5xMIC ◊.
Figure 2. Efficacy of NZ2114, vancomycin (VAN) and daptomycin (DAP) in an experimental IE model. MRSA densities in target tissues after three-day NZ2114, VAN or DAP treatment in the IE model. The values are shown as mean $\log_{10}$ CFU/g target tissue MRSA $\pm$ SD. Asterisks indicate statistical significance ($^* P < 0.05$; $^{**} P < 0.001$; and $^{***} P < 0.00001$ vs. untreated control groups). $P < 0.05$ = NZ2114 at 20 mg/kg as compared to VAN.
Figure 3. Treatment and relapse comparison of NZ2114, vancomycin and daptomycin in the IE model. The values shown are log_{10} CFU/g target tissue MRSA density ± SD. *P* < 0.05 for NZ2114 (at 10 and 20 mg/kg) and daptomycin vs. vancomycin relapse groups.
Figure 4. Free plasma pharmacokinetics of NZ2114 after a single iv administration.

Regimens: 5, 10 or 20 mg/kg bolus in animals with 24h-established experimental MRSA IE.

Each symbol represents the mean concentration from three animals. Error bars represent SDs.

<table>
<thead>
<tr>
<th>NZ2114 (mg/kg)</th>
<th>T_{1/2} (h)</th>
<th>Free C_{max} (µg/ml)</th>
<th>Free AUC_{0-12h} (µg.h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.13</td>
<td>1.78</td>
<td>3.09</td>
</tr>
<tr>
<td>10</td>
<td>2.67</td>
<td>3.30</td>
<td>4.26</td>
</tr>
<tr>
<td>20</td>
<td>2.00</td>
<td>8.44</td>
<td>10.30</td>
</tr>
</tbody>
</table>

T_{1/2}: apparent half-life of free plasma concentration; C_{max}: calculated as C_0, initial concentration, estimated by back extrapolation for bolus iv models; AUC_{0-12h} area under the free concentration-time curve from 0 to 12h.
Figure 5. Relationship between the NZ2114 PK/PD indices (fAUC/MIC, fC_{max}/MIC, and f%MIC, percent time above MIC) and efficacy against MRSA in an experimental IE model. Unbound (free drug) concentrations were used for index calculations. Efficacy is expressed as change in CFU/g. vegetation compared to organism burden at initiation of therapy. Each symbol represents an individual animal. The sigmoid line represents the best fit using the sigmoid E_{max} model. R^2 is the coefficient of determination, with values > 0.5 typically considered significant correlations.