Antimicrobial activity of daptomycin, vancomycin, and oxacillin in human monocytes, and daptomycin in combination with gentamicin and/or rifampin in human monocytes and in broth against *Staphylococcus aureus*

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Running Title: Daptomycin, *S. aureus* and human monocytes

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Abstract

We investigated the antistaphylococcal activity of daptomycin, vancomycin, oxacillin, gentamicin, and rifampin in human monocyte-derived macrophages. When compared with vancomycin and oxacillin, daptomycin had the most rapid and greatest antibacterial activity, but that of oxacillin was most sustained. The combination of daptomycin, gentamicin, and rifampin was most effective intracellularly, while daptomycin plus gentamicin and the three-drug combination were most effective extracellularly, completely eliminating viable Staphylococcus aureus.
*Staphylococcus aureus* is an important pathogen in hospital- and community-acquired infections (17). Because *S. aureus* often is an intracellular pathogen, infections caused by this microbe can be difficult to treat and can persist and recur (2,3,6,9,13). In order to be clinically effective, antibiotics must reach the target pathogen extracellularly in blood and tissues, as well as intracellularly in phagocytic cells, including their phagolysosomes (1,5,8,12). Because of increasing resistance to currently available antimicrobials such as β-lactams and vancomycin, studies with daptomycin are of interest (4,14). Daptomycin is a cyclic lipopeptide, known to be rapidly bactericidal against most Gram-positive pathogens, including *S. aureus* (10,15). Its mode of action is associated with rapid depolarization of the bacterial membrane, leading to inhibition of protein, DNA, and RNA synthesis and resulting in bacterial death (10,15). In addition, there is evidence indicating that daptomycin penetrates into human neutrophils, suggesting that it may be effective in killing intracellular bacteria (16).

The purpose of this study was to determine and compare the intracellular antibacterial effects of daptomycin, vancomycin, and oxacillin against intracellular methicillin-resistant and methicillin–susceptible *S. aureus* (MRSA and MSSA) in human monocyte-derived macrophages (MDM). In addition, activities of two- and three-drug combinations of daptomycin, gentamicin, and/or rifampin were evaluated against MRSA in human MDM and in broth.

*S. aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 43300 (MRSA), and *S. aureus* 8111 (MRSA) were used. MICs (Table 1) were determined using standard CLSI
methodology (11). Daptomycin (50 mg/L Ca\(^{2+}\) added for daptomycin), vancomycin, and oxacillin were used at 0.5, 1, 5 and 10xMIC. A clinical MRSA isolate obtained from a bacteremic patient, strain 8111, was used for drug-combination studies with daptomycin (1xMIC), rifampin (1xMIC), and gentamicin (0.5xMIC). For combination experiments, drug concentrations were chosen so that effects of combinations would not be obscured by the activities of single drugs. By using increasing drug concentrations in infected MDM monolayers we first determined the concentration which demonstrated a definite antimicrobial effect, but did not eliminate viable intracellular organisms over 24 h. For daptomycin and rifampin this concentration was 1xMIC, but for gentamicin it was 0.5xMIC. Monocytes were prepared from heparinized blood of healthy volunteers (consent form approved by the IRB of the Stratton VAMC). Monocytes were isolated by using Histopaque 1077 (Sigma Chemical Co, St. Louis, MO), suspended in RPMI 1640 containing 10% FBS (2x10^6 cells/ml; RPMI+), delivered (500 µl) to wells of 48-well tissue culture plates (Corning, Cambridge, MA), and incubated for 24 h at 35°C in 5% CO\(_2\). Media and non-adhered cells were removed and the monolayer washed with RPMI+. For phagocytosis (1 h), opsonized bacteria (500 µl; 2x10^7 CFU/ml) were added to wells containing mainly adherent monocyte-derived macrophages (MDM). Medium with remaining bacteria was removed and MDM were washed with RPMI+. Repeated washing of MDM monolayers did not change the number of CFU/ml in the lysate, and staining with fluorescent monoclonal antibody indicated that >85% of the bacteria were intracellular. Antibiotics were added. At 0, 2, 4, and 24 h supernatants were removed, MDM were lysed with distilled H\(_2\)O, and
numbers of viable bacteria were determined using MH-II agar plates (lower limit of
detection was 20 CFU/ml). Extracellular time-kill assays were performed in MH-II broth
(2x10^6 CFU/ml at 0 h). Sampling times after antibiotic addition were as noted above.
The limit of detection was 10 CFU/ml. Each experiment was performed three times in
duplicate. Viable counts are expressed as percentages of counts at 0 h. There was no
evidence of antibiotic carryover when 100 µl of MDM lysate or bacterial suspension in
broth was centrifuged for 5 min at 13,000 x g, resuspended in 100 µl PBS, and the
viable counts were compared with those of unwashed samples. For data analysis, the
analysis of variance methodology was used. The level of significance was 0.01.

The intracellular activities of daptomycin, vancomycin, and oxacillin against MRSA
and MSSA strains in human MDM are shown in Table 2. At the highest drug
concentrations (5 and 10xMIC) for all three drugs and both strains, the antimicrobial
activity, compared to the controls, was early, rapid, and evident for 24 h (P <0.01).
The degree of the antimicrobial activity was concentration, time, and strain dependent.
Daptomycin caused the greatest and most rapid decrease in bacterial viability at 2 and
4 h, followed by oxacillin (P <0.01). At lower concentrations (0.5 and 1xMIC), there
was regrowth to the same cell density as the control for daptomycin and vancomycin,
while oxacillin demonstrated continued antibacterial activity for 24 h with either stable
suppression or continued net decline in the number of viable bacteria (P <0.01). It is
noted, that the levels of oxacillin effective against MRSA strains in these experiments
cannot be safely maintained in humans.
Results of intracellular experiments in MDM indicated that all two-drug combinations reduced the number of viable bacteria from 0 to 4 h (Figs. 1A & B; \(P < 0.01\)), but only the combinations containing gentamicin (0.5xMIC) suppressed growth for 24 h (\(P < 0.01\); Fig. 1A). The most effective antimicrobial activity was seen with the three-drug combination (Fig. 1B). At lower concentrations of all three antibiotics (0.5xMIC) regrowth occurred at 24 h (data not shown). Small colony variants (SCV) were observed in experiments with gentamicin (viable counts in the reported results include all colonies).

Results of the extracellular experiments with two-drug combinations similar to those used in MDM are shown in Figs. 2 A & B. Daptomycin plus gentamicin and gentamicin plus rifampin killed rapidly such that no viable bacteria were detected at 4 h (Fig. 2A). While killing was slower with daptomycin plus rifampin, it continued for 24 h. Between 4 and 24 h there was regrowth with gentamicin plus rifampin, but no regrowth occurred with daptomycin plus gentamicin or daptomycin plus rifampin. With the three-drug combination (Fig. 2B), rapid and complete killing of MRSA occurred. With the three-drug combination (0.5xMIC of each drug) 99.99% killing was also observed (data not shown). For single drugs, only daptomycin at 1xMIC suppressed the number of viable bacteria below that of the starting inoculum for 24 h (Figs. 2A &B).

It is known that intracellular conditions may influence the activity of the drugs (e.g. gentamicin has poor activity at the low pH usually present in phagolysosomes), and may change the metabolism of the pathogen by making it less susceptible to the antibiotic (5, 8), or even select for the emergence of SCV, which can be induced by
gentamicin and was observed in this study (13). Antibiotics are known to bind to proteins (7). However, a recent study indicated that telavancin and daptomycin (>90% protein-bound) maintain their cidal activity even though their susceptibilities decrease when tested in serum (7). As described by Barcia-Macay et al. for THP-1 macrophages, our study also demonstrates that intracellular antibiotic activity in human MDM depends on the concentration of the extracellular antibiotics and the duration of the MDM exposure to that concentration; furthermore, intracellular activities of antibiotics are lower than extracellular activities (1).

In conclusion, we have demonstrated that the five anti-staphylococcal drugs studied have intracellular antimicrobial activity, although the degree and duration of their activities varies. Although daptomycin demonstrated the most rapid and greatest activity, oxacillin activity was most sustained. The combination of daptomycin, gentamicin, and rifampin was most effective intracellularly in human MDM, while daptomycin plus gentamicin and the three-drug combination were most effective extracellularly in eliminating viable *S. aureus.*
This work was supported by Cubist Pharmaceuticals and in part by resources and facilities of the Samuel S. Stratton Department of Veterans Affairs Medical Center, Albany, New York.
TABLE 1. Antimicrobial susceptibilities of *S. aureus* ATCC strains 29213 and 43300 and clinical strain 8111 to daptomycin, vancomycin, oxacillin, gentamicin, and rifampin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Daptomycin</th>
<th>Vancomycin</th>
<th>Oxacillin</th>
<th>Gentamicin</th>
<th>Rifampin</th>
</tr>
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<tbody>
<tr>
<td>MRSA ATCC 43300</td>
<td>0.5</td>
<td>2</td>
<td>16</td>
<td>128</td>
<td>0.015</td>
</tr>
<tr>
<td>MSSA ATCC 29213</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.015</td>
</tr>
<tr>
<td>MRSA 8111</td>
<td>0.5</td>
<td>2</td>
<td>64</td>
<td>2</td>
<td>0.015</td>
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TABLE 2. Intracellular antibacterial activities of daptomycin, vancomycin and oxacillin against MRSA ATCC 4330 and MSSA ATCC 29213

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (x MIC)</th>
<th>MRSA</th>
<th>MSSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>0</td>
<td>+0.86</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>+0.14</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-0.59</td>
<td>0.00</td>
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<td></td>
<td>5</td>
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<td>-1.48</td>
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<td></td>
<td>10</td>
<td>-1.52</td>
<td>-1.80</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5</td>
<td>+0.60</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-0.39</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-0.38</td>
<td>-0.88</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-0.51</td>
<td>-0.84</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.5</td>
<td>-0.85</td>
<td>-1.28</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-0.85</td>
<td>-1.35</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-0.94</td>
<td>-1.32</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-1.27</td>
<td>-1.75</td>
</tr>
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*Change in Log$_{10}$ viable counts between 0 h and indicated times (a negative value indicates net killing and a positive value indicates net growth).*
**Figure Legends**

**Figure 1.** Intracellular killing of *S. aureus strain* 8111 (MRSA) in human MDM using two-drug combinations (A) and a three-drug combination (B). The drug concentrations are expressed as multiples of the MIC. Single drug concentrations are depicted in dotted lines. Lower limit of detection was 20 CFU/ml (0.004%).

**Figure 2.** Extracellular killing of *S. aureus strain* 8111 (MRSA) in MH-II broth using two-drug combinations (A) and a three-drug combination (B). The drug concentrations are expressed as multiples of the MIC. Single drug concentrations are depicted in dotted lines. Lower limit of detection was 10 CFU/ml (0.01%).
References


Two-drug Combinations in MDM

1A

% CFU/ml at 0 hour

Hours

- Control
- Dap 1x
- Gent 0.5x
- Rif 1x
- Dap 1x+Rif 1x
- Gent 0.5x+Rif 1x
- Dap 1x+Gent 0.5x
Three-drug Combination in MDM

- Gent 0.5x
- Rif 1x
- Control
- Dap 1x
- 1x Dap + 0.5 x Gent + 1x Rif
Two-drug Combinations in MH-II Broth

% CFU/ml at 0 hour

Hours

Control
Dap 1x
Gent 0.5x
Rif 1x
Dap 1x+Gent 0.5x
Dap 1x+Rif 1x
Gent 0.5x+Rif 1x
Dap 1x+Gent 0.5x
Three-drug Combination in MH-II Broth

Hours

% CFU/ml at 0 hour

0 2 4 2 4

0.001

0.01

0.1

1

10

100

1000

10000

100000

Control
Dap 1x
Gent 0.5x
Rif 1x
Dap 1x+Gent 0.5x+Rif 1x

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