In vitro and in vivo activities of echinomycin against clinical isolates of Staphylococcus aureus

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Objectives: To verify in vitro and in vivo activities of echinomycin against clinical isolates of Staphylococcus aureus, we compared antistaphylococcal activities of echinomycin with those of vancomycin.

Methods: In vitro activities (MICs and MBCs) of oxacillin, vancomycin and echinomycin against 18 isolates of methicillin-susceptible S. aureus (MSSA) and 118 isolates of methicillin-resistant S. aureus (MRSA) were compared. Using four representative isolates of S. aureus, time–kill assay and in vivo antistaphylococcal activities were assessed. Echinomycin and vancomycin were compared in an in vivo mouse infection model.

Results: Echinomycin demonstrated higher in vitro activities against MSSA and MRSA strains, exhibiting 2-fold lower MIC90s and 4-fold lower MBC90s than vancomycin. Additionally, time–kill assay indicated that echinomycin is more potent than vancomycin against MSSA and MRSA strains in the context of MICs and MBCs. Using an in vivo protection model, it was shown that the 50% effective doses of echinomycin were at least 7-fold lower than those of vancomycin. Therefore, echinomycin displayed excellent protection in mice against acute peritoneal infections caused by both MSSA and MRSA strains.

Conclusions: Collectively, these data indicate that the activity of echinomycin against S. aureus strains is at least equivalent to that of vancomycin, regardless of the methicillin resistance of these strains. These promising activities of echinomycin might justify its potential use against infections with S. aureus strains resistant to vancomycin. This might be the first report to show that echinomycin possesses antipathogenic staphylococcal activity.

Keywords: antistaphylococcal activities, vancomycin, methicillin resistance

Introduction

Staphylococcus aureus has been recognized as one of the most significant pathogens in community-acquired1,2 and hospital-acquired infections. In particular, methicillin-resistant S. aureus (MRSA) can cause serious nosocomial infections in patients admitted to the intensive care unit.3,4 Nevertheless, the number of antibiotics available for treating nosocomial infections caused by MRSA is very limited, because these organisms are frequently resistant to many current antibiotics. To date, vancomycin is the drug of choice for treating infections with multidrug-resistant MRSA strains,5 although some antibiotics such as linezolid, daptomycin and quinupristin/dalfopristin are under investigation as alternatives to vancomycin.6 Unfortunately, since 1997 MRSA strains that have a reduced susceptibility to vancomycin have been reported worldwide; eventually, MRSA strains with high-level resistance to vancomycin, vancomycin-resistant S. aureus (VRSA), emerged.7 VRSA retained the vancomycin resistance gene vanA that previously only vancomycin-resistant enterococci (VRE) had possessed.8 Consequently, the development of novel therapeutic antibiotics against MRSA strains with higher resistance is urgently needed.

While screening anti-MRSA substances against clinical isolates of MRSA, we discovered that echinomycin might be active against pathogenic strains of S. aureus. Echinomycin has been one of the leading candidates for antitumour agents isolated from the culture broth of Streptomyces sp.9 Echinomycin is the prototypical bis-intercalator, a quinoxaline molecule that binds to DNA by inserting two planar chromophores between the base-pairs of duplex DNA, placing its cyclic depsipeptide backbone in the minor groove.10 Emerging evidence has shown that echinomycin or DNA bis-intercalator owns novel medicobiological activities such as HIF
suppression,\textsuperscript{11} transactivator of transcription of HIV binding,\textsuperscript{12} anti-VRE activity\textsuperscript{13} and antithrombotic activity.\textsuperscript{14} In particular, echinomycin showed distinct antimicrobial activities against a variety of Gram-positive bacteria (\textit{Bacillus anthracis}, \textit{S. aureus}, \textit{Streptococcus pneumoniae}, \textit{Listeria monocytogenes} and \textit{Enterococcus faecalis}) and Gram-negative bacteria (\textit{Shigella dysenteriae}),\textsuperscript{15} viruses (poliomyelitis virus, HIV and bacteriophage)\textsuperscript{12,16,17} and malarial parasite (\textit{Plasmodium falciparum}).\textsuperscript{15} Previously, we reported that echinomycin is active against clinical isolates of VRE.\textsuperscript{13} Synthesizing this evidence, we rationally hypothesized that echinomycin would be active against MRSA and methicillin-susceptible \textit{S. aureus} (MSSA). To address this issue, we examined the \textit{in vitro} and \textit{in vivo} activities of echinomycin against clinical isolates of \textit{S. aureus} by employing the MIC and MBC assay, the time–kill assay, as well as an \textit{in vivo} protection model.

\section*{Materials and methods}

\subsection*{Bacterial strains}

A total of 136 clinical isolates of \textit{S. aureus} were collected from three hospitals in Korea. Forty-four of them were obtained from the clinical microbiology laboratory of Yonsei University Medical Center in Seoul, 58 isolates were from the Wonju Christian Hospital in Gangwon and 34 isolates were from the Kyungpook National University Hospital in Daegu. All of the isolates had been identified as \textit{S. aureus} by routine laboratory methods in each hospital and were preserved as suspensions in 50\% glycerol broth at \(-80\,^{\circ}\text{C}\).

\subsection*{Antimicrobial agents}

Oxacillin, vancomycin and echinomycin were employed to test antistaphylococcal activities. Antimicrobial agents were freshly prepared on the day of use. Oxacillin and vancomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA) and echinomycin was provided by Dr Y. H. Kim (KAIST, Korea).

\subsection*{Animals}

Animal experiments were approved by and conducted under the guidelines of the Animal Care and Use established by The Ethics Review Committee of Yonsei Wonju College of Medicine, Wonju, Gangwon, South Korea.

\subsection*{Determination of MICs}

The MICs were determined by the 2-fold serial agar dilution method using Mueller–Hinton medium (Difco Laboratories, Detroit, MI, USA).\textsuperscript{18} All clinical isolates of \textit{S. aureus} were grown overnight in tryptic soy broth (TSB; Difco) at 37\,\^{\circ}\text{C}. Overnight broth cultures of bacterial suspension were diluted to a concentration of \(\approx 10^8\) cfu/mL using fresh TSB. A portion of the dilution was inoculated with a loop onto Mueller–Hinton agar plates containing various concentrations of antimicrobial agents. The final inoculum size was \(\approx 10^3\) cfu per spot. When the isolates were tested against vancomycin and echinomycin, the plates were incubated at 37\,\^{\circ}\text{C} for 18\,h; however, when they were tested against oxacillin, the Mueller–Hinton medium was supplemented with 4\% NaCl and the plates were incubated at 35\,\^{\circ}\text{C} for 24\,h. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth.

\subsection*{Determination of MBCs}

The MBCs were determined by a broth microdilution method.\textsuperscript{18} Overnight broth cultures of the clinical isolates of \textit{S. aureus} were diluted with fresh Mueller–Hinton, and a portion of the dilution was added to the wells containing various concentrations of antimicrobial agents in a 96-well microplate. The final inoculum size of all organisms was \(\approx 1 \times 10^6\) cfu/mL, and the plates were incubated at 37\,\^{\circ}\text{C} for 18\,h and inspected for turbidity. Growth-negative broth was subcultured onto antimicrobial agent-free plates, which were then incubated for 48\,h at 37\,\^{\circ}\text{C} for colony formation. The MBC was defined as the lowest concentration that exhibited 99.9\% or more reduction of the numbers of colonies compared with the cfu in the initial inoculum.

\subsection*{Time–kill assay}

\emph{In vitro} bactericidal activities of vancomycin and echinomycin against four representative isolates of \textit{S. aureus} were determined by a time–kill study.\textsuperscript{19,20} Overnight broth cultures of test organisms in TSB were diluted to a concentration of 10\(^7\) cfu/mL using fresh broth and then incubated at 37\,\^{\circ}\text{C} with shaking. After 2\,h of incubation, antimicrobial agents were added to a final concentration of 1/4\,\(\times\), 1\,or\,4\,\(\times\) the MIC. At 2, 4, 6 and 8\,h during the incubation period, duplicate 0.1\,mL aliquots were removed from the flasks for the determination of viable cells. Serial 10-fold dilutions of the sample in sterile PBS were spread on drug-free Mueller–Hinton agar plates, and the number of colonies was determined after incubation for 24\,h at 37\,\^{\circ}\text{C}. Time–kill curves were constructed over 24\,h as the \(\log_{10}\) cfu/mL versus time.

\subsection*{Determination of in vivo antistaphylococcal activity}

The \emph{in vivo} efficacies of vancomycin and echinomycin against four representative isolates of \textit{S. aureus} were determined by using a mouse model of acute bacterial infection. Test organisms were cultured overnight in TSB at 37\,\^{\circ}\text{C}. The bacterial cells grown to the late exponential phase in TSB were harvested and washed with sterile PBS. Subsequently, the cells were resuspended in sterile PBS, appropriately diluted with the PBS, and mixed with the same volume of 10% gastric mucin (Difco). Four-week-old female ICR mice (Orient Bio Inc., SeongNam, Kyonggi, South Korea) weighing 20–22\,g were injected intraperitoneally with a portion of a 0.5\,mL dose of the bacterial suspension, which corresponds to 100 times the 50\% lethal dose (LD\(_{50}\)). Within 3\,days after receiving the 100\,\times\,LD\(_{50}\) challenge inoculum, a mortality rate of 100\% was produced in all groups of untreated mice. The test antimicrobial agents were administered subcutaneously in a single dose 1\,h after inoculation, with 10 mice in each dosage group of antimicrobial agents. The number of surviving mice for each dosage was recorded 7\,days after the infection, and the mean effective dose sufficient to protect 50\% of the mice (ED\(_{50}\))\textsuperscript{11,23} was determined from the final survival rates using GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA, USA).

\section*{Results}

\subsection*{In vitro antistaphylococcal activity}

One hundred and thirty-six clinical isolates of \textit{S. aureus} collected from three hospitals were initially screened by dot-blot hybridization using a DNA probe specific for the \textit{mecA} gene. Of these isolates, 118 isolates had a positive reaction to the \textit{mecA}-specific
probe whereas 18 did not react. We regarded the mecA-negative isolates as MSSA and the mecA-positive isolates as MRSA.

In vitro activities of oxacillin, vancomycin and echinomycin against 18 isolates of MSSA and 118 isolates of MRSA were compared (Table 1). The MICs of vancomycin ranged from 0.25 to 1 mg/L for MSSA isolates and 0.25 to 2 mg/L for MRSA isolates. The MICs of echinomycin for both MSSA and MRSA isolates, which ranged from 0.06 to 0.5 mg/L, were lower than those of vancomycin. Fifty percent of strains were inhibited by a vancomycin concentration of 0.5 mg/L and 90% of strains were inhibited by 1 mg/L for both MSSA and MRSA. Meanwhile, echinomycin inhibited the same proportions of isolates at 4- and 2-fold lower concentrations than vancomycin, respectively.

The MBCs of vancomycin (1–2 mg/L) for both MSSA and MRSA isolates were higher than those of echinomycin (0.125–0.5 mg/L). The MBC50s and MBC90s of vancomycin were 0.25 and 0.5 mg/L, respectively, whereas those of echinomycin were 0.25 and 0.5 mg/L, respectively.

Paired t-test analyses of Table 1 showed that the mean MIC and MBC of echinomycin for testing MRSA and MSSA are significantly different from those of vancomycin, verifying that echinomycin is more active than vancomycin (data not shown).

Two of the 18 MSSA isolates (W5 and K110) and 2 of the 118 MRSA isolates (Y7563 and K141) were selected so that the bactericidal activities of vancomycin and echinomycin could be further evaluated by time–kill assay (Figures 1–4). Vancomycin at 1/4 × the MIC was clearly inactive against W5, a MSSA isolate, and Y7563, a MRSA isolate (Figures 2a and 4a). By contrast, echinomycin was active even at 1/4 × MIC (Figures 2a and 4a) against both strains in a time-dependent manner. It was statistically clear that the time–kill activity of echinomycin is superior to that of vancomycin at the same MIC. When K110 (another MSSA isolate) and K141 (another MRSA isolate) were tested by the time–kill assay, vancomycin and echinomycin displayed similar bacterial kill patterns as against W5 and Y7563 (data not shown). No significant differences were observed between the MSSA and MRSA isolates with respect to the bactericidal activities of echinomycin.

In vivo antistaphylococcal activity

The protective effects of echinomycin against experimental systemic infections caused by clinical isolates of S. aureus in mice were compared with those of vancomycin (Table 2). The same isolates used in the time–kill study were employed for the in vivo experimental infection model. Vancomycin was administered to each dosage group of mice at serial doses ranging from 0.2 to 100 mg/kg; echinomycin was administered at serial doses

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**Table 1.** MICs and MBCs of oxacillin, vancomycin and echinomycin for 136 clinical isolates of *S. aureus*

<table>
<thead>
<tr>
<th>Organism (no. of tested isolates)</th>
<th>Antimicrobial agent</th>
<th>MIC (mg/L)ᵃ</th>
<th>MBC (mg/L)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin-susceptible <em>S. aureus</em> (18)</td>
<td>oxacillin</td>
<td>1–4 2 2</td>
<td>4–8 8 8</td>
</tr>
<tr>
<td></td>
<td>vancomycin</td>
<td>0.25–1 0.5 1</td>
<td>1–2 1 2</td>
</tr>
<tr>
<td></td>
<td>echinomycin</td>
<td>0.06–0.5 0.125 0.5</td>
<td>0.125–0.5 0.25 0.5</td>
</tr>
<tr>
<td>Methicillin-resistant <em>S. aureus</em> (118)ᶜ</td>
<td>oxacillin</td>
<td>64–&gt;512 256 &gt;512</td>
<td>512–&gt;1024 &gt;1024 &gt;1024</td>
</tr>
<tr>
<td></td>
<td>vancomycin</td>
<td>0.25–2 0.5 1</td>
<td>1–2 1 2</td>
</tr>
<tr>
<td></td>
<td>echinomycin</td>
<td>0.06–0.5 0.125 0.5</td>
<td>0.125–0.5 0.25 0.5</td>
</tr>
</tbody>
</table>

ᵃ50% and 90%, MICs at which 50% and 90% of isolates are inhibited, respectively.
ᵇ50% and 90%, MBCs at which 50% and 90% of isolates are inhibited, respectively.
ᶜBoth homogeneous- and heterogeneous-resistant organisms are included.

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**Figure 1.** Time–kill curves of MSSA (W5) at 1/4 ×, 1 × or 4 × the MIC of vancomycin (a) and echinomycin (b). Untreated control, open squares; 1/4 × MIC, filled triangles; 1 × MIC, filled circles; 4 × MIC, filled squares. Values are the averages of duplicate experiments and error bars indicate the range of values.
ranging from 0.02 to 10 mg/kg because its LD50 was calculated as 12.3 mg/kg. The ED50s of vancomycin and echinomycin against infections by W5 were 9.6 and 1.3 mg/kg, respectively, which showed that the ED50 of echinomycin was ~7-fold lower than that of vancomycin. The ED50s of echinomycin against infections by K110, Y7563 and K141 were ~11-, 16- and 13-fold lower than those of vancomycin, respectively. There were no significant differences between the MSSA and MRSA isolates regarding the ED50s of echinomycin for therapy of acute systemic infections.

Discussion

This study clearly demonstrates through three ways that echinomycin is active against clinical isolates of S. aureus (MRSA and MSSA) both in vitro and in vivo. First of all, comparing MIC and MBC values of echinomycin against S. aureus demonstrated that echinomycin has excellent activity. Table 1 clearly indicates that in terms of MBC50s and MBC90s of vancomycin and echinomycin, the bactericidal activity of echinomycin against S. aureus is 2-fold higher than that of vancomycin. Even though there were small numbers of MSSA in this study, echinomycin showed consistent, excellent bacteriostatic and bactericidal activities compared with vancomycin.

Additionally, the time–kill assay employing two representative strains (W5 and Y7563) clearly showed that echinomycin inhibits bacterial growth even at 1/4 MIC, whereas vancomycin fails to inhibit growth at such a low concentration (Figures 1 and 3). This time–kill effect of echinomycin increased in a time- and dose-dependent fashion. Moreover, these growth-inhibitory patterns were very similar to those of other test strains, K110 and K141 (data not shown). These data strongly support our hypothesis that echinomycin is at least equivalent to vancomycin in terms of MIC and MBC. However, cumulative data have suggested that echinomycin might be less active due to its chemical hydrophobicity and to having a short in vivo half-life.23,24 In that regard, proving the in vivo protection or therapeutic efficacy of echinomycin is crucial for translating this study into a clinical trial. To this end, we established a systemic, intraperitoneal infection model using the same strains that were used for the in vitro time–kill assay. This model has several advantages, including fair reproducibility and easy manipulation. Even though the strains used in the in vivo assay are not standard strains, using clinical isolates may be suitable for comparing the correlation

Figure 2. Bactericidal activities of vancomycin and echinomycin against MSSA W5 at 1/4× (a), 1× (b) and 4× (c) the MIC of each drug. The error bars indicate 95% CIs. Vancomycin, open squares; echinomycin, filled squares. Values are the averages of duplicate experiments and error bars indicate the range of values. *P < 0.05; **P < 0.01.

Figure 3. Time–kill curves of MRSA (Y7563) at 1/4×, 1× or 4× the MIC of vancomycin (a) and echinomycin (b). Untreated control, open squares; 1/4× MIC, filled triangles; 1× MIC, filled circles; 4× MIC, filled squares. Values are the averages of duplicate experiments and error bars indicate the range of values.
between in vitro and in vivo data and for translating this study into the clinic in the future. In light of excellent antitumour activity and tolerable toxicity in preclinical and Phase 1 trials, echinomycin has been used in a Phase II clinical trial for the treatment of solid types of cancer such as colon cancer and ovarian cancer.\(^{25,26}\)

Therefore, we investigated in vivo applicability.

Finally, the in vivo ED\(_{50}\) of echinomycin against three test strains were \(~4\)–\(16\)-fold lower than those of vancomycin (Table 2). These in vivo efficacy data might provide a scientific basis for the usage of echinomycin in intractable staphylococcal infection. Despite excellent antistaphylococcal activities of echinomycin, the underlying germicidal mechanism of echinomycin is still veiled. Considering the in vitro and in vivo data, the antistaphylococcal activity of echinomycin against MRSA and MSSA is the same, thus suggesting that echinomycin might hold a novel mechanism to circumvent existing resistance mechanisms.

Collectively, these data indicate that the activity of echinomycin against \(S\). \(aureus\) strains is at least equivalent to that of vancomycin, regardless of the methicillin resistance of the strains. The excellent activity of echinomycin might justify its potential use against infections with \(S\). \(aureus\) strains that are resistant to vancomycin. This might be the first report to show that echinomycin possesses antipathogenic staphylococcal activity. One of the limitations of this study is the lack of data comparing MICs of echinomycin and anti-MRSA drugs except vancomycin. Further studies to resolve these issues are underway.

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**Transparency declarations**

None to declare.

**References**


