Comparison studies of the bactericidal, morphological and post-antibiotic effects of arbekacin and vancomycin against methicillin-resistant *Staphylococcus aureus*

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Arbekacin, an aminoglycoside antibiotic, has antibacterial activity against both Gram-positive and Gram-negative bacteria and is stable in the presence of aminoglycoside-inactivating enzymes produced by methicillin-resistant *Staphylococcus aureus* (MRSA). In this report, the antibacterial activity of arbekacin was compared with that of vancomycin, a glycopeptide antibiotic that also has potent antibacterial activity against MRSA. Arbekacin showed concentration-dependent bactericidal activity against MRSA strain 1936 (0.5–2 x MIC), but vancomycin showed only slight bactericidal activity, even at high concentrations of 19 x MIC. Arbekacin showed a longer post-antibiotic effect (2.3–3.8 h) than vancomycin (0–1.3 h) against MRSA strain 1936. Arbekacin induced marked morphological changes at 0.5 x MIC and the changes remained for 2 h after removal of the agent. When exposed to 0.5 x MIC of vancomycin, no notable morphological change was observed in the treated cells. Since arbekacin has broad-spectrum activity, these findings suggest that it may be a useful agent against MRSA infection, especially for polymicrobial MRSA infection with Gram-negative bacilli, such as *Pseudomonas aeruginosa*.

**Introduction**

Strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were detected in 1961, shortly after methicillin came into clinical use. However, such strains occurred sporadically and were resistant only to β-lactam antibiotics, causing no major problems. By contrast, the resistant strains that appeared in Australia in the late 1970s were entirely different; they were resistant to other types of antibiotics, such as aminoglycosides, macrolides, tetracyclines and, more recently, quinolones, and have spread worldwide, while retaining their virulence. In Japan, most strains of MRSA now exhibit high-level resistance to various antibiotics and therefore pose a serious problem clinically and epidemiologically, among compromised in-patients in particular, as the causative pathogen of nosocomial infection. Arbekacin is a broad-spectrum aminoglycoside antibiotic and is active against MRSA that have a variety of aminoglycoside-inactivating enzymes. In this study, the bactericidal activity and post-antibiotic effects (PAEs) of arbekacin were compared with those of vancomycin against MRSA strain 1936, a clinical isolate. The morphological changes of the strain caused by exposure to these agents were also examined.

**Materials and methods**

**Antibiotics**

Arbekacin (Meiji Seika Kaisha, Ltd, Tokyo, Japan) and vancomycin (Sigma Chemical Co., St Louis, MO, USA) were used in this study.

**Organism**

MRSA strain 1936 was a clinical isolate from Japan and was used throughout this study. This strain was resistant to methicillin (MIC > 800 mg/L), erythromycin (MIC > 800 mg/L) and gentamicin (MIC = 100 mg/L), and is considered to be representative of MRSA in Japan.

**Determination of MICs**

The MICs of MRSA strain 1936 were determined by one
of two methods; a two-fold serial agar dilution method according to the ‘Standard Method’ recommended by the Japan Society of Chemotherapy, and a two-fold serial broth dilution method with the shaking culture described below. A few colonies from an overnight culture were inoculated into Mueller–Hinton broth (MHB) (Difco Laboratories, Detroit, MI, USA), and cultured overnight at 35°C. This culture was then diluted to a density of about 10^6 cfu/mL in MHB and 10 mL portions were distributed into L-tubes without antibiotic. These cultures were incubated for 1 h and then either arbekacin or vancomycin was added at concentrations in the range of 0.05–100 mg/L (two-fold serial dilutions). The endpoint was determined by turbidity after overnight incubation at 35°C.

**Bactericidal activities**

A few colonies from an overnight culture were inoculated into MHB and allowed to grow at 35°C until an optical density of 0.3 (660 nm) was reached. This culture was then diluted to a density of about 10^6 cfu/mL in MHB, and 10 mL portions were distributed into L-tubes without antibiotic. These cultures were incubated for 1 h and then either arbekacin or vancomycin was added at concentrations of 0.78, 1.56 or 3.13 mg/L. Concentrations of 4 mg/L of arbekacin and 20 mg/L of vancomycin, which correspond to half of the peak serum found in humans receiving the usual clinical dose of each agent (arbekacin: 100 mg; vancomycin: 500 mg), were added to the bacterial suspensions to assess the killing activity in clinical practice. Incubation was conducted at 35°C in a shaking waterbath. Each culture suspension was diluted with saline containing 0.05% agar at serial ten-fold dilution, and 50 µL of each dilution was seeded on to an antibiotic-free agar plate at intervals determined previously. The bacterial count on these agar plates was determined after incubation at 35°C for 18–24 h.

**Post-antibiotic effects**

A rbekacin or vancomycin was added at concentrations of 0.78, 1.56 and 3.13 mg/L to 10 mL of the MRSA strain 1936 bacterial suspension and an antibiotic-free control was also prepared. The suspensions were incubated at 35°C for 1 or 2 h, and filtered through a 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA) to collect the bacteria free of antibiotic. Sterile MHB was added and the filtration was repeated to ensure complete elimination of the antibiotic. The bacteria on the filter were carefully resuspended and dispersed with a tube mixture in 10 mL of fresh MHB. The number of cells in each culture was determined as described above.

The bacterial suspensions were separately exposed to 4 mg/L of arbekacin for 1 h, or to 20 mg/L of vancomycin for 2 h. These exposure times had previously been determined to be the times required to keep the concentration above the serum levels in vivo. These agents were then removed by filtration, and viable cells in these suspensions were counted using the method described above.

The PAEs were calculated using the following equation: PAE = T – C, where T is the time required for the count in the test culture to increase by 1 log_{10} above the count observed immediately after drug elimination, and C is the time required for the count in the untreated control culture to increase by 1 log_{10} above the count immediately after completion of the same procedure used on the test culture for drug removal.

**Ultrastructural change in MRSA exposed to arbekacin or vancomycin**

Bacterial suspensions were exposed to 0.78 mg/L of arbekacin or 0.78 mg/L of vancomycin for 2–4 h, and cultivated for 2 h after removal of each agent. The treated bacteria were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, then treated with 1% osmium tetroxide in phosphate buffer. After dehydration in a graded alcohol series, the organisms were cut with an LKB 2088 Ultron V, and stained with Reynolds’ double stain (uranyl acetate and lead citrate). The ultrastructural findings were observed with a Hitachi H-600 electron microscope (Tokyo). Other bacterial suspensions that had been exposed to 4 mg/L of arbekacin for 1 h or 20 mg/L of vancomycin for 2 h were cultivated for 2 h after removal of each drug, and were observed with a transmission electron microscope as described above.

**Results**

**MICs**

The MICs of arbekacin and vancomycin for MRSA strain 1936 were found to be 0.78 and 1.56 mg/L, respectively, by the agar dilution method and 1.56 and 1.56 mg/L, respectively, by the broth dilution method.

**Bactericidal activities**

A rbekacin showed potent bactericidal activity against MRSA strain 1936. Exposure to concentrations of 0.78 (0.5 × MIC), 1.56 (1 × MIC) and 3.13 mg/L (2 × MIC) of arbekacin, killed 90%, 99% and 99.9% of the inoculum within 8 h, respectively. When exposed to 0.78 mg/L (0.5 × MIC) of vancomycin, the viable cell numbers did not decrease, and the organisms regrew after 4 h. The vancomycin bactericidal activity was slight at 1 × MIC and 2 × MIC (Figure 1). When exposed to drug concentrations that correspond to half the peak serum levels found in humans receiving the usual clinical dose (4 mg/L for arbekacin and 20 mg/L for vancomycin) (Figure 2).
Effects of arbekacin and vancomycin on MRSA

Arbekacin showed bactericidal activity, but vancomycin showed poor bactericidal activity.

Post-antibiotic effects

Arbekacin and vancomycin at concentrations of 0.5–2 × MIC showed respective PAEs of 2.3–3.2 and 0–0.4 h when exposed for 1 h. The PAEs were extended to 3.0–3.8 and 0.4–1.3 h, respectively, when exposure was increased to 2 h (Table I). Arbekacin and vancomycin showed PAEs of 3.9 and 1.1 h, respectively, when exposed to the drug concentration corresponding to half the maximum serum level of these agents attainable in vivo (Table II).

Ultrastructural findings

When bacteria were exposed to 0.78 mg/L of arbekacin for 2 h, the ultrastructure of MRSA strain 1936 showed undulating thick cell walls with mesosomes and thick cross walls of decreased electron density. In some cells, thick, multilayered cell walls were partly destroyed and cytoplasm was extruded. These findings remained for at least 2 h after arbekacin removal (Figure 3). When exposed to 0.78 mg/L of vancomycin for 2–4 h, the treated cells appeared similar to normal cells and the cellular structure did not change for 2 h after the removal of vancomycin (Figure 3). However, undulating cell walls and decreased electron density cross walls were observed when the cells were exposed to 20 mg/L of vancomycin.
Discussion

At present, arbekacin and vancomycin are the most effective agents against MRSA infection. However, relatively few reports describe the direct comparison of both antibiotics in fundamental research. When compared with vancomycin, the most notable differences of arbekacin are its bactericidal action and its longer PAE. Arbekacin binds to both 50S and 30S ribosomal subunits, inhibits protein synthesis at bacterial ribosomes and causes codon misreading. The high stability of arbekacin in the presence of inactivating enzymes produced by MRSA, such as 2'-aminoglycoside phosphotransferase, 4'-aminoglycoside adenyllyltransferase and 3'-aminoglycoside phosphotransferase causes MRSA to be sensitive to the antibiotic. Most other aminoglycoside antibiotics, such as gentamicin, tobramycin and kanamycin, do not show antibacterial activity against MRSA, because of inactivation by these enzymes. Vancomycin is active against Gram-positive cocci and bacilli by inhibiting cell wall synthesis. It has also been reported that vancomycin prevents the polymerization of the phosphodisaccharide–pentapeptide–lipid complex during the second stage of cell wall synthesis. It binds very tightly to precursor peptides that contain D-alanyl-D-alanine at the free carboxyl end, and causes steric hindrance of transglycosylase and transpeptidase enzymes. Thus peptidoglycan synthesis is blocked, and membrane-bound lipid intermediates accumulate in the presence of vancomycin. The bacteriostatic action of vancomycin against MRSA, observed in this study and by others, is consistent with the mode of action, but the bactericidal action of arbekacin could not be explained by the inhibition of protein synthesis and miscoding.

It has been reported that aminoglycoside antibiotics show potent PAEs against Gram-positive and Gram-negative bacteria. In this report, the PAEs of arbekacin were longer than those of vancomycin when compared at the same concentrations and the same exposure times. Furthermore, arbekacin showed a longer PAE than vancomycin at concentrations corresponding to serum levels attainable in vivo. Thickened cell walls with multiple layers were observed after exposing S. aureus to protein synthesis inhibitory antibiotics, such as macrolides and tetracyclines. In this study, the morphological changes in strain 1936, after exposure to arbekacin, were similar to those caused by protein synthesis inhibitors described previously. Interestingly, the ultrastructural change of this strain persisted during the PAE phase of arbekacin. It is known that aminoglycoside antibiotics inhibit an initiation step of DNA replication inhibiting bacterial division, but that they do not completely block bacterial protein synthesis. It is suggested, therefore, that the formation of thick cell walls in the MRSA strain treated with arbekacin may be because of the continuation of cell wall synthesis for a short period in which cell division was inhibited. However, there have been no reports of ultrastructural changes in Gram-positive bacteria induced by the action on aminoglycoside antibiotics. This is the first report on induced cell wall thickening by an aminoglycoside and the mechanism will be the subject of future study. It has been reported that when S. aureus was exposed to vancomycin, the most pronounced ultrastructural change was the irregular thickness and frequent absence of the cell wall. However, in this study, we found that these changes were slight in bacteria exposed to 0.5 × MIC (0.78 mg/L) of the agent for 2–4 h. Only after exposure to 20 mg/L of the agent for 4 h was severe damage found. The morphological change of the strain

Table I. Post-antibiotic effects of arbekacin and vancomycin against MRSA strain 1936 after exposure to half-peak concentration levels of the agents usually used in humans

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Drug concentration (mg/L)</th>
<th>PAE (h) arbekacin</th>
<th>PAE (h) vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.78 (0.5 × MIC)</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.56 (1 × MIC)</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>3.13 (2 × MIC)</td>
<td>3.2</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>0.78 (0.5 × MIC)</td>
<td>3.8</td>
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<td></td>
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<td>3.13 (2 × MIC)</td>
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<td>1.0</td>
</tr>
</tbody>
</table>
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Figure 3. Ultrastructure of MRSA strain 1936 exposed to arbekacin or vancomycin; (a) no drug control; (b) exposed to 0.78 mg/L of arbekacin for 2 h; (c) cultivated for 2 h after removal of arbekacin after 2 h exposure to 0.78 mg/L of arbekacin; (d) exposed to 0.78 mg/L of vancomycin for 4 h; (e) cultivated for 2 h after removal of vancomycin after 2 h exposure to 0.78 mg/L of vancomycin.

Figure 4. Ultrastructure of MRSA strain 1936 exposed to vancomycin under conditions considered to represent the serum levels in humans; (a) exposed to 20 mg/L of vancomycin for 2 h; (b) cultivated for 2 h after removal of vancomycin after 2 h exposure to 20 mg/L of vancomycin.
exposed to vancomycin was consistent with the poor bactericidal activity of vancomycin. Based on observations of M.R.S.A strain 1936, arbekacin appeared superior to vancomycin in bactericidal activity, PAE and in causing morphological changes.

There are many reports describing infections with M.R.S.A., accompanied by other pathogens, such as P. aeruginosa and other Gram-negative bacilli.

Considering these facts, arbekacin seems to be a more useful agent than vancomycin against polymicrobial M.R.S.A. infection with Gram-negative bacilli.

References


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