Influences of dosage regimen and co-administration of low-molecular weight proteins and basic peptides on renal accumulation of arbekacin in mice

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Objectives: The objectives of this study were to characterize renal accumulation of arbekacin, an aminoglycoside antibiotic for treatment of infections with methicillin-resistant Staphylococcus aureus, and to modulate renal uptake of arbekacin, leading to prevention of arbekacin-induced nephrotoxicity.

Methods: In vivo renal uptake studies were performed using mice. Renal concentrations of arbekacin after a bolus intravenous administration at various doses were analysed by HPLC. In addition, renal concentrations were investigated 24 h after an injection of arbekacin alone or in combination with low-molecular weight proteins and basic peptides.

Results: When administered by bolus injection at various doses, renal accumulation of arbekacin showed saturation kinetics with increasing dose. Renal concentration of arbekacin after a bolus administration remained constant from 4 to 24 h and subsequently decreased by a first-order process with a half-life of 42.7 h. The influences of three dosage regimens (a single injection of 4 mg/kg, two injections of 2 mg/kg and three injections of 1.33 mg/kg) were investigated. A single injection resulted in lower renal level of arbekacin than the multiple administrations. Co-administration of cytochrome c, lysozyme and N-WASP181–200 decreased renal accumulation of arbekacin in a dose-dependent manner. N-W(N1n), N-W(N1n,I2i,S3s) and N-W(N1n,K20k), in which the N- and/or C-terminal regions of N-WASP181-200 were substituted by one to three D-isomers, more potently decreased renal arbekacin accumulation than N-WASP181-200.

Conclusions: These data may be useful for prevention of arbekacin-induced nephrotoxicity owing to reduction of renal accumulation of the aminoglycoside.

Keywords: aminoglycoside, renal uptake kinetics, once-daily dosing, megalin, nephrotoxicity

Introduction

Arbekacin, an aminoglycoside antibiotic, has antibacterial activity against both Gram-positive and Gram-negative bacteria. Since arbekacin is stable in the presence of aminoglycoside-inactivating enzymes produced by methicillin-resistant Staphylococcus aureus (MRSA), the antibiotic is used for the treatment of patients infected with MRSA in Japan. The typical clinical dose of arbekacin for the treatment of MRSA infection is 150–200 mg per day for adult patients or 4–6 mg/kg per day for child patients, which is intravenously administered in a twice-daily divided dose. The antibacterial activity of arbekacin against MRSA is reported to be superior to that of vancomycin, another glycopeptide antibiotic. Like other aminoglycosides, most of the intravenously injected arbekacin is excreted into the urine, whereas a fraction is selectively accumulated in the renal proximal tubular cells. The concentrated accumulation of arbekacin in the kidney is involved in the incidence of nephrotoxicity, which is the major dose-limiting side effect of arbekacin.

It was suggested early on that acidic phospholipids of the plasma membrane of the proximal tubular cells are the initial binding site for aminoglycosides. Subsequently, megalin, a multiligand endocytic receptor abundantly expressed in the renal proximal tubule, was reported to bind gentamicin. Later, our and other studies revealed that megalin plays an important role in uptake of gentamicin and amikacin in the kidney. These findings indicated that aminoglycoside binding receptors such as...
acidd phospholipids and megalin are a potential target for preventing aminoglycoside nephrotoxicity. In a previous report, we showed that co-administration of a low-molecular weight protein cytochrome c, a ligand of megalin, decreased gentamicin-induced nephrotoxicity as well as renal accumulation of gentamicin. Like cytochrome c, a low-molecular weight protein, lysozyme, is reported to be a ligand of megalin. As expected from that observation, we demonstrated that lysozyme and gentamicin interact with each other in their re-absorption process in the renal proximal tubules. Furthermore, we found that several basic peptide fragments, which bind to phosphoinositides, inhibited in a dose-dependent manner the renal accumulation of gentamicin injected intravenously. Among the fragments examined, N-WASP181-200 from neural Wiskott–Aldrich syndrome protein (N-WASP) was the most effective antagonist under in vitro conditions. On the other hand, under in vivo conditions, N-WASP181-200 inhibited renal accumulation of gentamicin less than was expected from the in vitro inhibitory effect, which is possibly due to rapid degradation in plasma by proteases. Since α-amin acid substitutions have been reported to make peptides resistant to proteolysis, partial α-amino acid substitution may be a potent approach for decreasing the dose of the peptidic inhibitors for renal accumulation of aminoglycosides.

Previously, fosfomycin, a broad-spectrum antibiotic that contains a phosphonate group, was reported to decrease nephrotoxicity induced by aminoglycosides including dibekacin and arbekacin. However, the mechanism underlying the protective effect of fosfomycin against gentamicin-induced nephrotoxicity has not been fully clarified, though the inhibition of gentamicin-induced lipid peroxidation is suggested to be involved in the protection by fosfomycin against gentamicin-induced nephrotoxicity.

In this study, we first characterized the renal accumulation of arbekacin in mice. In addition, the effect of co-administration of cytochrome c, lysozyme and N-WASP181-200 on renal accumulation of arbekacin was investigated. To examine whether or not partial α-amino substitution is useful for decreasing the dose, the effects of three analogues with a D-amino acid as the N- and/or C-terminal flanking residue, N-W(N1n), N-W(N1n,I2i,S3s) and N-W(N1n,K20k), were analysed. Furthermore, we examined the effect of fosfomycin on renal arbekacin concentration in order to clarify whether or not a change in the renal accumulation of arbekacin is involved in the protective effect of fosfomycin against nephrotoxicity induced by aminoglycosides including arbekacin.

Materials and methods

Materials

Arbekacin sulphate was from Meiji Seika Kaisha, Ltd (Tokyo, Japan). Cytochrome c from horse heart, lysozyme chloride from egg white and 2,4,6-trinitrobenzenesulphonic acid sodium salt were purchased from Nacalai Tesque (Kyoto, Japan). Fosfomycin was obtained from Wako Pure Chemical Industries (Osaka, Japan). All chemicals used for the experiments were of the highest purity available.

Animals

Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University. Male ddY mice (18–34 g) were administered arbekacin via the tail vein.

Tissue distribution of arbekacin in mice

At 24 h after a bolus administration of arbekacin at a dose of 3.5 mg/kg via the tail vein, blood samples were withdrawn by cardiac puncture and mice were sacrificed by cervical dislocation under deep anaesthesia with diethyl ether. Then, brain, heart, liver, lung, spleen, pancreas, jejunum, ileum and kidney were excised. The tissues were weighed and then homogenized with an IKA T25 Basic dispenser (IKA Labortechnik, Germany) in 2 mL of 30 mM phosphate buffer (pH 7.5). After shaking for 20 min, the homogenate was mixed with 400 μL of 20% trichloroacetic acid and was further shaken for 40 min. After centrifugation at 3000 rpm for 10 min, the arbekacin concentration in the supernatant was determined by HPLC as described below.

Renal concentration of arbekacin after a bolus injection at various doses

At 24 h after a bolus administration of arbekacin at various doses (0.25, 0.5, 1, 2, 4 and 8 mg/kg) via the tail vein, both kidneys were processed as described earlier.

Disappearance of arbekacin from kidney

At a stated time (4, 6, 8, 24, 48, 72, 96, 120 and 144 h) after a bolus administration of arbekacin at a 4 mg/kg via the tail vein, both kidneys were processed as described earlier.

Renal concentration of arbekacin after successive doses

Mice were given a single dose, and two, four and seven successive doses of arbekacin of 4 mg/kg via the tail vein. At 24 h after the final injection, both kidneys were processed as described earlier.

Renal concentration of arbekacin in different dosing regimens

Mice were administered three different dosage regimens yielding a total daily dose of 4 mg of arbekacin (a single injection of 4 mg/kg, two injections of 2 mg/kg and three injections of 1.33 mg/kg) via the tail vein. At 24 h after the first injection, both kidneys were processed as described earlier.

Effect of co-administration of fosfomycin, peptides and low-molecular weight proteins on arbekacin accumulation in kidney

Mice were administered arbekacin alone or in combination with the indicated compound via the tail vein. Fosfomycin was administered to mice within a few minutes just before injection of arbekacin. The doses of fosfomycin in this study (84 and 168 mg/kg) were set based on the human dose of 2–4 g per day for adult patients or 100–200 mg/kg/day for children. Cytochrome c, lysozyme, N-WASP181-200, N-W(N1n), N-W(N1n,I2i,S3s) or N-W(N1n,K20k) was simultaneously given as a mixture with arbekacin, which was prepared just before the experiment. At 24 h after the final injection, both kidneys were processed as described earlier.
Preparation of peptide fragments

Synthetic peptide fragments were produced with the peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan). The peptide fragments were synthesized chemically and their amino acid sequences were as follows (the lowercase letters indicate the amino acids replaced by D-amino acids): N-WASP181-200 (NISHTKEKKKGKAKKKRLTK); N-W(N1n) (nISHTKEKKKGKAKKKRLTK); N-W(N1n,I2i,S3s) (nisHTKEKKKGKAKKKRLTK); and N-W(N1n,K20k) (nISHTKEKKKGKAKKKRLTk). The peptide sequence of these peptides prepared by the synthesizer was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems, Tokyo, Japan).

Analytical methods

Quantitative determination of arbekacin was performed by using HPLC (PU-980, Jasco, Tokyo, Japan) equipped with an ultraviolet spectrophotometric detector (UV-970, Jasco; wavelength 350 nm) after derivatization of the drug by reaction with 2,4,6-trinitrobenzenesulphonic acid. The conditions were as follows: column 4.6 × 100 mm (TSK gel ODS-80TM Tosoh, Tokyo, Japan); mobile phase 0.03 M phosphate buffer (pH7.5)/acetonitrile/methanol 1.1:2:1 (vol/vol); flow rate 1.0 mL/min; and room temperature. The elimination rate constant $k$, Michaelis–Menten constant $K_m$ and 50% inhibitory concentration (IC50) values were calculated based on the first-order equation, the Michaelis–Menten equation and the Hill equation, respectively. The KaleidaGraph™ program (version 3.08, Synergy Software, PA, USA) was used for the curve-fitting, and the linear and non-linear regression analysis was done without weighting. Goodness of fit was assessed by the correlation coefficient ($r$) and chi-square ($\chi^2$) values. Statistical analysis was performed by the one-way analysis of variance with the Scheffé test for post hoc analysis. $P < 0.05$ for a difference was considered statistically significant.

Results

Tissue distribution of arbekacin 24 h after a bolus intravenous administration of the drug to mice

Arbekacin was abundantly accumulated in the kidney after its intravenous injection (12.3 ± 1.5% of dose, $n = 3$), while no or a very faint accumulation was observed in other tissues including brain, heart, liver, lung, spleen, pancreas, jejunum, ileum and plasma (data not shown).

Renal concentration of arbekacin 24 h after administration at various doses

Figure 1 shows renal concentration of arbekacin after a bolus intravenous administration of arbekacin at various doses. It was apparent that the renal uptake of arbekacin was increased in a saturable manner. Curve-fitting was performed by non-linear regression analysis, using the Michaelis–Menten equation ($r = 0.992, \chi^2 = 3.763$), to determine the apparent $K_m$ value for renal uptake of arbekacin. The analysis yielded an apparent $K_m$ value of 4.86 mg/kg body weight.
Effects of dosing schedules on renal accumulation of arbekacin

The influences of three dosage regimens on the renal accumulation of arbekacin were investigated (Figure 4). A single injection resulted in a lower renal concentration of arbekacin than administration of the same total dose over two or three injections. These results would not be due to the differences in periods after the final injection (24 h for single injection, 12 h for two injections and 8 h for three injections) since the renal arbekacin level was constant over the period from 4 to 24 h after a bolus injection as shown in Figure 2.

Effect of co-administration of fosfomycin on renal accumulation of arbekacin

To investigate whether fosfomycin inhibits renal uptake of arbekacin, the effect of co-administration of fosfomycin on renal accumulation of arbekacin was examined. As shown in Figure 5, co-administration of fosfomycin at doses of 84 and 164 mg/kg with arbekacin (4 mg/kg) had no effect on renal accumulation of arbekacin at 24 h after injection. Therefore, it is likely that the protective effect of fosfomycin against arbekacin-induced nephrotoxicity is due to mechanisms other than the inhibition of renal accumulation of arbekacin.

Effect of co-administration of cytochrome c, lysozyme, N-WASP181-200 and its partial d-amino acid-substituted peptides on renal accumulation of arbekacin

The effects of cytochrome c, lysozyme and N-WASP181-200 on renal accumulation of arbekacin were examined. Like 3H-labelled gentamicin,9 the accumulation of arbekacin was inhibited by co-administration of cytochrome c, lysozyme or N-WASP181-200 in a dose-dependent fashion (Figures 6 and 7). It has been reported that partial d-amino acid substitution is a useful approach to improve the stability of peptides administered in vivo.11–13 Therefore, we further studied the effects of d-amino acid substitutions within the N- and/or C-terminal flanking regions of N-WASP181-200 on renal accumulation of arbekacin. When arbekacin was co-administered with N-W(N1n), which contains only one d-amino acid on the N terminus, N-W(N1n) inhibited renal accumulation of arbekacin in a dose-dependent manner and its dose–response curve was shifted to the left, compared with that of N-WASP181-200 (Figure 7). In addition, N-W(N1n,I2i,S3s) with three d-amino acids on the N terminus...
and N-W(N1n,K20k) with one D-amino acid each on the N and C termini decreased the renal accumulation of arbekacin more potently than N-WASp181-200 (Figure 7). The inhibitory potencies of N-W(N1n,I2i,S3s) and N-W(N1n,K20k) were almost the same as that of N-W(N1n). The IC50 values of proteins and peptides tested in this study were determined by the Hill equation and are summarized in Table 1.

### Table 1. Arbekacin renal accumulation IC50 values of cytochrome c, lysozyme, N-WASp181-200, N-W(N1n), N-W(N1n,I2i,S3s) and N-W(N1n,K20k)

<table>
<thead>
<tr>
<th>Protein</th>
<th>IC50 (mg/kg body weight)</th>
<th>IC50 (µmol/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>15.2</td>
<td>1.23</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>437.8</td>
<td>30.8</td>
</tr>
<tr>
<td>N-WASp181-200</td>
<td>46.1</td>
<td>19.6</td>
</tr>
<tr>
<td>N-W(N1n)</td>
<td>16.6</td>
<td>7.04</td>
</tr>
<tr>
<td>N-W(N1n,I2i,S3s)</td>
<td>17.0</td>
<td>7.22</td>
</tr>
<tr>
<td>N-W(N1n,K20k)</td>
<td>19.4</td>
<td>8.25</td>
</tr>
</tbody>
</table>

The IC50 values of proteins and peptides were determined by fitting the data shown in Figures 6 and 7 to the Hill equation. Each estimated IC50 value was expressed in two units: mg/kg body weight and µmol/kg body weight. The r value and χ2 value for each compound obtained in the non-linear regression analysis were as follows: cytochrome c (0.970, 2.774); lysozyme (0.878, 4.461); N-WASp181-200 (0.933, 6.279); N-W(N1n) (0.885, 8.042); N-W(N1n,I2i,S3s) (0.991, 0.319) and N-W(N1n,K20k) (0.938, 1.787).

### Figure 7. Effect of co-administration of N-WASp181-200 or its peptides with D-amino acid substitutions on arbekacin accumulation. Mice were administered arbekacin alone or in combination with N-WASp181-200 (open circles), N-W(N1n) (filled circles), N-W(N1n,I2i,S3s) (open squares) or N-W(N1n,K20k) (filled triangles). Each point represents the mean ± SE of three mice.

#### Discussion

MRSA, which is a major cause of hospital-acquired infection, has acquired resistance to most clinically available antibiotics. Although vancomycin has been very widely used for the treatment of patients infected with MRSA, many cases of vancomycin-intermediate *S. aureus* (VISA) infections have been reported since the first report of VISA with a vancomycin MIC of 8 mg/L. Subsequently, some strains of vancomycin-resistant *S. aureus* with MICs of more than 32 mg/L have been isolated. Arbekacin is a broad-spectrum aminoglycoside developed in Japan and has been shown to be effective against most isolates of MRSA. The bactericidal activity of arbekacin against MRSA has been reported to be greater than that of vancomycin. Therefore, arbekacin has attracted attention as a therapeutic drug for the treatment of patients infected with MRSA, but nephrotoxicity tends to be a dose-limiting factor in arbekacin therapy, similar to other aminoglycosides such as gentamicin and amikacin. Therefore, therapeutic drug monitoring is often performed to achieve the optimal serum concentration of arbekacin as well as other aminoglycosides, but not enough studies on prevention of arbekacin-induced nephrotoxicity have been performed. In this study, the renal uptake of arbekacin was characterized in detail from the point of view of attempts to decrease renal accumulation of arbekacin.

Nephrotoxicity of aminoglycosides is considered to be closely associated with their accumulation in the kidney. While nephrotoxicity is the most common adverse reaction during administration of aminoglycosides, Giuliano et al. reported that kinetics for renal uptake differ among aminoglycosides. According to their report, the renal accumulation of gentamicin and netilmicin increased non-linearly with increasing steady-state serum concentrations. In contrast, the renal uptake of tobramycin was linearly related to elevations in serum concentrations. In the case of amikacin, a biphasic pattern was observed: saturation kinetics at low serum concentrations and a linear pattern at high serum concentrations. Such differences in renal uptake kinetics among these aminoglycosides may explain the influence of dosage schedules on renal accumulation of aminoglycosides. Continuous infusion of gentamicin in rats resulted in much higher renal concentrations compared with single injection in rats at the same daily dose, whereas renal concentrations of tobramycin were not affected by the dosage regimen. In humans, a single dose of amikacin (15 mg/kg) resulted in significantly lower renal concentrations than continuous infusion and twice-daily dosing, whereas renal concentrations of tobramycin were independent of dosage regimens. In the present study, we observed that renal uptake of arbekacin was a saturable process with an apparent K_m value of 4.86 mg/kg body weight. As expected from the result, a single injection resulted in lower renal concentrations of arbekacin than repeated administrations of the same total dose. Therefore, these findings suggest that administration of arbekacin by once-daily dosing may be less nephrotoxic than the multiple daily dosing. Furthermore, considering that arbekacin shows concentration-dependent bactericidal activity and a post-antibiotic effect against MRSA, once-daily dosing may be preferred to increase the bactericidal activity against MRSA and to decrease the renal accumulation and subsequent nephrotoxicity of arbekacin, as well as other aminoglycosides.

Our previous study showed that co-administration of cytochrome c, a low-molecular weight protein, with gentamicin decreased not only renal accumulation of gentamicin but also gentamicin-induced nephrotoxicity. In this study, renal accumulation of arbekacin was shown to be inhibited in a dose-dependent manner by co-administration of cytochrome c. Lysozyme, another low-molecular weight protein, also decreased renal accumulation of arbekacin, but the inhibitory potency of lysozyme was much weaker than that of cytochrome c. In our previous *in vitro* binding study, lysozyme inhibited the binding...
of gentamicin to isolated rat renal brush-border membrane to nearly the same extent as cytochrome c.8 Since it has been reported that renal uptake rates of lysozyme and cytochrome c after intravenous injection were similar,29 it is likely that there is little difference in concentration–time profiles of these proteins in renal proximal tubular fluid after a bolus injection. Therefore, cytochrome c may efficiently decrease renal accumulation of arbekacin under in vivo conditions, not only by inhibiting binding to the receptors expressed in the apical membrane of renal proximal tubular cells but also by modulating the subsequent internalization. However, further investigation will be required to clarify the differences in the in vivo inhibitory potencies.

In this study, co-administration of N-WASP181-200 inhibited arbekacin accumulation in the kidney, consistent with our previous finding that N-WASP181-200 inhibited renal accumulation of gentamicin.9 In previous in vitro binding studies, the IC50 value of N-WASP181-200 for gentamicin binding to renal brush-border membrane (0.041 mM) was much lower than that of cytochrome c (3.20 mM).8 However, in the present in vivo uptake studies, the IC50 value of N-WASP181-200 (19.6 μmol/kg) was found to be higher than that of cytochrome c (1.23 μmol/kg). One of the main reasons for the apparent inconsistency between in vitro and in vivo studies may be the rapid degradation by proteases of N-WASP181-200 in plasma under in vivo conditions. Therefore, here we investigated the inhibitory effects of analogues with α-amino acids on the N- and/or C-termini of N-WASP181-200 on renal accumulation of arbekacin since such α-amino acid substitutions have been reported to make peptides resistant to proteolysis.11–13 As a result, three α-amino acid-substituted analogues tested in this study, N-W(N1n), N-W(N1n,I2i,S3s) and N-W(N1n,K20k), were found to be more potent inhibitors than N-WASP181-200. Therefore, partial α-amino acid substitution may be a potential approach for decreasing the dose of peptidic inhibitors necessary to modulate the renal accumulation of aminoglycosides.

In conclusion, the present study suggests that arbekacin is taken up in the kidney in a saturable manner, resulting in lower accumulation of arbekacin in a single injection than that in repeated injections of the same daily dose. In addition, basic peptides partially substituted with α-amino acid(s) may be a potent protectant against aminoglycoside-induced nephrotoxicity.

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Transparency declarations
None to declare.

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