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Efficacy of High-Dose Meropenem (6 g/day) in the Treatment of Experimental Murine Pneumonia Induced by Meropenem-resistant Pseudomonas aeruginosa

Running title: High-dose Meropenem for Murine Pneumonia

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ABSTRACT

High-dose meropenem (MEPM; 6 g/day) has been approved as a treatment for purulent meningitis; however, little is known regarding its in vivo efficacy in refractory lower respiratory tract infection. The purpose of this study was to evaluate the efficacy of 6 g/day MEPM in a murine model of severe pneumonia caused by MEPM-resistant Pseudomonas aeruginosa. Experimental pneumonia induced by MEPM-resistant P. aeruginosa was treated with normal-dose MEPM (150 mg/kg, simulating 3 g/day regimen in humans) or high-dose MEPM (500 mg/kg, simulating 6 g/day regimen in humans). Mice treated with high-dose MEPM showed significantly restored survival compared to that of untreated mice, and tended to show a higher survival rate compared to that of mice treated with normal-dose MEPM. The viable bacteria counts in the lungs significantly decreased in mice treated with high-dose MEPM compared with that of untreated mice ($P < 0.001$) and mice treated with normal-dose MEPM ($P < 0.01$ and $P < 0.05$). The number of inflammatory cells in the BALF was also significantly lower in mice treated with high-dose MEPM than in untreated mice. The free MEPM concentration in the epithelial lining fluid (ELF) exceeded 16 $\mu$g/mL for 85 min in mice treated with high-dose MEPM, but not in mice treated with normal-dose MEPM. Our results demonstrate that high-dose MEPM (6 g/day) might provide superior protection...
against pneumonia caused by MEPM-resistant strains of *P. aeruginosa* compared to that by the normally administered dose (less than 3 g/day).
Introduction

*Pseudomonas aeruginosa* is one of the major causes of hospital-acquired pneumonia (HAP) and opportunistic infection (1). HAP and bacteremia caused by *P. aeruginosa* can be fatal. A mortality rate of 47% for *P. aeruginosa* pneumonia patients has been reported. Mortality as a result of *P. aeruginosa* pneumonia is associated with a delay in initiating effective antimicrobial therapy and multidrug-resistant *P. aeruginosa* (2,3).

MEPM is an antimicrobial drug that is potent against *P. aeruginosa*; however, the incidence of multidrug-resistant *P. aeruginosa* (MDRP) has increased recently (4), and *P. aeruginosa* is the most common multidrug-resistant bacterial cause of hospital-acquired pneumonia and ventilator-associated pneumonia (1). Approximately 13% of all healthcare-associated *P. aeruginosa* infections in the United States are caused by MDRP (5). Although MDRP is responsible for only 0.6% of *P. aeruginosa* infections detected in Japan, MEPM-resistant *P. aeruginosa* and imipenem-resistant *P. aeruginosa* are responsible for 13.8% and 16.3% of *P. aeruginosa* infections, respectively, indicating an increase in the rate of infection by carbapenem-resistant *P. aeruginosa* (6). Carbapenem-resistant *P. aeruginosa* infections generally have a poorer prognosis than carbapenem-sensitive *P. aeruginosa* infections do (7). In addition, the efficacy of colistin against carbapenem-resistant *P. aeruginosa* infections has not been established
Appropriate use of antimicrobial drugs is required to control the emergence of drug-resistant bacterial pathogens. Pharmacokinetics (PK) parameters, such as time above MIC (TAM), $C_{\text{max}}$/MIC, and AUC/MIC, are used to predict the effectiveness of antimicrobial drugs. However, PK parameters used as efficacy indicators differ among antimicrobial drugs. TAM is an efficacy indicator for carbapenems, including MEPM. Because the mutant prevention concentration and mutant selection window influence the emergence of drug-resistant bacterial pathogens, high doses of antibiotics can minimize the emergence of drug-resistant bacterial pathogens. In addition, high doses of antibiotics can lead to better outcomes compared to that observed with normal doses of antibiotics.

Several reports have shown the efficacy of high-dose antibacterial drugs against severe pneumonia. The efficacy of 3 g/day sulbactam/ampicillin for 4 days on intermediate-to-severe community-acquired pneumonia has been reported. High-dose tigecycline and colistin are effective against pneumonia caused by carbapenem-resistant *Klebsiella pneumoniae* in liver transplant patients. While the indications of high-dose MEPM (6 g/day) are currently limited to purulent meningitis and cystic fibrosis (CF), high-dose MEPM (6 g/day) is used for acute exacerbation of CF. In addition, high-dose MEPM (6 g/day) reduces the sputum bacterial burden and
improves clinical status (19). High-dose MEPM (6 g/day) might be effective against pneumonia caused by MEPM-resistant strains because high drug concentrations reach the lungs.

This study was designed to evaluate the efficacy of high-dose MEPM (6 g/day) in comparison to that of normal-dose MEPM as a treatment for severe pneumonia caused by MEPM-resistant and low susceptibility \( P. aeruginosa \) in mice.

**MATERIALS AND METHODS**

**Bacterial isolates**

Clinical isolates of MEPM-resistant (MIC 16 μg/ml) \( P. aeruginosa \) were utilized in this study. MEPM MIC was determined by broth microdilution method according to CLSI guidelines. Isolate stored in trypticase soy broth with 10% glycerol stocks maintained at -80°C at Nagasaki University Hospital was spread on LB agar (SIGMA, Tokyo) and incubated overnight at 37°C under 5% \( CO_2 \) prior to use in the experiments. The mechanism of MEPM resistance was not investigated in this study; however, we confirmed that these strains did not produce metallo-beta-lactamase.
Laboratory animals

Pathogen–free, ddY mice (7 weeks old, female) weighing about 30 g were purchased from SLC Inc., Shizuoka, Japan. All of the animals were housed in a pathogen-free environment and received sterile food and water at laboratory of the Animal Center for Biomedical Science at Nagasaki University (Nagasaki, Japan). The Ethics Review Committee for Animal Experimentation at Nagasaki University approved all experimental protocols used in this study.

Pharmacokinetic (PK) studies and determination of dosing regimen

Plasma concentrations of MEPM were measured after intraperitoneal administration of 100 mg/kg MEPM combined with 100 mg/kg cilastatin in mice, and those in humans after intravenous administration of 1 g of MEPM over 30 minutes were obtained from previous data (20,21). The PK parameters were calculated using a two-compartment model with the MULTI program (22). The percent time that free-drug concentrations remained above the MIC (fTAM) was calculated using the PK parameters, protein binding, and MIC. The level of protein binding of MEPM in mice is 10% (23). Because
the plasma protein binding of MEPM in human plasma is very low (2%) (24), total plasma concentrations in humans were used as free-drug concentrations. Table 1 shows the fTAM of the MEPM regimen for humans and mice. The fTAM of the MEPM for humans was calculated when 1 g or 2 g of MEPM was administered to humans for 30 minutes, three times a day. Four-dose intraperitoneal administration of MEPM (10 ml/kg/dose) at 2-hour intervals was chosen to alleviate the pain of the mice. The dose regimens for mice infected with the MEPM-resistant strain to achieve fTAM using human regimens of 3 g/day and 6 g/day were 150 mg/kg×4/day and 500 mg/kg×4/day, respectively (Table 1). The half-life of meropenem/cilastatin in mice is short (12 minutes) and plasma concentrations of meropenem/cilastatin at 2 h after administration were less than 1/500 compared to that at 5 minutes after administration. Therefore, the PK of the first dose of meropenem was equal to that of other doses of meropenem, and TAM in the regimen with 2-hour intervals was the same as that in the regimen with 6-hour intervals.

**Murine models of pneumonia caused by *P. aeruginosa***

*P. aeruginosa* was cultured on LB agar and incubated overnight at 37°C under 5% CO₂, and the organisms were suspended in normal saline. For the pneumonia with
bacteremia study, 20 µl of the suspended MEPM-resistant strain (clinical isolate 1: $3 \times 10^7$ CFU and clinical isolate 2: $1 \times 10^8$ CFU) was inoculated intranasally with anesthesia. For the pneumonia study, 20 µl of the suspended MEPM-resistant strain (clinical isolate 1: $3 \times 10^6$ CFU and clinical isolate 2: $1 \times 10^7$ CFU) was inoculated intranasally with anesthesia. MEPM was administered intraperitoneally 3 h after inoculation in the pneumonia with bacteremia model and 14 h after inoculation in the pneumonia model. MEPM at 150 mg/kg and 500 mg/kg was administered 4 times/day at 2-hour intervals in combination with 100 mg/kg cilastatin to yield PK similar to that in humans (3 g/day and 6 g/day, respectively). The treatment lasted for 2 days and 1 day in the pneumonia with bacteremia model and in the pneumonia model, respectively. The pneumonia with bacteremia model was used to evaluate the survival rate and the viable bacterial counts in blood, whereas the pneumonia model was used to evaluate the viable bacterial counts in the lungs, the number of inflammatory cells in BALF, free drug concentrations of MEPM in epithelial lining fluid (ELF), and for histopathological analysis of the lungs.

**Lung preparation for CFU determination and histopathological analysis**
Whole lungs were removed under aseptic conditions and homogenized in 1.0 ml phosphate-buffered saline (PBS). *P. aeruginosa* was quantified by placing serial dilutions of the lung homogenates onto LB agar plates and incubating them at 37°C in a 5% CO₂ atmosphere. For histopathological analysis, lung specimens were fixed in 10% formalin-buffered solution, and then the lung tissue sections were paraffin embedded and stained with hematoxylin and eosin (HE) using standard procedures (25).

**Bronchoalveolar lavage fluid (BALF) cell analysis**

BAL analysis was performed with different mice from the mice used for CFU determination and histopathological analysis to assess inflammatory cell accumulation in the airspace. The chest was opened to expose the lungs after the mice were anesthetized, and a disposable sterile feeding tube (Toray Medical Co., Chiba, Japan) was inserted into the trachea. BAL was performed using 1.0 ml of PBS, and the recovered fluid was pooled for each mouse. Total cell counts were performed by Turk staining with a hemacytometer (25,26).

**Measurement of MEPM concentrations in ELF**

BALF samples were mixed with 4 volumes of methanol, vortex mixed, and
centrifuged at 10,000 g for 10 min at 4°C. The supernatants were stored at -80°C until the measurement of MEPM concentrations by HPLC. The supernatants (50 μl) were separated on a Xterra MS C₁₈ reverse phase column (3.5 μm, 4.6 × 20 mm; Nihon Waters K.K., Tokyo, Japan) with methanol-5 mM sodium dihydrogenphosphate (pH 7.0) (3:17) as the mobile phase delivered at 1.0 ml/min. The HPLC system (LC-2010C; Shimadzu Co., Kyoto, Japan) was controlled by a CLASS-VP workstation (Shimadzu), and the wavelength for MEPM detection was 300 nm. Five-point standard curves (0.1–10 μg/ml) were linear with r² > 0.98. The lower limit of quantitation was 0.1 μg/ml. The MEPM concentrations in ELF were calculated using the following formula:

concentration in ELF = concentration in BALF × (urea in serum/urea in BALF). The fTAM in ELF was calculated as described above. Serum samples were also collected just before BAL was obtained from the same mice used for the urea assay.

Urea assay

The rate of decline of NADH levels induced by NH₃ in the samples was measured. Urea was hydrolyzed by urease to produce NH₃. The produced NH₃ reacted with α-ketoisohexanoic acid and NADH by the action of leucine dehydrogenase to form leucine and NAD. The rate of decline of NADH levels at this point was measured
optically, and the urea content in the sample was calculated by subtracting the rate of

decline resulting from the endogenous ammonia reaction.

Statistical analysis

All data were analyzed by using Prism 5 GraphPad Software and expressed as the
mean ± standard error of the mean (SEMs). Survival analysis was performed using the
log-rank test, and the survival rate was calculated by the Kaplan-Meier method.

Differences between groups were examined using the Kruskal-Wallis test and Dunn’s
Multiple Comparison Test. P < 0.05 was considered to indicate a statistically significant
difference.

RESULTS

High-dose MEPM treatment protects mice from pneumonia induced by
MEPM-resistant P. aeruginosa

Survival of the mice was observed for 7 days after infection. As shown in Fig. 1, the
survival of mice treated with high-dose MEPM was significantly restored compared
with that of untreated mice in the MEPM-resistant strain-induced pneumonia and
bacteremia model. In addition, the survival of mice treated with high-dose MEPM was
higher than that of mice treated with normal-dose MEPM; however, no significant
difference was observed.

**Superior bactericidal activity of high-dose MEPM compared to that of the normal
dose in the blood and lungs**

The viable bacteria counts in blood were evaluated 4 h after infection (1 h after the
first dose of MEPM) in the MEPM-resistant strain-induced pneumonia and bacteremia
model (Fig. 2A,C). The viable bacteria counts in blood significantly decreased in the
500 mg/kg × 4/day group compared to those in both the untreated and 150 mg/kg ×
4/day groups [clinical isolate 1: 500 mg/kg × 4/day group vs. untreated group = (1.72 ±
0.12 vs. 4.37 ± 0.17) log cfu/ml, $P < 0.001$, clinical isolate 2: 500 mg/kg × 4/day group
vs. untreated group = (1.97 ± 0.23 vs. 4.23 ± 0.14) log cfu/ml, $P < 0.001$] and [clinical
isolate 1: 500 mg/kg × 4/day group vs. 150 mg/kg × 4/day group = (1.72 ± 0.12 vs 3.27
± 0.32) log cfu/ml, $P < 0.05$]. However, there was no significant difference between the
untreated and the 150 mg/kg × 4/day groups. The viable bacteria counts in the lungs
were evaluated 36 h after infection (24 h after 1st dose of MEPM) in the pneumonia
model (Fig. 2B,D). The viable bacteria counts in the lungs significantly decreased in the
500 mg/kg × 4/day group compared to those in both the untreated and 150 mg/kg ×
4/day groups [clinical isolate 1: 500 mg/kg × 4/day group vs. untreated group =

(2.61±0.33 vs. 5.11 ± 0.30) log cfu/ml, $P < 0.001$, clinical isolate 2: 500 mg/kg × 4/day
group vs. untreated group = (3.56±0.15 vs. 5.28 ± 0.19) log cfu/ml, $P < 0.001$] and

[clinical isolate 1: 500 mg/kg × 4/day group vs. 150mg/kg × 4/day group = (2.61 ± 0.33
vs 4.28 ± 0.31) log cfu/ml, $P < 0.01$, clinical isolate 2: 500 mg/kg × 4/day group vs.
150mg/kg × 4/day group = (3.56± 0.15 vs. 4.41 ± 0.15) log cfu/ml, $P < 0.05$]. However,

there was no significant difference between the untreated and the 150 mg/kg × 4/day
groups.

High-dose MEPM treatment inhibits the pulmonary inflammation induced by

MEPM-resistant *P. aeruginosa*

Lung quantitative cultures and BALF granulocyte counts over time in the control group
(Fig. 3A,B) was evaluated. The number of bacteria in the lungs and BALF granulocytes
at the evaluation point (38 h after infection) were higher than the numbers at the
previous 2 time points (6 and 14 h after infection). The number of inflammatory cells in
the BALF (Fig. 3C) was evaluated 38 h after infection in the pneumonia model. The
number of inflammatory cells in the BALF significantly decreased in the 500 mg/kg ×
4/day group compared to that in the untreated group [500 mg/kg × 4/day group vs.
untreated group = (5.44 ± 0.11 vs. 6.13 ± 0.13) log cells/ml, P < 0.01]. However, there was no difference between the 150 mg/kg × 4/day and untreated groups.

Histopathological examination

As shown in Fig 4, histopathological analysis of the lungs stained with HE at 38 h after infection revealed that the 500 mg/kg × 4/day treatment was more effective than the 150 mg/kg × 4/day treatment was.

The kinetics of free drug concentrations in the ELF of mice administered high-dose MEPM

Free drug concentrations of MEPM in ELF were evaluated in both infected and uninfected mice and were found to not exceed 16 μg/ml even when a dose of 500 mg/kg was administered to uninfected mice (Fig. 4A). Conversely, in infected mice, the free drug concentrations exceeded 16 μg/ml for 85 min after administration at 500 mg/kg; however, it never exceeded 16 μg/ml after administration of MEPM at 150 mg/kg. The fTAMs of infected mice in the 500 mg/kg × 4/day and 150 mg/kg × 4/day groups were 23.6% and 0%, respectively (Fig. 4B). These data suggest that increased penetration in the airway by high-dose MEPM might underlie the protection against MEPM-resistant P.
DISCUSSION

The efficacy of β-lactam drugs, including MEPM, is generally predicted by comparison between MIC for causative bacteria and unbound drug concentration in the extracellular fluid. The efficacy of MEPM can be discussed by considering the unbound drug concentration in the extracellular fluid (intracellular substance) as the total plasma concentration because the plasma protein binding rate of MEPM in humans is as low as 2%. In contrast, the plasma protein binding rate of MEPM is as high as 10% in mice; thus, the efficacy of MEPM should be discussed by taking this difference into consideration. In addition, a number of reports have suggested that the antibacterial drug concentration in the topical infected site reflects the efficacy of the drug. Although the topical infected site is indicative of intracellular substances in the lungs in pneumonia because of the presence of extracellular respiratory tract pathogens, including *P. aeruginosa*, bacteria can exist on the alveolar surface as well. Thus, the drug concentration in the ELF is also an important factor that needs to be considered when discussing therapeutic efficacy (27-29). Hence, we measured the changes in MEPM concentration in ELF over time to evaluate its association with therapeutic efficacy.
A non-infection mouse model and a mouse model of *P. aeruginosa* infection were used to measure the MEPM concentration in the ELF. Because the migration of MEPM into the ELF was found to be poor in the non-infection model, the unbound MEPM concentration in ELF did not exceed 16 μg/ml in both the 150 mg/kg × 4/day group and 500 mg/kg × 4/day group. In the *P. aeruginosa* infection model, however, the unbound MEPM concentration in the ELF did not exceed 16 μg/ml in the 150 mg/kg × 4/day group and fTAM was 0%, whereas fTAM was 23.6% in the 500 mg/kg × 4/day group. Carbapenems, including MEPM, have been reported to exert a bacteriostatic effect at TAM of 20% to 30% and an antimicrobial effect at TAM of 40% to 50% (30,31). The TAM in the 500 mg/kg × 4/day group was 23.6%, supporting the following effects of MEPM in the 500 mg/kg × 4/day group: trend toward improved survival rate, significant reduction of viable bacteria counts in the lungs, and improvement of inflammatory cell infiltration in the lungs in comparison with that in BALF and as observed by pathological images. In this study, a trend toward improved survival rate and reduction of viable bacteria counts in the lungs was observed in the 150 mg/kg × 4/day group compared to those observed in the untreated group. The following factors may have potentially contributed to the reduction of viable bacteria counts in the lungs and improvement of survival rate: the plasma MEPM concentration achieved a TAM of...
17.2%, a certain antimicrobial effect of MEPM was obtained at the sub-MIC level (32), and MEPM itself enhanced phagocytosis of bacteria by macrophages (33). The results of this study indicate that high-dose MEPM (6 g/day) was more effective against pneumonia caused by MEPM-resistant *P. aeruginosa*. *P. aeruginosa* is a typical causative bacteria in HAP and healthcare-associated pneumonia with high mortality (1). Because delayed administration of effective antimicrobial therapy and failure of initial therapy result in poor prognosis, clinicians should select antibacterial drugs and initiate therapy without waiting for drug sensitivity test results for treatment of patients suspected to have *P. aeruginosa* pneumonia. Carbapenem antibiotics, including MEPM, are first-line drugs for treatment of *P. aeruginosa* pneumonia. In this study, the drug concentration at the topical infection site (both plasma and ELF) of MEPM-resistant *P. aeruginosa* (MIC of MEPM = 16 μg/ml) was unsatisfactory on administration of normal-dose MEPM (3 g/day), and this may have potentially led to treatment failure. However, high-dose MPEM (6 g/day) achieved TAM (both plasma and ELF), which enabled a sufficient treatment effect against MEPM-resistant *P. aeruginosa* (MIC of MEPM =16 μg/ml). The MIC$_{90}$ of MEPM against *P.aeruginosa* is equal to or less than 16 μg/ml in Japan and the other countries (34-41); thus, according to PK-PD theory, high dose MEPM (6g/day) might be able to achieve a clinical efficacies greater than
90% against *P. aeruginosa*. In clinical practice, high-dose MEPM may be indicated in patients suspected to have severe *P. aeruginosa* pneumonia for whom failure of initial therapy is not permissible. Moreover, high-dose (6 g/day) MEPM may be highly effective in patients with MEPM-sensitive *P. aeruginosa* pneumonia and in immunocompromised patients with neutropenia, lung abscess, cystic fibrosis, or idiopathic pulmonary fibrosis (IPF) that limits drug migration into the topical infected site. Therefore, further validation, including clinical studies, are necessary.

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Figure legends

Figure 1. Survival of mice infected with $3 \times 10^7$ (clinical isolate 1) or $1 \times 10^8$ (clinical
isolate 2) CFU of MEPM-resistant *P. aeruginosa* treated with MEPM at 500 mg/kg or 150 mg/kg, or PBS (untreated group), four times/day (n = 9-10). Statistical differences compared to the untreated group were determined by the Kaplan-Meier log-rank test. *

*P* < 0.05, **P* < 0.01 (versus untreated group).

**Figure 2.** Dose-dependent bactericidal effect of MEPM. Number of viable bacteria in blood (A,C). Mice were inoculated with 3×10^7 (clinical isolate 1) or 1×10^8 (clinical isolate 2) CFU of MEPM-resistant *P. aeruginosa*. At 4 h after infection (1 h after the first dose of MEPM), mice treated with MEPM at 500 mg/kg and 150 mg/kg and untreated mice were compared (A). At 5 h after infection (2 h after the first dose of MEPM), the mice in each group were compared (C). The number of viable bacteria was significantly lower in the 500 mg/kg treatment group (n=9). ***P* < 0.001 (versus untreated group), *P* < 0.05 (versus 150 mg/kg treatment group). Number of viable bacteria in the lungs (B,D). Mice were inoculated with 3×10^6 (clinical isolate 1) and 1×10^7 (clinical isolate 2) CFU of MEPM-resistant *P. aeruginosa*. At 38 h after infection (24 h after 1st dose of MEPM), mice treated with MEPM at 500 mg/kg × 4/day and 150 mg/kg × 4/day and untreated mice were compared. The number of viable bacteria in the lungs was significantly lower in the 500 mg/kg × 4/day treatment group ((B): n=13-15,
Figure 3. Number of inflammatory cells in BALF. Mice were inoculated with $3 \times 10^6$ CFU of MEPM-resistant \textit{P. aeruginosa}. At 38 h after infection (24 h after the first dose of MEPM), mice treated with MEPM at 500 mg/kg × 4/day and 150 mg/kg × 4/day and untreated mice were compared. Lung quantitative cultures and BALF granulocyte counts over time in the control group (A,B). The number of bacteria in the lungs and BALF granulocytes at the evaluation point (38 h after infection) were higher than the numbers at the previous 2 time points (6 and 14 h after infection). The number of inflammatory cells in BALF was significantly lower in the 500 mg/kg × 4/day treatment group (n=7)(C). **$P < 0.01$ (versus untreated group or 6h after infection).

Figure 4. Histopathological analysis of the lungs of mice inoculated with $3 \times 10^6$ CFU of MEPM-resistant \textit{P. aeruginosa} and treated with high-dose MEPM. At 38 hours after infection (24 hour after the first dose of MEPM), mice treated with MEPM at 500 mg/kg × 4/day and 150 mg/kg × 4/day and mice with no treatment were compared. HE-stained tissue sections were observed at magnifications of ×40 and ×200.
No-treatment group (A), 150 mg/kg×4/day group (B), 500 mg/kg×4/day group (C). The inflammation of lungs decreased in a dose-dependent manner. The accumulation of inflammatory cells, hemorrhage in the lungs, and destruction of alveoli were limited in mice treated with MEPM at 500 mg/kg × 4/day.

**Figure 5.** Free drug concentrations of MEPM in ELF at 5, 15, 30, and 60 minutes after 4 doses of MEPM in non-infected mice (A) and mice infected with $3 \times 10^6$ CFU of MEPM-resistant *P. aeruginosa* (B). The free drug concentrations of MEPM in the ELF did not reach 16 μg/ml even when MEPM was administered at 500 mg/kg in non-infected mice (A). The free drug concentrations of MEPM in the ELF were higher than 16 μg/ml for 85 min in the mice treated with 500 mg/kg of MEPM; however, they never exceeded 16 μg/ml in the mice treated with 150 mg/kg of MEPM.
Table 1

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<td>Human</td>
<td>2 g × 3/day</td>
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<td>Human</td>
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PK/PD parameters. fTAM of MEPM dose regimens for humans and mice with pneumonia caused by MEPM-resistant *P. aeruginosa*. *fTAM: the percent time that free-drug concentrations remain above the MIC.*
Figure 1. The survival of mice infected with $3 \times 10^7$ (clinical isolate 1) or $1 \times 10^8$ (clinical isolate 2) CFU of MEPM-resistant *P. aeruginosa* treated with MEPM at 500 mg/kg or 150 mg/kg, or PBS (untreated group), four times/day (n = 9-10). Statistical differences compared to untreated group were determined by the Kaplan-Meier log-rank test. *$P < 0.05$, **$P < 0.01$ (versus untreated group).
Figure 2. Dose-dependent bactericidal effect of MEPM. Number of viable bacteria in blood (A,C). Mice were inoculated with $3 \times 10^7$ (clinical isolate 1) or $1 \times 10^8$ (clinical isolate 2) CFU of MEPM-resistant *P. aeruginosa*. At 4 h after infection (1 h after 1st dose of MEPM), mice treated with MEPM at 500 mg/kg and 150 mg/kg and untreated mice were compared (A,C). The number of viable bacteria was significantly lower in the 500 mg/kg treatment group (n=9). ***$P < 0.001$ (versus untreated group), *$P < 0.05$ (versus 150 mg/kg treatment group).

Number of viable bacteria in the lungs (B,D). Mice were inoculated with $3 \times 10^6$ (clinical isolate 1) and $1 \times 10^7$ (clinical isolate 2) CFU of MEPM-resistant *P. aeruginosa*. At 38 h after infection (24 h after 1st dose of MEPM), mice treated with MEPM at 500 mg/kg x 4/day and 150 mg/kg x 4/day and untreated mice were compared. The number of viable bacteria in the lungs was significantly lower in the 500 mg/kg x 4/day treatment group ((B):n=13-15, (D):n=10-11). ***$P < 0.001$ (versus untreated group), **$P < 0.01$, *$P < 0.05$ (versus 150 mg/kg treatment group).
Figure 3. The number of inflammatory cells in BALF. Mice were inoculated with $3 \times 10^6$ CFU of MEPM-resistant *P. aeruginosa*. At 38 h after infection (24 h after 1st dose of MEPM), mice treated with MEPM at 500 mg/kg $\times$ 4/day and 150 mg/kg $\times$ 4/day and untreated mice were compared. The number of viable bacteria in lung (A) and the number of inflammatory cells in BALF obtained from untreated mice (B) were elevated time-dependently. The number of inflammatory cells in BALF was significantly lower in the 500 mg/kg $\times$ 4/day treatment group (n=7) (C). **$P < 0.01$** (versus untreated group or 6h after infection).
Figure 4. Effect of high-dose MEPM on histopathological analysis in lungs of mice inoculated with $3 \times 10^6$ CFU of MEPM-resistant *P. aeruginosa*. At 38 hours after infection (24 hour after 1st dose of MEPM), mice treated with MEPM at 500mg/kg × 4/day and 150mg/kg × 4/day and no treatment mice were compared. HE stained tissue sections were at magnifications of ×40 and ×200. No treatment group (A), 150mg/kg × 4/day group (B), 500mg/kg × 4/day group (C). The inflammation of lungs becomes mild by dose-dependent. The accumulation of inflammatory cells, hemorrhage in lungs and destruction of alveoli are limited in the mice treated with MEPM at 500mg/kg × 4/day.
Figure 5. Free drug concentrations of MEPM in ELF at 5, 15, 30, and 60 minutes after 4 doses of MEPM for non-infected mice (A), and mice infected with $3 \times 10^6$ CFU of MEPM-resistant *P. aeruginosa* (B). Free drug concentrations of MEPM in ELF could not reach 16 μg/ml even at 500 mg/kg of MEPM in non-infected mice (A). Free drug concentrations of MEPM in ELF were more than 16 μg/ml for 85 min in the mice treated with 500 mg/kg of MEPM; however, it never exceeded 16 μg/ml in the mice treated with 150 mg/kg of MEPM.