Efficacy of colistin combination therapy in a mouse model of pneumonia caused by multidrug-resistant Pseudomonas aeruginosa

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Received 7 September 2008; returned 18 October 2008; revised 8 December 2008; accepted 10 December 2008

Objectives: Multidrug-resistant Pseudomonas aeruginosa (MDRP) is becoming a serious problem in hospitals, especially in patients on ventilators. Recent data demonstrate that colistin may be effective for these patients, although limited in vitro and in vivo data are available. Our aim was to identify further characteristics of colistin for the therapy of pneumonia caused by MDRP.

Methods: The effects of colistin on clinical strains of MDRP were examined by susceptibility test, time–kill assay, lipopolysaccharide (LPS)-blocking assay and a mouse pneumonia model, alone or in combination with other antibiotics. For the pneumonia model, mice were intranasally infected with bacteria and kept in hyperoxic conditions to mimic ventilator-associated pneumonia.

Results: As a single agent, colistin exhibited the strongest activity of the antimicrobial agents tested. In combination, maximum synergy was observed with colistin plus rifampicin. As expected, co-incubation of bacterial culture supernatants with colistin significantly reduced LPS activities with an associated decrease in cellular cytotoxicity. In the pneumonia model, intranasal, but not intravenous, colistin combined with rifampicin produced maximum survival protection. Pharmacokinetic analysis of colistin demonstrated the superiority of intranasal administration, judging from the compartmentalized high concentration and the long half-life in the lungs. Moreover, colistin therapy significantly decreased both production of inflammatory cytokines and LPS activity, even at a dose effecting no change in the bacterial burden in the lung.

Conclusions: These data strongly suggest that colistin may be an important option for combination therapy against critical MDRP infections. For pneumonia especially, intranasal colistin with rifampicin may be beneficial not only for synergistic antibacterial activity, but also for blocking LPS.

Keywords: rifampicin, lipopolysaccharide, pharmacokinetics, cytokines

Introduction

Pseudomonas aeruginosa is a leading cause of life-threatening infectious disease, such as ventilator-associated pneumonia (VAP), and is associated with a high mortality rate.1,2 The estimated prevalence of VAP within the intensive care unit setting ranges from 5% to 67% and the mortality rates are 24% to 76%, depending on the study population.3,4 It is also well known that P. aeruginosa is intrinsically resistant to a variety of antibiotics, and moreover tends to acquire resistance during and after antimicrobial treatment.5,6 Emergence and spread of multidrug-resistant P. aeruginosa (MDRP) has become a serious problem worldwide. Against this backdrop, there is renewed interest in one of the original anti-pseudomonal antimicrobials, colistin.7–9

Colistin is an old antimicrobial belonging to the polymyxin family. It acts primarily on the cell wall of Gram-negative bacteria, causing rapid changes in the permeability of the cytoplasmic membrane and, ultimately, cell death.8 The use of colistin as parenteral therapy has been limited because of concerns...
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about adverse reactions, such as nephrotoxicity and neurotoxicity. Recently, investigators have reported that this drug is highly active against antibiotic-resistant organisms, including MDRP and *Acinetobacter* spp. In addition, colistin also has unique anti-endotoxin activity, probably through binding and neutralizing of the bacterial cell wall component lipopolysaccharide (LPS), which may provide potential benefits in clinical conditions. Recently, aerosolized colistin has been successfully used in combination with other antibiotics for treatment of multidrug-resistant organisms. The efficacy of colistin may be due to multiple factors including synergistic combination with other antimicrobials, pharmacological characteristics and LPS-neutralizing activity.

The aim of this study was to examine the efficacy and mechanisms of colistin and colistin combinations against MDRP pulmonary infection. Several experiments, including MIC determination, time–kill study, determination of LPS-blocking activity and a hyperoxic mice pneumonia model (mimicking the conditions of VAP), were performed. The data suggest a promising activity of colistin against MDRP infections, especially when this agent is locally administered with certain antibiotics.

**Materials and methods**

**Bacterial strains used**

We used the *P. aeruginosa* PAO1 strain (a gift from Barbara H. Iglewski, University of Rochester, Rochester, USA) and six strains of clinical isolates, including five strains of MDRP, collected between February 2006 and March 2007 from different hospitals in Japan. These clinical isolates were resistant to several anti-pseudomonal antibiotics, such as ceftazidime, imipenem, amikacin or ciprofloxacin. For these clinical isolates were resistant to several anti-pseudomonal antibiotics, such as ceftazidime, imipenem, amikacin or ciprofloxacin. For bioassay of colistin concentrations, *Escherichia coli* 7437 was used as an indicator (a gift from Shionogi & Co., Ltd, Osaka, Japan).

**Antibiotics**

Imipenem and amikacin were obtained from Banyu Pharmaceutical Co. (Tokyo, Japan) and ceftazidime was obtained from GlaxoSmithKline (Tokyo, Japan). Imipenem/cilastatin was used for the *in vivo* experiments. Ciprofloxacin was obtained from Bayer Yakuhin Ltd (Osaka, Japan). Tobramycin was obtained from Shionogi & Co., Ltd. Piperacillin was obtained from Toyama Chemical Co., Ltd (Toyama, Japan). Rifampicin and colistin sulphate were obtained from Sigma-Aldrich S. r. l. (Tokyo, Japan). In the animal model, the dosages of colistimethate were expressed as colistin base activity (1 mg/kg colistin base activity is equivalent to 2.66 mg/kg colistimethate). Powders were dissolved in accordance with the manufacturer’s recommendations.

**Susceptibility test and synergy studies**

Antibacterial activities of colistin, imipenem, rifampicin, amikacin and ciprofloxacin were examined alone or in combination. Susceptibility testing was performed by a microbroth dilution method according to the recommendations of the CLSI. Combination effects of antibiotics were investigated using checkerboard titration methods in 96-well microplates. The fractional inhibitory concentration index (FICI) was interpreted as follows: FICI ≤ 0.5, synergistic; 0.5 < FICI ≤ 4, indifferent; and FICI > 4, antagonistic. *P. aeruginosa* ATCC 27853 was used as a quality control strain. *In vitro* bactericidal activity was also evaluated with time–kill synergy studies. Growth-phase cultures in cation-adjusted Mueller–Hinton broth were used for this experiment. Each tube contained bacteria at an inoculum of 5 × 10^5 CFU/mL, with serial concentrations of antimicrobial agents. At the selected timepoints, aliquots obtained from tubes were inoculated on Mueller–Hinton agar for colony counts after serial 1:10 dilutions.

**LPS assay**

LPS, an important virulence factor associated with endotoxin shock, was measured using an LPS-specific chromogenic *Limulus* test (Endospecty test; Seikagaku Co., Tokyo, Japan) as described previously. Sterile, LPS-free specimen containers and pipette tips were used for the LPS assay. Samples were centrifuged at 5000 g for 15 min and filtered through an LPS-free filter (EB-DISK 25, 0.2 μm; Kanto Chemical Co., Inc., Tokyo, Japan) in order to remove LPS associated with bacteria. The supernatants were used for the assay.

**Cell viability assay**

The human lung alveolar epithelial cell line A549 was obtained from ATCC, and the cells were seeded in the wells (2–3 × 10^5 cells/well) of a 96-well plate. The cells were incubated in a chamber containing 5% CO₂ or 5% CO₂ plus 90% O₂ (ASTEC, Ltd, ACL-165D/ACM-165D, Japan) at 37°C for 48 h, with or without 10% bacterial culture supernatants. Cell viability was examined using the MTT assay using TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan).

**Animal models**

BALB/c mice (female, 6 weeks old) were purchased from Charles River Japan. Mice were quarantined for 1 week after reception. They were housed in separate cages under constant temperature (23°C) with a 12 h light/dark cycle and given standard laboratory food and water *ad libitum*. All animal experiments were performed under the approval of the animal centre of Toho University (approval number: #169). Mice were anaesthetized intramuscularly with 50 mg/kg ketamine and 10 mg/kg xylazine, and then 30 μL of bacterial suspension was administered intranasally. Two inoculum doses were used in this model. The higher dose was 2–2.5 × 10^6 CFU/mouse, whereas the lower inoculum size was 1–1.5 × 10^6 CFU/mouse. For hyperoxic exposure, mice were kept in hyperoxic conditions for the required duration in an airtight chamber. The oxygen concentration in the chamber was maintained by a constant flow of oxygen, which was monitored with an in-line oxygen analyser (model D2; Beckman, Fullerton, CA, USA). Mice were divided into six or seven groups according to the therapy regimen, and each group contained 14–16 mice (six of each group were randomly chosen for analysis of lung bacterial burden). Therapies were initiated 2 h after induction of pneumonia. Total daily doses of imipenem and colistimethate were divided into two doses and administered every 12 h, except for rifampicin, which was administered as a once-daily dose regimen. Imipenem (60 mg/kg) was injected subcutaneously, and rifampicin (25 mg/kg) was orally administered. Pharmacokinetic data of imipenem and rifampicin have been described previously. Colistimethate was administered subcutaneously (20 mg/kg) or intranasally (10 mg/kg). Treatments or placebo were continued until 48 h after inoculation, and the survival was monitored twice a day.
In some experiments, the lungs were removed and homogenized for analysis. At designated timepoints, mice were sacrificed by CO₂ asphyxiation. After removal, whole lungs were homogenized in 1 mL of saline using tissue homogenizer (Omni International). Homogenates (10 μL) were inoculated on Mueller–Hinton agar after serial 1:10 dilutions. The remaining homogenates were centrifuged at 1000 g for 15 min. The supernatants were used for cytokine and LPS assays.

**Pharmacokinetics of colistimethate**

Colistimethate (250 mg/kg) was intravenously administered to one group of mice. A second group received colistimethate (25 mg/kg) intranasally. After the indicated timepoints (5, 10, 30, 60 and 120 min), the mice were sacrificed by CO₂ asphyxiation, and the blood samples and the entire lungs were taken. The serum was separated from the blood by centrifugation. The lung was homogenized and centrifuged at 1000 g for 15 min for preparation of the supernatant. Colistimethate concentrations in the serum and the lung were determined by a bioassay using *E. coli* 7437, as reported previously. Pharmacokinetic parameters were calculated with the non-linear least-squares regression program MULTI. The area under the concentration–time curve (AUC), the mean residence time (MRT) and the elimination half-life (t1/2) were calculated by means of moment analysis.

**Cytokine assay**

The levels of cytokines in the lung homogenates were determined using an ELISA Kit (Duo Set, ELISA Development System; R&D Systems), according to the manufacturer’s instructions.

**Statistical analysis**

All bacterial counts and cytokine and LPS assay data are presented as means ± SD. Statistical significance was determined using the unpaired, two-tailed t-test. Survival curves were constructed by the Kaplan–Meier method, and were analysed by log-rank tests. For all tests, differences were considered to be statistically significant when P values were <0.05.

**Results**

**Susceptibility test and synergy study**

As a single agent, colistin demonstrated the strongest activity against the *P. aeruginosa* examined (MIC range 0.5–2 mg/L) (Table 1). Although these clinical isolates were resistant to three or more agents examined, there were no strains resistant to colistin, according to the CLSI breakpoint (MIC ≤2 mg/L). In clinical cases, colistin is often used in combination therapy and thus we checked the synergic effects with other antibiotics (Table 2). In combination, maximum synergy was observed with colistin plus rifampicin (FICI ranged from 0.375 to 0.5), followed by colistin plus imipenem (FICI ranged from 0.5 to 1). Colistin plus amikacin showed no synergic effect, but was additive or indifferent. The only combination that was synergistic for all the strains examined was colistin plus rifampicin.

**Time–kill synergy test**

The synergic effect was evaluated in *P. aeruginosa* strain 10 by a time–kill study (Figure 1). We used 1× MIC of colistin plus 1/2× MIC of other drugs, 1× MIC of colistin plus 1/4× MIC of other drugs and 1/2× MIC of colistin plus 1/2× MIC of other drugs. The data for the time–kill synergy test correlated well with those in Table 2. Colistin plus rifampicin or imipenem had striking effects, whereas ciprofloxacin and amikacin had no detectable synergy with colistin. For example, 1/2× MIC of colistin plus 1/2× MIC of rifampicin resulted in no detectable bacteria at 16 h, whereas 1/2× MIC of colistin plus 1/2× MIC of ciprofloxacin or amikacin resulted in bacterial growth equivalent to 1/2× MIC of colistin alone.

**LPS release in time–kill study**

Next, we examined whether sub-MICs of colistin at doses having no effect on bacterial counts (Figure 2a) reduced the amounts of LPS released into the culture supernatants. Sub-MICs of colistin significantly reduced the release of LPS in a concentration-dependent manner (Figure 2b). The amounts of LPS released were <10% of the control when the bacteria were grown in the presence of 1 mg/L colistin, despite no change in the viable bacterial number (88.1 ± 10.0 ng/mL for control versus 8.1 ± 3.2 ng/mL with colistin; P<0.001).

**Cell viability assay**

Next, we examined the effects of colistin on LPS-neutralizing activity in the cell viability assay. An addition of culture supernatant to A549 cells induced changes in cell morphology, such as rounding and detachment. Cell viability decreased to 27.1%...
of the control in the presence of culture supernatant, while cell viability was restored to 49.1% in the supernatants pretreated with 8 mg/L colistin ($P = 0.001$). Although the cytotoxic activity of culture supernatants was exaggerated in the setting of hyperoxia, substantially similar cell viability-protecting effects were observed by pre-incubation with colistin in a dose-dependent fashion. Although the culture supernatants may also include various secreted factors, such as exotoxin A and proteases, these data demonstrated that pre-incubation of supernatants with colistin protected cells from a decrease in viability, probably through colistin’s LPS-neutralizing activity.

**Mouse pneumonia model**

To assess how these characteristics of colistin would affect an *in vivo* model, survival of mice in a VAP model was examined. Considering previous reports using colistimethate for treatment of infections with multidrug-resistant organisms, such as *P. aeruginosa* and *Acinetobacter baumannii*, 14–16, 29–31 10 and 20 mg/kg colistimethate were used in the present study. First, we looked at how hyperoxia exaggerates the lethality of *P. aeruginosa* PAO1 pneumonia in mice. As shown in Figure 3(a), an increase in lethality was observed in a manner that was dependent on the length of time of hyperoxia. No death was observed in either infection in normoxic conditions or without infection in hyperoxia even after 80 h of exposure (data not shown). We also tested several concentrations of O₂, such as 40%, 70% and 80%, and observed an increase in lethality in a manner that was dependent on oxygen concentration (data not shown).

As shown in Figure 3(b), all control and rifampicin-, imipenem- or colistimethate (subcutaneous or intranasal)-treated mice died within 42 h after infection. In contrast, rifampicin and

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ND, not determined.

**Figure 1.** Time–kill curves of *P. aeruginosa* strain 10 for the combinations with colistin. (a) Colistin was tested at concentrations of 0.5× (open triangles), 1× (open circles), 2× (open squares) and 4× MIC (crosses in circles), and saline (open diamonds) was used as a control. (b) Colistin at 1× MIC (open circles) was tested with 0.5× MIC amikacin (filled diamonds), ciprofloxacin (filled triangles), imipenem (filled squares) or rifampicin (filled circles). (c) Colistin at 1× MIC (open circles) was tested with 0.25× MIC amikacin, ciprofloxacin, imipenem or rifampicin. (d) Colistin at 0.5× MIC (open triangles) was tested with 0.5× MIC amikacin, ciprofloxacin, imipenem or rifampicin.
imipenem combined with intranasal colistimethate strikingly increased the survival to 75% and 62.5%, respectively ($P<0.05$). Substantially similar results were observed at a milder challenge dose, in which the control mice died 60–72 h after infection (Figure 3c and d). A clear discrepancy was observed in survival between mice treated with rifampicin + colistimethate (intranasal) and rifampicin + colistimethate (subcutaneous) (100% versus 14%; $P<0.01$). Figure 3(d) also shows the superiority of imipenem + colistimethate (intranasal) over imipenem + colistimethate (subcutaneous). The survival data were well correlated with the bacterial burden in the lungs (Figure 4). We observed substantially similar trends of synergy in mice lungs when strain 6 was used as a challenging organism. These results demonstrated the therapeutic efficacy of intranasal administration of colistimethate in combination with certain antibiotics, such as rifampicin and imipenem.

For further understanding of the efficacy of intranasal administration of colistimethate, we examined the pharmacokinetic profiles of colistimethate in the lungs and serum (Figure 5). Although the doses of colistimethate used were different from those of the animal experiments, the data demonstrate that intranasal colistimethate produces compartmentalized delivery of this

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**Figure 2.** LPS release in time–kill study of PAO1 with sub-MICs of colistin. Chosen concentrations of colistin were added to the tubes containing PAO1 culture at a crude size of $10^6$ cfu/mL. After incubation for 4 h at 37°C with constant shaking, viable bacterial counts and LPS in the culture supernatants were measured. *$P<0.05$, considered statistically significant versus without colistin. **$P<0.01$, versus 0.5 mg/L colistin.

**Figure 3.** Mouse pneumonia model in the setting of hyperoxia. (a) Mice were inoculated with PAO1 ($6 \times 10^8$ cfu/mouse). One group was kept in room air, whereas the other groups were placed in hyperoxic conditions for 40, 60 or 80 h ($n=10$). (b, c and d) Mice were inoculated with *P. aeruginosa* strain 10 [2–2.5 × $10^6$ cfu/mouse in (b) and 1–1.5 × $10^6$ cfu/mouse in (c) and (d)] and kept in hyperoxic conditions (80%) for 48 h. Therapies were initiated 2 h after induction of pneumonia and continued until 48 h after inoculation. Total daily doses of imipenem, rifampicin, subcutaneous (sc) colistimethate or intranasal (in) colistimethate were 60, 25, 20 or 10 mg/kg, respectively. (b) *$P<0.01$ compared with colistimethate or rifampicin monotherapy. **$P<0.05$ compared with colistimethate or imipenem monotherapy. (c) *$P<0.01$ compared with colistimethate monotherapy, rifampicin monotherapy or combination therapy of colistimethate (sc) and rifampicin. (d) *$P<0.01$ compared with colistimethate or imipenem monotherapy.
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![Image](https://via.placeholder.com/150)

**Figure 4.** Advantage of intranasal colistimethate combination therapy regarding bacterial levels in lungs. Mice were inoculated with *P. aeruginosa* strain 10 (2–2.5 × 10⁶ cfu/mouse) and kept in hyperoxic conditions (80%); 2 h after induction of pneumonia, rifampicin (25 mg/kg), colistimethate [subcutaneous (sc)] (10 mg/kg) and colistimethate [intranasal (in)] (5 mg/kg) were administered. After 8 h of administration, mice were sacrificed, and whole lung homogenates were used for assay. *P < 0.05 compared with colistimethate (in), rifampicin or rifampicin plus colistimethate (sc). *P < 0.05 compared with colistimethate (in), imipenem or imipenem plus colistimethate (sc).

**Effects of colistin on cytokine production in lungs**

Next, we examined the effect of colistin on the suppression of cytokine activity in the infected lungs. As shown in Figure 6(a), 0.5 mg/kg of intranasal colistimethate did not alter lung bacterial burdens at 2 and 4 h after inoculation of colistimethate (4 and 6 h after infection), compared with the control. Intranasal administration of colistimethate produced significant reduction in lung interleukin (IL)-6 and IL-1β at the timepoints examined (Figure 6b: *P < 0.05*). The reduction in inflammatory cytokines that was not through an antibacterial effect was evaluated by a further experiment that looked at the effects of supernatants of *P. aeruginosa* PAO1 on the induction of lung inflammation. Supernatants (45 μL aliquots) were inoculated into the lungs, and then various doses of colistimethate were intranasally administered 1 h after inoculation (Figure 7). The bacterial supernatants were prepared by centrifugation (at 5000 g for 15 min) and filter-sterilization of incubated broth cultures of PAO1 incubated for 24 h. Administration of colistimethate significantly reduced lung inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in a dose-dependent manner. Associated with the suppression of cytokines, LPS activities in the lungs were also decreased in a manner that was dependent on the dose of colistimethate (1258.7 ± 55.6 ng/mL with 1.25 mg/kg, 997.6 ± 243.1 ng/mL with 2.5 mg/kg and 706.8 ± 107.6 ng/mL with 5 mg/kg colistin).

**Discussion**

In the present study, we evaluated the efficacy of colistin alone and in combination with other antimicrobials against clinical isolates of MDRP using several different experiments, including MIC determination, FICI determination, time–kill assay, determination of LPS-blocking activity and a mice pneumonia model. Our data are consistent with previous reports indicating that colistin may be one of the options for the therapy of MDRP infections. Furthermore, maximum synergistic effects were achieved when colistin was simultaneously administered with rifampicin in the time–kill assay and VAP model. Finally, the *in vitro* and *in vivo* data suggest that the LPS-blocking activity of colistin may play a role in protecting pulmonary epithelial cells from the cytotoxic effects of *P. aeruginosa* culture supernatants and modulating host cytokine responses in the lungs of mice with MDRP pneumonia.

The MICs of colistin in this study were consistent with those in previous reports.7–9 Gales et al.32 reported from the SENTRY antimicrobial surveillance programme that polymyxin showed excellent potency against 8705 *P. aeruginosa* (MIC₅₀ ≤ 1 mg/L.

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**Pharmacokinetic data in the lungs and serum after colistimethate administration through intravenous or intranasal routes.** Mice were administered with colistimethate through intravenous (250 mg/kg) or intranasal routes (25 mg/kg), and then sacrificed at the indicated timepoints. The concentrations of colistin in the lungs (open squares) and serum (open circles) were determined by a microbiological bioassay as described in the Materials and methods section. *Each pharmacokinetic parameter was determined from time 0 to 120 min, and was expressed in AUC (mg/L-min for serum; μg/g wet wt-min for lungs), MRT (min) or t₁/₂ (min), respectively.

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and MIC$_{90}$ 2 mg/L). Even for carbapenem-resistant _P. aeruginosa_ or MDRP, the polymyxin resistant rates were 2.7% or 3.3%, respectively. In combination, synergy was observed with rifampicin, imipenem and ciprofloxacin in seven, two and one of seven strains examined, respectively. Maximum synergism was produced with colistin + rifampicin, for which an FICI of 0.375 was demonstrated in five of seven strains. Consistent with these MIC and FICI data, the time–kill assay confirmed a clear synergism between colistin and rifampicin. Other investigators have reported synergy with different antibiotic combinations, such as amikacin + aztreonam and meropenem + ciprofloxacin. Although the potential therapeutic cost of colistin + rifampicin against MDRP was reported by several investigators, the response of an infecting organism may be strain-dependent. Since colistin is known to induce damage to cell wall structures of Gram-negative bacteria, it is likely that accelerated penetration of macromolecular rifampicin may explain at least in part the synergy between colistin and rifampicin.

It is known that polypeptide antibiotics, such as colistin and polymyxin B, bind and block the LPS activity of Gram-negative organisms, although the practical significance is less understood. The present data clearly demonstrate that from a concentration of 0.25 mg/L, colistin strongly suppressed LPS activity in a dose-dependent manner. LPS is a cell wall component of Gram-negative bacteria, and released LPS at the site of infection exaggerates host responses, such as inflammation and coagulation, which may be associated with cytokine production, cellular damage and consequently, septic shock. On this point, Dubot et al. showed that polymyxin prevented induction of plasminogen activator by LPS in endothelial cell culture. Importantly, the present study demonstrated that colistin modulated _in vivo_ production of inflammatory cytokines, such as IL-6 and IL-1β, which were well associated with suppression of LPS activities. These results suggest that colistin may be beneficial for hosts, especially for dysregulation of cytokine production and cellular/tissue damage by released LPS. Since neutralization
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of LPS is an attractive therapeutic strategy, it may be necessary to re-evaluate the role and significance of colistin binding and blocking of LPS in combination therapy.

The present data from a hyperoxic mice pneumonia model demonstrated a dramatic survival benefit of intranasal, but not systemic, administration of colistimethate simultaneously with systemic rifampicin or imipenem. The discrepancy in survival rates observed for different routes of colistimethate administration, intranasal or systemic, suggests the importance of pharmacokinetic characteristics of colistimethate. In this regard, Levin et al.40 reported that intravenous colistimethate was less effective against pneumonia (25% cure) than other sites of infection (75% cure). Similarly, using intravenous colistimethate therapy, response rates were 57% to 61% in hospital-acquired pneumonia caused by P. aeruginosa and A. baumannii.41–44 While aerosolized colistimethate showed efficacy of 76% to 100%14–16,31,45 Although there is little information on the pharmacokinetics of colistimethate or colistin at infection sites after intravenous administration of colistimethate, it is suggested that colistimethate is not extensively distributed outside plasma.7–9 In 1973, Craig and Kunit46 reported that the concentration of free colistin in lung homogenates was extremely low in rabbits who received intramuscular administration. Recently, Wootton et al.25 have reported a novel microbiological assay using E. coli as an indicator, in which more accurate and sensitive measurements than the method used previously became available. We have applied this assay to determine and compare the levels of colistin in the lungs and serum of mice after intravenous or intranasal administration. As shown in Figure 5, intranasal delivery was associated with more compartmentalization and a longer half-life of colistin in the lungs, which may be related to the higher efficacy of intranasal colistimethate in the pneumonia model. Although human pharmacokinetics of drugs are different from those of rodents, it is likely that the present data support the superiority of direct administration of colistin. Human pharmacokinetic profiles of colistin in organs and tissues, in addition to comparative combination effects in patients, are warranted for future investigations.

For reasons relating to experimental equipment, we have used the intranasal route, but not inhalation. Inhalation of antibiotics, including colistimethate, is theoretically attractive for the prevention and treatment of hospital-acquired pneumonia.37 Although inhalation may be better than intranasal administration, there is still a concern of delivery of the drug to the sites of infection, especially to areas with less air flow. In adverse reactions, several clinical data suggest that aerosolized administration of colistimethate may be less toxic to kidneys and neurological systems compared with systemic administration.14,16,31,45

Together, our data are in line with previous reports and further support a rationale for aerosolized colistimethate therapy with other antibiotics, such as rifampicin and imipenem, to treat critical MDRP pneumonia including VAP. Further investigations are warranted to confirm the efficacy of colistin combination therapy, not only its synergistic effects with other agents and pulmonary pharmacokinetic/pharmacodynamic profiles, but also the frequency/severity of adverse reactions and clinical roles of LPS-blocking activity.

Acknowledgements

We thank Barbara H. Iglewski for the gift of P. aeruginosa PAO1. We would also like to thank Tse-Hsien Koh for his careful review of this manuscript prior to submission and his valuable comments.

Funding

No specific funding was received for this study.

Transparency declarations

None to declare.

References

8. J Respir Crit Care Med
13. Pristovsek P, Kidric J. Solution structure of polymyxins B and E as an indicator, in
Aoki et al.


