**In vitro** pharmacodynamics of colistin against *Acinetobacter baumannii* clinical isolates

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Received 31 July 2006; returned 6 September 2006; revised and accepted 20 November 2006

**Background:** Colistin is being increasingly used for treatment of infections caused by multidrug-resistant Gram-negative bacteria, including *Acinetobacter baumannii*.

**Methods:** The in vitro pharmacodynamic properties of colistin (sulphate) were investigated by studying the time–kill kinetics and the post-antibiotic effect (PAE) against multidrug-resistant, including colistin heteroresistant, *A. baumannii*. Time–kill was studied with four multidrug-resistant clinical isolates at concentrations ranging from 0.5 to 64\(\times\) MIC. The PAE was examined after 20 min exposure with five clinical isolates, including the four in the time–kill study, plus ATCC 19606.

**Results:** Colistin showed extremely rapid killing in a concentration-dependent manner; but re-growth was observed as early as 3 h and substantial re-growth at 24 h even at concentrations up to 32\(\times\) MIC or 64\(\times\) MIC for some isolates. Colistin exhibited modest PAE of 1.0, 2.3 and 3.5 h at 16, 32 and 64\(\times\) MIC, respectively, against ATCC 19606. Surprisingly, negative PAE (range: –0.8 to –8.15 h) was observed for all of the five clinical isolates.

**Conclusions:** These findings suggest that monotherapy with colistin methanesulphonate, the parenteral form of colistin, and long dosage intervals (e.g. 24 h) may be problematic for treatment of infections caused by colistin heteroresistant *A. baumannii*.

Keywords: killing kinetics, heteroresistance, post-antibiotic effect

**Introduction**

One of the greatest accomplishments of modern medicine has been the development of antibiotics for the treatment of infections that were fatal. However, this has inevitably been followed by the acquisition of resistance towards their antimicrobial activity. Unfortunately, the past two decades have seen a remarkable increase in resistance to the currently available antibiotics and a marked decline in the discovery and development of novel antibiotics.1,2 In particular, multidrug-resistant Gram-negative bacteria are a difficult target for new antibiotic development, mainly due to the poor permeability posed by their outer membranes and efflux pumps. Indeed, no new antibiotic classes are expected to be commercially available within the next several years against multidrug-resistant Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. It is precisely this scenario to which the IDSA refers in its ‘Bad bugs, no drugs’ campaign.1

*A. baumannii* is an opportunistic Gram-negative pathogen that may cause pneumonia, bacteraemia, infection in burn wounds, meningitis and urinary tract infections.3 Infections caused by *A. baumannii* in war-related injuries have been documented.4 The mortality rates for *A. baumannii* infections have been reported to be 52% for bacteraemia and 23–73% for pneumonia.5 Unfortunately, resistance to aminoglycosides, carbapenems, cephalosporins and fluoroquinolones in multidrug-resistant *A. baumannii* clinical isolates has substantially increased worldwide in the last decade and it is regarded as one of the most difficult nosocomially-acquired Gram-negative pathogens to treat and control.5 The recently approved tigecycline is a therapeutic option for adults with complicated skin and skin structure infections caused by multidrug-resistant *A. baumannii*; however, the potential toxicities similar to tetracycline and its unavailability in many countries have limited its clinical applications.5 In this setting of increasing resistance and diminishing therapeutic options, the ‘old’ antibiotic colistin is now being used more extensively.8

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Colistin, also known as polymyxin E, is a multi-component polypeptide antibiotic that was discovered in the 1950s. Owing to its significant (currently high) activity against Gram-negative superbugs, including P. aeruginosa and A. baumannii, and uncommon resistance, colistin is now being administered systemically as ‘salvage’ therapy in patients where none of the other available antibiotics are active against a patient’s isolate. With increased use in recent years, it has been possible to more thoroughly assess colistin’s potential for causing toxicity; interestingly, it appears to be less nephrotoxic than the aminoglycosides, which were believed to be ‘less’ toxic and replaced colistin in the 1970s. Because colistin was developed before contemporary drug development and approval procedures were introduced, there are substantial gaps in knowledge of its pharmacokinetics, pharmacodynamics and toxicodynamics. Worryingly, resistance to colistin in clinical isolates has been reported recently in Europe. Also of very substantial concern is the observation of colistin heteroresistance in 15 out of 16 clinical isolates of ‘colistin-susceptible’ (colistin MICs ≤2 mg/L) but multidrug-resistant A. baumannii. Clearly, a comprehensive understanding of its pharmacology is urgently required and will form the basis for strategies to prevent or minimize the development of resistance. The aim of the present study was to examine the in vitro pharmacodynamic properties, namely bacterial killing and the post-antibiotic effect (PAE), of colistin against multidrug-resistant A. baumannii and to compare the magnitude of pharmacodynamic properties with concentrations achieved in vivo. Because sodium colistin methanesulphonate (CMS), the form of colistin for parenteral use, is a non-active pro-drug, only colistin (sulphate) was employed in the current study.

Materials and methods

Bacterial strains and antibiotics

The five A. baumannii clinical isolates, all of which were multidrug-resistant, were obtained from patients at the Alfred Hospital (Melbourne, Australia). The samples were taken from sputum (isolates 3, 6, 7 and 10) or bronchoalveolar lavage (isolate 8) between 12 December 2002 and 17 November 2004 and identified with VITEK2 GN card (bioMérieux, Australia). A. baumannii ATCC 19606 (VA, USA) was employed as a reference strain. All strains were stored at −80°C and subcultured onto horse-blood agar plates (Medium Preparation Unit, University of Melbourne, Australia) before the experiments.

Colistin stock solutions were prepared by dissolving powdered colistin sulphate (Lot123K1382, Sigma-Aldrich, Castle Hill, NSW, Australia) with Milli-Q water (Millipore, Australia) and sterilizing by passage through 0.20 μm syringe filters (Sartorius, Australia).

Time–kill kinetics

The time–killing kinetics of four clinical isolates (isolates 3, 7, 8 and 10) by colistin (sulphate, MICs 1, 0.5, 2 and 1 mg/L, respectively) was examined. The antibiotic was added to a logarithmic-phase broth culture of approximately 10^6 cfu/mL (5.73–5.91 log_{10} cfu/mL) to yield concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32 and 64× MIC of the respective isolate. Viable counting was performed on samples collected at 0, 20, 40, 60 min and 2, 3, 4, 6 and 24 h after antibiotic addition. After appropriate dilutions with saline, samples of bacterial cell suspension (50 μL) were spirally plated on nutrient agar plates (Medium Preparation Unit) using a Whitley automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted by a ProtoCOL automated colony counter (Symbiosis, Cambridge, UK) after 24 h of incubation of subcultures at 35°C. The lower limit of counting was 20 cfu/mL. The killing effect of colistin at different concentrations was quantified by calculation of the mean survival time over 4 h (MST_{4h}).

Post-antibiotic effect (PAE)

The in vitro PAE was determined by a previously described method for the four clinical isolates noted above, plus isolate 6 and ATCC 19606; the time–kill kinetics of the latter two strains had been examined in a recent study. The five clinical isolates and the reference strain belonged to six different PFGE pattern groups. For each PAE experiment, A. baumannii (~10^6 cfu/mL) in logarithmic phase growth was exposed for 20 min in CAMHB to colistin (sulphate) at concentrations of 0.5, 1, 2, 4, 8, 16, 32 and 64× MIC. Exposure of 20 min was used for colistin due to its very rapid bactericidal effect. Antibiotic was removed by centrifuging three times at 3000 g for 10 min, decanting the supernatant and resuspending in pre-warmed broth. Viable counts were performed at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h, onto nutrient agar. A growth control was performed in the same fashion, but without exposure to the antibiotic. The colonies were counted after 24 h of incubation at 35°C. PAE was determined by comparing regrowth of treated and growth control cultures, using the standard formula of the time for the control culture to increase 10-fold subtracted from the time for the treated culture to do the same.

Results

Time–kill kinetics

In time–kill studies, colistin was bactericidal in a concentration-dependent manner (Figure 1); results for isolates 3 and 7 were similar to those for isolates 8 and 10 shown in the figure. At the highest multiples of the MIC, the bacterial killing for all the strains was so rapid that at 64× MIC, no bacteria could be detected (lower limit of counting 20 cfu/mL) within 20 min. Re-growth was observed as early as 3 h and substantial re-growth occurred at 24 h in all clinical isolates at multiples of the respective MICs. For isolate 3, regrowth occurred at 24 h even at 64× MIC, while for isolates 7, 8 and 10 it was observed at concentrations up to 32× MIC, 16× MIC and 4× MIC, respectively (Figure 1).

Mean survival times (MST_{240min}) were calculated at different multiples of the MIC (Figure 2). Both the abscessa and the ordinate of this figure are plotted on a logarithmic scale to assist in comparisons across strains. The killing kinetics of colistin against ATCC 19606 and isolate 6 were reported previously, and, in the current study, their MST_{240min} was calculated and compared with the four clinical isolates reported here. At 0.5 and 1× MIC, all five of the clinical isolates and ATCC 19606 had mean survival times in the range of 82–122 min. At concentrations between 2 and 32× MIC, there were substantial differences among these strains (Figure 2). With the exception...
of ATCC 19606 which had an MST$_{240\text{min}}$ less than 10 min at 4× MIC and above, the MST$_{240\text{min}}$ of all the clinical isolates decreased to less than 10 min only at concentrations of at least 16 or 32× MIC.

Post-antibiotic effects (PAEs)
Figure 3 illustrates the PAEs of colistin on the five clinical isolates and the reference strain. Substantially different profiles were obtained among the strains. Modest PAE was observed for ATCC 19606 (1.0–3.5 h) only at concentrations of 16× MIC and above, and for isolates 6 and 8 (0.3 h and 1.2 h, respectively) at 64× MIC. Surprisingly, substantial negative PAEs ranging from −0.8 to −8.15 h were observed for all the clinical isolates at various multiples of MICs (Figure 3).

Discussion
Many clinicians have already been confronted with the reality of infections with Gram-negative bacteria, including A. baumannii resistant to all the currently available antibiotics except colistin. Unfortunately, emergence of resistance to colistin has been recently reported in multidrug-resistant A. baumannii. Equally worrying, heteroresistance to colistin was discovered recently in multidrug-resistant but ‘colistin-susceptible’ (based on MICs) A. baumannii. Our knowledge on the pharmacology of colistin is still limited and there has been no systematic investigation on the in vitro pharmacodynamics of colistin against A. baumannii. In the

Figure 1. Time–kill curves for colistin against A. baumannii isolates 8 (a) and 10 (b).

Figure 2. Bactericidal activities of colistin against A. baumannii isolates as measured by MST$_{240\text{min}}$. Due to the rapid killing, MST$_{240\text{min}}$ values could not be calculated for isolate 7 at 16× MIC and above, for ATCC 19606 and isolates 8 and 10 at 32× and above, and for isolates 3 and 6 at 64× MIC.

Figure 3. PAE of colistin against A. baumannii isolates.
current study, the killing kinetics and PAE of colistin were examined against multidrug-resistant \textit{A. baumannii} clinical isolates.

Colistin was very active in the initial killing (Figure 1); even with $0.5 \times \text{MIC}$, the log$_{10}$ cfu/mL decreased at least 1.5 (with maximum decrease of 4.26 for isolate 3) within 1–4 h for the four clinical strains tested. Arguably, the most important finding in this study was the substantial regrowth observed at 24 h even at 64$\times$ MIC. This is consistent with our recent observation of heteroresistance to colistin in these isolates.\textsuperscript{15} The more resistant sub-populations probably led to the substantial regrowth at 24 h. Considering that achieved plasma colistin concentrations are in the range of 1–4 mg/L after intravenous administration of CMS in humans,\textsuperscript{18,19} the concentrations required to inhibit any regrowth at 24 h (64$\times$ MIC, 16–128 mg/L) are definitely unachievable \textit{in vivo}, even without considering plasma protein binding. Therefore, this study strongly suggests that care is needed when CMS is administered parenterally as monotherapy for infections caused by multidrug-resistant \textit{A. baumannii}.

The MST$_{240}$min patterns of the tested isolates are slightly different (Figure 2). For all the tested isolates except number 7, decreases in the MST$_{240}$min were observed at concentrations above 1 or 2$\times$ MIC. The MST$_{240}$min of isolate 7 did not decrease until the concentration reached 8$\times$ MIC, above which the killing was so rapid that the MST$_{240}$min could not be calculated. The values of MST$_{240}$min at 0.5$\times$ MIC for clinical isolates 3, 7, 8 and 10, plus ATCC 19606 and isolate 6,\textsuperscript{15} were in the range of 97–122 min (Figure 2), which were longer than those observed previously for \textit{P. aeruginosa} (3.7–84 min).\textsuperscript{17} In addition, unlike \textit{P. aeruginosa} in which the MST$_{240}$min of all the tested strains fell below 10 min at colistin concentrations of 1$\times$ MIC and higher,\textsuperscript{17} the \textit{A. baumannii} clinical isolates reported here required at least 16$\times$ MIC for the MST$_{240}$min to be shorter than 10 min. These differences are likely due to more extensive bacterial regrowth for \textit{A. baumannii} than \textit{P. aeruginosa}, resulting from the resistant sub-populations in the former species.

Colistin showed only modest positive PAEs against ATCC 19606 at relatively high concentrations ($\geq$16$\times$ MIC) which are not achievable clinically.\textsuperscript{18,19} In contrast, all the clinical isolates exhibited negative PAEs at the tested concentrations; isolates 3 and 7 showed substantial negative PAE (–5.30 to –8.15 h, respectively) at 0.5$\times$ MIC (Figure 3). Such negative PAEs for colistin were not observed against \textit{P. aeruginosa}.\textsuperscript{17} Negative PAE is not commonly observed with any antibiotic/bacterium combinations; it has been reported with meropenem against \textit{P. aeruginosa}, \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} (–0.2 to –0.8 h).\textsuperscript{20} In imipenem-treated \textit{E. coli}, negative PAE (–0.1 to –3.7 h) was suggested to be due to the lysis of osmotically fragile spheroplasts, which were formed by exposure to imipenem, on agar plates thereby influencing viable counting.\textsuperscript{21} A short negative PAE was also observed with the antimicrobial peptide lactoferrin against \textit{Staphylococcus aureus} (range: –6 to –63 min) and \textit{E. coli} (range: –3 to –12 min).\textsuperscript{22} The mechanism(s) of the colistin-associated negative PAE against \textit{A. baumannii} clinical isolates is unknown and warrants further investigation.

Once-daily administration of CMS in cystic fibrosis patients for infections caused by multidrug-resistant \textit{P. aeruginosa} may provide satisfactory effectiveness and toxicity profiles.\textsuperscript{23} The use of such extended dosage intervals against \textit{P. aeruginosa} requires a greater understanding of the pharmaco kinetic/pharmacodynamic determinants for efficacy and comparative clinical trials against more conventional dosage intervals (8 or 12 h). For infections caused by colistin-heteroresistant \textit{A. baumannii}, in view of the lack of positive PAE at clinically achievable concentrations of colistin,\textsuperscript{18,19} as observed in the current study, and the relatively short half-life (approximately 4 h) of colistin after intravenous CMS,\textsuperscript{18,19} it is unlikely that once-daily administration of CMS would be a good option for the treatment of such infections. Further pre-clinical pharmacodynamic investigation is required.

In summary, the current study demonstrated initial concentration-dependent bacterial killing against \textit{A. baumannii}, which was not surprising given that all of the strains studied were colistin-susceptible on the basis of MIC values. Undoubtedly, the most significant findings were the substantial regrowth that occurred even at colistin concentrations up to 64$\times$ MIC and the minor or negative PAE of colistin. These findings send a strong warning that monotherapy with CMS and extended-interval (e.g. 24 h) dosage regimens may be problematic for treatment of infections caused by colistin-heteroresistant \textit{A. baumannii}.

\section*{Acknowledgements}

We thank the Infectious Diseases Unit, Alfred Hospital, Australia for technical support in identification of the isolates, and Professor John Turnidge, Women’s and Children’s Hospital, Adelaide, Australia for helpful discussion. This study was presented in part at the Seventh Annual Meeting of the Australian Society for Antimicrobials, Sydney, Australia, 2006. Disclosure of all financial support: we acknowledge the partial financial support of the Australian National Health and Medical Research Council.

\section*{Transparency declarations}

Statement of proprietary interest: we do not have any financial, commercial or proprietary interest in any drug, device or equipment mentioned in this paper.

\section*{References}


Pharmacodynamics of colistin


