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Determining β-lactam exposure threshold to suppress resistance development in Gram-negative bacteria

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Objectives: β-Lactams are commonly used for nosocomial infections and resistance to these agents among Gram-negative bacteria is increasing rapidly. Optimized dosing is expected to reduce the likelihood of resistance development during antimicrobial therapy, but the target for clinical dose adjustment is not well established. We examined the likelihood that various dosing exposures would suppress resistance development in an *in vitro* hollow-fibre infection model.

Methods: Two strains of *Klebsiella pneumoniae* and two strains of *Pseudomonas aeruginosa* (baseline inocula of $\sim 10^8$ cfu/mL) were examined. Various dosing exposures of cefepime, ceftazidime and meropenem were simulated in the hollow-fibre infection model. Serial samples were obtained to ascertain the pharmacokinetic simulations and viable bacterial burden for up to 120 h. Drug concentrations were determined by a validated LC–MS/MS assay and the simulated exposures were expressed as C_{min}/MIC ratios. Resistance development was detected by quantitative culture on drug-supplemented media plates (at 3× the corresponding baseline MIC). The C_{min}/MIC breakpoint threshold to prevent bacterial regrowth was identified by classification and regression tree (CART) analysis.

Results: For all strains, the bacterial burden declined initially with the simulated exposures, but regrowth was observed in 9 out of 31 experiments. CART analysis revealed that a C_{min}/MIC ratio $\geq$3.8 was significantly associated with regrowth prevention (100% versus 44%, $P = 0.001$).

Conclusions: The development of β-lactam resistance during therapy could be suppressed by an optimized dosing exposure. Validation of the proposed target in a well-designed clinical study is warranted.

Introduction

The prevalence of antibiotic resistance among Gram-negative bacteria (e.g. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) is rising at an alarming rate, rendering many first-line agents ineffective. Infections due to these antibiotic-resistant bacteria have been associated with less favourable outcomes.[1–3] New agents are unlikely to be developed fast enough to solve this crisis, thus available agents must be used judiciously and optimally to prolong their clinical utility.

β-Lactams (e.g. cephalosporins and carbapenems) are the most commonly used agents against serious nosocomial infections. They exhibit time-dependent bactericidal activity and prolonged/continuous infusions have been proposed to improve outcomes of infections due to pathogens with low to intermediate levels of resistance.[4,5] In addition, there is also experimental evidence to suggest that resistance development during therapy could be delayed using more aggressive dosing strategies.[6,7] However, substantial inter-subject variability in β-lactam pharmacokinetics has been reported in critically ill patients, which could impact the likelihood of clinical success using standard dosing regimens.[8]

Therapeutic drug monitoring of β-lactams has been reported in different ICUs worldwide as part of routine medical care. While optimized dosing is expected to improve outcome and/or reduce the likelihood of resistance development during antimicrobial therapy, there are no consensuses on the target for dose adjustment.[9]

To optimally support the design of a clinical study involving dosing adjustment based on serial prospective feedbacks, we examined the likelihood of various drug exposures suppressing resistance development in an *in vitro* hollow-fibre infection model. For the ease of dosing adjustment (without the need for multiple samples over time), we focused on the relationship between C_{min} (trough concentration) measurements and bacterial susceptibility.

Materials and methods

Antimicrobial agents

Three representative β-lactam agents were studied. Cefepime and ceftazidime powder were obtained from Chem-Impex International (Wood Dale, IL, USA). Meropenem powder was purchased from TCI America (Portland, OR, USA). Stock solutions of each agent were prepared in sterile water, aliquoted and stored at −80°C. On the day of experiment, working solutions of each agent were diluted to the desired concentrations.

Bacteria and antimicrobial susceptibility testing

Two clinical strains of *K. pneumoniae* and two clinical strains of *P. aeruginosa* with known mechanisms of resistance were obtained from an academic reference microbiology laboratory (Madrid, Spain). The susceptibilities (MIC) of the bacteria to the above antibiotics and a panel of standard first-line antibiotics were determined by a broth microdilution method (MicroScan, Beckman, Sacramento, CA, USA) and interpreted using the most recently updated EUCAST criteria. The molecular typing and specific mechanisms of resistance of these strains were determined as detailed previously.[10,11]

Hollow-fibre infection model

The schematics of the experimental setup and details of the procedures have been detailed previously.[12] Fresh growing cultures in late log-phase growth (20 mL) were adjusted to $\sim 1 \times 10^8$ cfu/mL based on absorbance at 630 nm. Various unbound clinically relevant dosing exposures of cefepime, ceftazidime and meropenem given every 8 h were simulated for up to 120 h; each dose of antibiotics was given over 30 min. Protein bindings of 19%, 19% and 2% were used for cefepime, ceftazidime and meropenem, respectively.[13,14] Initially, standard clinical dosing regimens (e.g. 2000 mg of cefepime every 8 h and 1000 or 2000 mg of meropenem every 8 h) were examined. Once a preliminary working target was established, we explored additional C_{min}/MIC ratios to refine the target exposure to suppress resistance development. Atypical variables representing patients with augmented/impaired drug clearance (e.g. elimination half-lives of 1 or 4 h for cefepime) and/or supra-physiological experimental variables (e.g. higher peak concentrations to simulate doses of 3000 or 4000 mg of ceftazidime) were considered to improve achievement of a specific target.[15] Serial (four or more for each dosing interval) samples were obtained over up to six dosing intervals to ascertain the pharmacokinetic simulations. Based on our pilot stability studies, the samples were stored at −20°C upon collection and analysed within 1 week. Additionally, to determine the viable bacterial burden, samples were also obtained serially in duplicate. The samples were centrifuged at 10 000 **g** for 15 min, reconstituted with sterile normal saline to their original volumes in order to minimize the drug carry-over effect, diluted 10-fold serially and quantitatively plated onto media plates. The emergence of resistant isolates during drug exposure was detected by quantitative culturing on drug-supplemented media plates (at 3× the corresponding baseline MIC regardless of the resistance phenotype). The experiments could be terminated prior to 120 h if the total population had reached a saturation level (>9 log cfu/mL) or was below the limit of detection (<2 log cfu/mL) on more than one sampling occasion; a meaningful change in the outcome would not be anticipated by extending the duration of the experiments.

Drug assay and pharmacokinetics

Drug concentrations were determined by a validated LC–MS/MS assay. Briefly, 5 μL of thawed sample was added to 895 μL of MS-grade water (EMD Millipore Corporation, Darmstadt, Germany) and 100 μL of doripenem (320 ng/mL) was added as internal standard. The tubes were mixed by vortexing for 15 s and centrifuged at 18 000 **g** for 15 min before injection. The LC–MS/MS system consisted of a Waters Acquity™ UPLC with a Waters BEH C_{18} column (1.7 μm, 2.1 × 50 mm) and an API 5500 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with Turbo-Ion-Spray™ source. Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. The injection volumes were 5 μL for cefepime, ceftazidime and meropenem. The analytes were separated by gradient elution at 45°C, using a flow rate of 0.35 mL/min. The gradient was: 0–0.5 min, 95% A; 0.5–0.7 min, 95%–84% A; 0.7–1.2 min, 84%–76% A; 1.2–1.7 min, 76%–70% A; 1.7–2.1 min, 70%–50% A; 2.1–2.5 min, 50%–5% A; 2.5–3.0 min, 5% A; 3.0–3.2 min, 5%–95% A; and 3.2–5 min, 95% A. Multiple reaction monitoring (MRM) scan type in positive mode was used in the mass spectrum. The transitions of *m/z* 481.2 → 86.0, 547.2 → 468.1, 384.2 → 141.0 and 421.2 → 274.1 were used for quantifying cefepime, ceftazidime, meropenem and doripenem, respectively. The intra-day variability for all the drugs was <9.4%, while the inter-day variability was <13.4%. The concentration–time profiles were analysed by fitting a one-compartment model with zero-order infusion to the data. The best-fit model parameter estimates were used to determine the clinically relevant dose exposure and the simulated exposures were expressed as C_{min}/MIC ratios. The pharmacokinetic simulations were considered acceptable if the best-fit peak concentrations and elimination half-lives were within 20% of the target values.

Statistical analysis

The C_{min}/MIC ratios stratified by outcomes (i.e. suppression versus regrowth) were compared using Student's *t*-test. The C_{min}/MIC breakpoint threshold to prevent bacterial regrowth associated with resistance development was identified by classification and regression tree (CART) analysis. Subsequently, the relationship between drug exposures (above and below the breakpoint threshold) and regrowth was compared using Fisher's exact test. A *P* value of <0.05 was considered statistically significant.

Confirmation of resistance

Selected isolates were randomly recovered from drug-supplemented media plates. To confirm resistance phenotypically, MICs of the exposed agents as well as a panel of standard first-line antibiotics were determined and compared with those for the parent strains. Subsequently, different molecular approaches were used to characterize the resistance mechanisms in different isolates. For *K. pneumoniae*, the outer membrane proteins (OMPs) were examined by SDS–PAGE and western blot analysis, as previously described.[16] For *P. aeruginosa*, molecular characterization of resistance mechanisms was performed as follows. The expression of the genes encoding the AmpC cephalosporinase (*ampC*) and major efflux pumps (*mexB*, *mexD* and *mexY*) was determined by RT–PCR. PAO1 (*P. aeruginosa* with basal expression of these genes) was used as a control[17] according to published protocols.[18] All PCRs were performed in duplicate in at least two independent experiments and the mean values of mRNA expression were referred to PAO1 expressions. *P. aeruginosa* isolates were considered to overexpress *ampC*, *mexD* and *mexY* genes when the mRNA level was at least 10-fold higher than that of PAO1 control, negative if less than 5-fold higher and borderline if the mRNA levels were between 5- and 10-fold higher. However, for *mexB*, overexpression was considered when the corresponding mRNA level of an isolate was at least 3-fold higher than that of PAO1, negative if it was less than 2-fold higher and borderline if it was between 2- and 3-fold higher. In addition, sequencing of genes involved in

Table 1. Susceptibility (MIC in mg/L) and resistance mechanisms of the clinical strains used

Bacteria	Cefepime	Ceftazidime	Meropenem	MLST (ST)	Resistance mechanism
K. pneumoniae (Kp1)	0.25	0.5	0.06	ST678	WT
K. pneumoniae (Kp2)	**16**	**64**	0.06	ST16	CTX-M-15
P. aeruginosa (Pa1)	0.5	1	0.13	ST319	WT
P. aeruginosa (Pa2)	**16**	**64**	0.25	ST175	AmpC overexpression

Bold font indicates resistant phenotype according to EUCAST breakpoints.

Table 2. Resistance mutation frequency (at 3× baseline MIC) of the clinical strains used (determined at least twice on different days)

Bacteria	Cefepime	Ceftazidime	Meropenem
K. pneumoniae (Kp1)	2.2×10^{-8}–3.9×10^{-8}	3.9×10^{-9}–4.4×10^{-9}	1.1×10^{-9}–6.1×10^{-9}
K. pneumoniae (Kp2)	1.1×10^{-5}–1.8×10^{-5}	1.7×10^{-8}–6.4×10^{-8}	2.1×10^{-8}–2.5×10^{-8}
P. aeruginosa (Pa1)	1.3×10^{-8}–5.4×10^{-8}	6.1×10^{-9}–1.9×10^{-8}	1.9×10^{-8}–6.0×10^{-8}
P. aeruginosa (Pa2)	5.5×10^{-8}–1.1×10^{-7}	5.7×10^{-7}–1.9×10^{-6}	5.5×10^{-8}–7.4×10^{-8}

AmpC regulation [*ampC*, *ampR*, *ampD* and *dacB* (PBP4)] and *ftsI* (PBP3) was also performed.

Results

Bacteria

Two WT and two antibiotic-resistant bacterial strains were studied. Their susceptibility profiles to different β-lactam agents and known resistance mechanisms are shown in Table 1. Overall, these strains were selected based on their phenotypic resistance profiles and represent prevalent strains that clinicians would likely encounter in serious nosocomial infections (e.g. ventilator-associated pneumonia). Pilot studies revealed that the mutation frequency of resistance to the agents used (at 3× the baseline MIC) ranged from $\sim1\times10^{-5}$ to 6×10^{-9} (Table 2); thus, we anticipate that pre-existing resistant mutants would likely be present at baseline.

Pharmacokinetics

A total of 31 treatment courses (i.e. $n=10$ for cefepime, $n=8$ for ceftazidime and $n=13$ for meropenem) were simulated. Overall, the pharmacokinetic simulations were satisfactory ($r^2\geq0.93$). A typical mono-exponential profile simulated is as shown in Figure 1.

Resistance suppression

For all strains, the bacterial burden declined initially with the simulated treatment exposures. With sub-optimal drug exposures, regrowth associated with resistance development was observed in 9 out of 31 experiments (ceftazidime $n=3$, cefepime $n=4$ and meropenem $n=2$). No resistance development was observed for the WT *K. pneumoniae* (Kp1), presumably due to its low MICs of the agents used. Typical bacterial profiles over time are as shown in Figure 2. Resistance was observed to emerge from 24 to 120 h after initial drug exposure. The C_{min}/MIC ratios observed ranged from 0.2 to 20.8 (median 3.3). There was a significant difference in C_{min}/MIC ratio in experiments where sustained suppression of bacteria and regrowth were observed (7.5 ± 6.3 versus 1.6 ± 1.2, $P<0.001$), as shown in Figure 3. CART analysis revealed that a C_{min}/MIC ratio ≥3.8 was associated with regrowth prevention (100% versus 44%, $P=0.001$). Sub-group analyses by specific antibiotics revealed a reasonably consistent trend among the agents examined (Figure 4). Of note, we have also examined regrowth associated with C_{max}/MIC and AUC/MIC, but the correlations were not as high as those reported for C_{min}/MIC ratios (data not shown).

Mechanisms of resistance

Four *K. pneumoniae* mutants derived from Kp2 with an increase in MIC of cefepime (post-exposure) were selected for further resistance mechanism characterization. In addition to cefepime resistance (>16-fold elevation in MIC), cross-resistance to cefotaxime and in two cases also to cefoxitin was evident. No changes in gentamicin or ciprofloxacin susceptibilities were detected (Table 3). SDS–PAGE/western blot analysis revealed that resistance was likely attributable to porin loss (OmpK35 alone or with OmpK36) in mutants Kp2.2m and Kp2.4m (exhibiting resistance to cefoxitin). Interestingly, ertapenem MIC was not affected in all the mutants studied.

Using a similar experimental setup, we have previously shown that reduced expression of OprD and overexpression of efflux pumps (e.g. MexB) were associated with resistance development to meropenem in *P. aeruginosa*.[19] In this study, four additional *P. aeruginosa* mutants derived from Pa2 exposed to cefepime were examined. Similarly, cefepime resistance (≥16-fold elevation in MIC) and cross-resistance to ceftazidime (but not meropenem) were observed (data not shown). The parent strain (Pa2) had a moderate overexpression of AmpC (10.5 ± 3.2-fold increased expression compared with PAO1) at

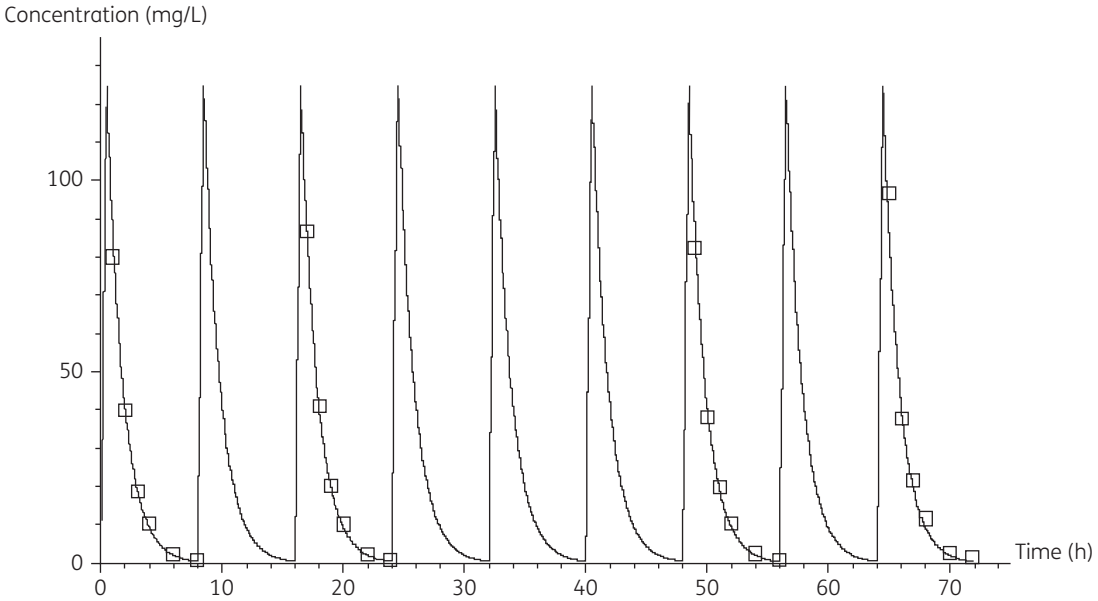

Figure 1. A typical simulated pharmacokinetic profile of 2 g of meropenem given every 8 h (each dose given over 30 min). $r^2 = 0.991$. Open squares depict observed concentrations and the continuous line represents the best-fit model. Target $C_{max} = 120$ mg/L, half-life = 1 h and $C_{min} = 0.66$ mg/L. Best-fit $C_{max} = 124$ mg/L, half-life = 0.93 h and $C_{min} = 0.51$ mg/L.

baseline that was due to a four-nucleotide (1268–1271) deletion in the *dacB* gene. Sequencing of *ampD* of the parent strain revealed two amino acid changes (G148A, D183Y), which were not related to AmpC overexpression.[20] *P. aeruginosa* mutants that regrew during hollow-fibre infection model experiments also overexpressed AmpC moderately at levels similar to the parent strain and showed identical *ampC*, *ampD*, *ampR* and *dacB* sequences. Moreover, overexpression of *mexB* or *mexD* was not detected in these isolates and *mexY* had a borderline overexpression both in the parent strain and in the mutants (range of expression compared with PAO1: 5.5–11.0). Since PBP3 modification may cause β-lactam resistance,[21] the *ftsI* gene was also sequenced, but *P. aeruginosa* isolates recovered from the hollow-fibre infection model showed no differences when compared with the parent strain.

Discussion

With the widespread and rapid rise in antibiotic resistance among Gram-negative bacteria, managing critically ill patients with severe infections is particularly challenging. Various first-line agents may not be effective and there are concerns that resistance may also emerge during therapy. In a clinical study when *P. aeruginosa* was recovered from initial respiratory tract cultures, a high rate of resistance development during therapy (33%–53%) was reported in severe pneumonia.[22] It is hoped that the emergence of resistance can be prevented/delayed by optimal dosing of antimicrobial agents using pharmacokinetic/pharmacodynamic principles.[23]

Several clinical studies have attempted to establish a relationship between pharmacokinetic/pharmacodynamic target exposures of β-lactams and outcomes in Gram-negative bacterial infections. Li *et al.*[24] showed that in patients receiving meropenem for the treatment of lower respiratory tract infections, an unbound meropenem C_{min}/MIC of >5 was associated with clinical efficacy. Aitken *et al.*[25] also reported that, in 33 patients with Gram-negative bacterial pneumonia who received cefepime monotherapy, an unbound C_{min}/MIC of >2.1 had a significantly lower risk of clinical failure. Moreover, Roberts *et al.*[8] reported that, in 361 evaluable critically ill patients across 68 hospitals, a positive clinical outcome was associated with free $T_{>MIC}$ of 100% (i.e. C_{min}/MIC ≥1). The use of the C_{min}/MIC ratio was not unprecedented in studies correlating drug exposures to outcomes. However, none of the studies mentioned above addressed the emergence of resistance as an outcome endpoint.

Many pharmacokinetic/pharmacodynamic studies focused on bacterial load reduction after a brief period of drug exposure (e.g. 24 h).[26] A prolonged duration of drug exposure (up to 120 h) was used in this study, which would more realistically represent a clinical treatment course. Furthermore, both WT (Kp1 and Pa1) and common drug-resistant phenotypes (Kp2 and Pa2) were investigated. Instead of focusing solely on the magnitude of bacterial load reduction, we examined the likelihood of resistance emergence during therapy. The drug exposure necessary to suppress resistance development is expected to be greater than that commonly cited for 1 log reduction.[27] Consequently, drug exposures were expressed as C_{min}/MIC in view of the lack of a ceiling effect (i.e. $\%T_{>MIC}$ cannot be expressed as >100%). Moreover, dosing adjustment based on C_{min} (trough concentrations) measurements at steady state is also more practical for the purpose of therapeutic drug monitoring.

In a dense bacterial population where the density exceeds the inverse of the natural mutation frequency of drug resistance, pre-existing resistant mutants are expected to be present

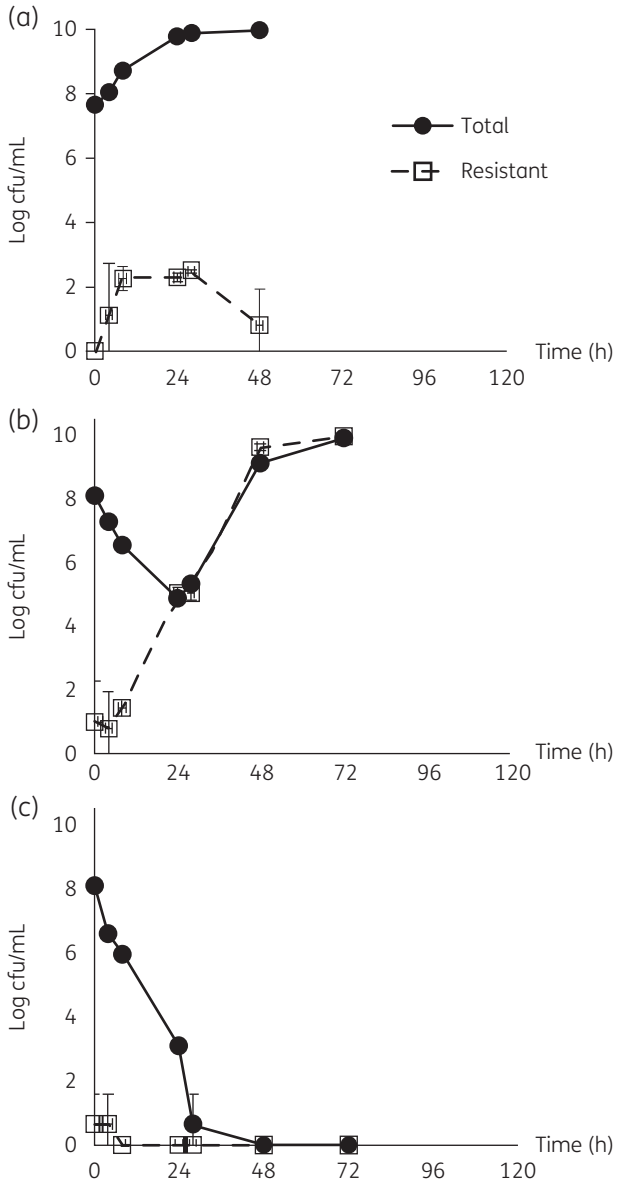

Figure 2. Typical bacterial profiles for WT *P. aeruginosa*. Placebo control (a). Ceftazidime at 500 mg every 8 h (C_{min}/MIC = 2.9) (b). Ceftazidime at 3000 mg every 8 h (C_{min}/MIC = 7.7) (c). Data are shown as mean ± SD.

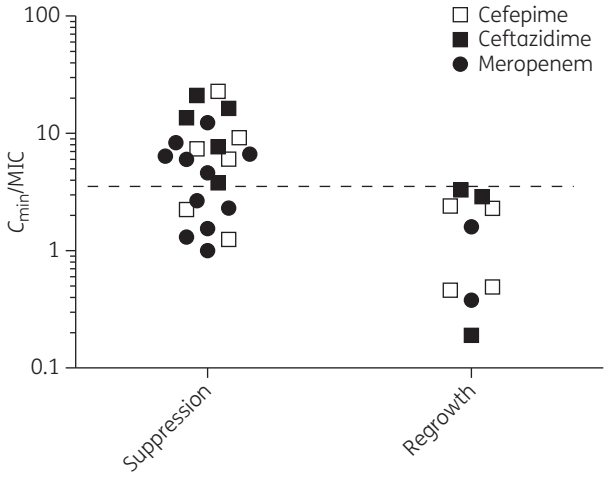

Figure 3. Drug exposures (C_{min}/MIC) stratified by outcomes. Each data point represents a hollow-fibre infection model experiment. The most significant threshold (C_{min}/MIC ≥3.8) is depicted by the horizontal broken line.

at baseline. With low dosing exposures, a selective pressure is exerted on the bacterial populations, facilitating selective enrichment of resistant mutant sub-population(s). The growth of the bacterial population can be controlled only once a threshold exposure is attained above which the preferential proliferation of the resistant mutants can be suppressed.[15,28–30] Key factors influencing the development of resistance *in vitro* have been discussed previously.[6] Using similar experimental setups, our group has previously demonstrated the same phenomenon with a quinolone and the aminoglycosides.[31,32] While the specific threshold exposure to suppress resistance development may not be identical among different drug classes, a reasonably consistent trend was observed using three representative β-lactam agents.

Taking the data collectively, we found a C_{min}/MIC ratio of ≥3.8 was associated with regrowth prevention (100% versus 44%, $P = 0.001$). Our data are consistent with the well-accepted belief that the bactericidal activity of the β-lactams is maximized at ∼4–5× MIC.[15,33–35] While there was a clear signal that unbound drug exposure was correlated to resistance emergence, the target was selected based on the threshold associated with the greatest statistical significance using a binary recursive partitioning technique. It should be acknowledged that other targets might also be useful with different positive/negative predictive values. For example, if C_{min}/MIC ≥1 was used as the cut-off, regrowth would have been prevented in 81% (when C_{min}/MIC ≥1) and 20% (when C_{min}/MIC <1) of the experiments, respectively ($P = 0.017$).

Unlike other studies in which WT strains were used, some strains we used were resistant to the antibiotic studied (i.e. extended-spectrum cephalosporins) and pre-existing mutants might have other resistance mechanisms than those we identified. In *K. pneumoniae*, the resistant mutants were associated with porin loss, but this mechanism could not explain the resistance profiles of Kp2.1m and Kp2.3m; other mechanisms, such as CTX-M-15 hyperexpression, could be responsible for the MIC elevation.[36] In contrast, higher levels of AmpC expression, overexpression of efflux pumps (i.e. *mexB*, *mexD* and *mexY*) or PBP3 modifications were not demonstrated in *P. aeruginosa* mutants. Whole-genome sequencing may allow us to identify the specific resistance mechanism(s) involved. Despite different MICs associated with different resistance mechanisms, our data suggest that selective amplification of (higher level) resistance can be prevented/reduced using an adequate drug exposure adjusted for the baseline MIC.

There are several limitations in our study. Firstly, we only examined intermittent administration of three representative β-lactam agents in this study. The selection was primarily based on availability in the anticipated trial sites for the subsequent clinical study. While the overall results were reasonably

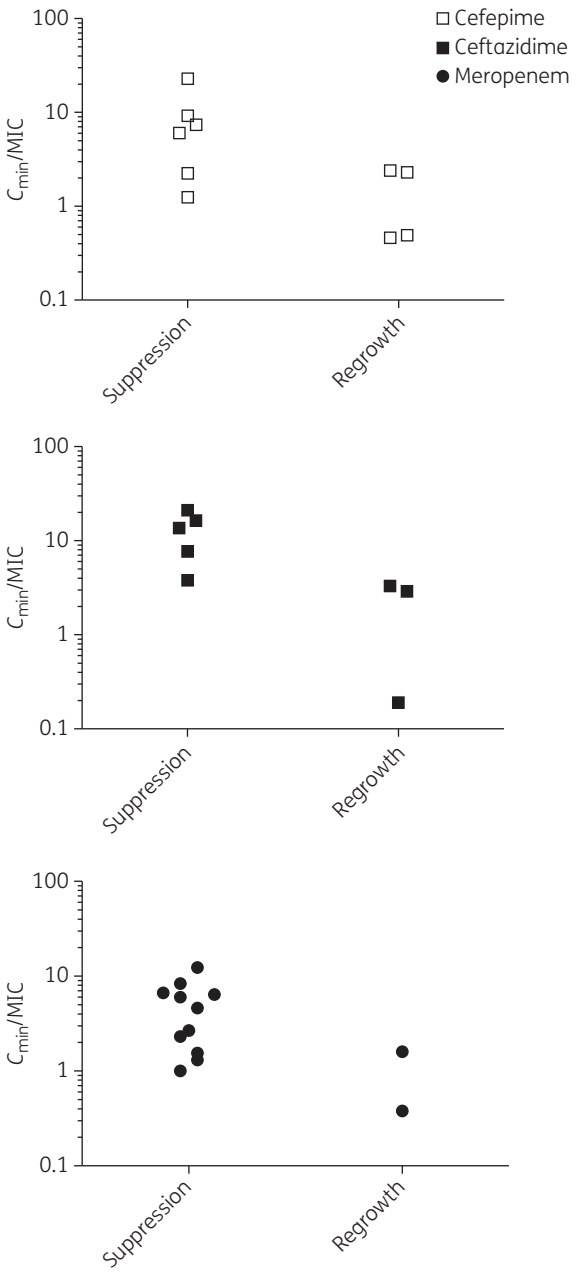

Figure 4. Sub-group analysis by antibiotic.

consistent among the agents, our data may not be directly applicable to other β-lactams (and alternative administration modes, such as continuous infusion) not investigated. Secondly, only a limited number of isolates with representative resistance mechanisms were tested. Our findings may also not be universally applicable to other bacteria with lower mutation frequency of resistance and/or with resistance mechanisms that have not been investigated. Thirdly, it is recognized that the *in vitro* infection models lack immune responses and resistance suppression could still be achieved with drug exposures below the target threshold identified (44%). Selected experiments were terminated prior to 120 h; resistance development (or enrichment of the resistant sub-population) is theoretically still possible after the observation period. Consequently, the drug target proposed would represent a conservative target for dosing adjustment. In addition to standard clinical dosing regimens, supra-physiological drug exposures (unlikely to be used clinically) were occasionally used against drug-resistant strains as a proof of concept. In practice, the toxicity profiles of various agents must also be considered during dosing selection. Finally, we simulated the systemic exposure of various antibiotics in the infection model in order to facilitate dosing adjustment by therapeutic drug monitoring. It should be noted that serum drug concentrations could be different from the drug concentration at the site(s) of infection and thus clinical judgement should be exercised when managing different infection types.

In conclusion, we demonstrated that the development of β-lactam resistance during therapy could be suppressed by elevated dosing exposures. A C_{min}/MIC ratio $\geq$3.8 was associated with regrowth prevention. In conjunction with prospective patient-specific susceptibility data, our results will be validated in an ongoing multicentre study of nosocomial pneumonia.

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Table 3. Susceptibility (MIC in mg/L) of selected resistant mutant *K. pneumoniae* strains recovered from the hollow-fibre infection model

Bacteria	Cefepime	Cefoxitin	Cefotaxime	Ceftazidime	Ertapenem	Ciprofloxacin	Gentamicin
K. pneumoniae parent Kp2 (CTX-M-15)	**16**	≤8	**96**	>**16**	≤0.5	>**2**	≤2
K. pneumoniae mutant (Kp2.1m)	>**256**	≤8	>**256**	>**16**	≤0.5	>**2**	≤2
K. pneumoniae mutant (Kp2.2m)	>**256**	>**16**	>**256**	>**16**	≤0.5	>**2**	≤2
K. pneumoniae mutant (Kp2.3m)	>**256**	≤8	**192**	>**16**	≤0.5	>**2**	≤2
K. pneumoniae mutant (Kp2.4m)	>**256**	>**16**	>**256**	>**16**	≤0.5	>**2**	≤2

Bold font indicates resistant phenotype according to EUCAST breakpoints.

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