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Activity of cefepime and carbapenems in experimental pneumonia caused by porin-deficient *Klebsiella pneumoniae* producing FOX-5 β-lactamase

C. Pichardo[1], M. del Carmen Conejo[2], M. Bernabéu-Wittel[1], A. Pascual[2,3], M. E. Jiménez-Mejías[1], M. de Cueto[3], M. E. Pachón-Ibáñez[1], I. García[2], J. Pachón[1,4] and L. Martínez-Martínez[2,3]

[1]Service of Infectious Diseases, Hospitales Universitarios Virgen del Rocío, [2]Department of Microbiology, Hospital Universitario Virgen Macarena, [3]Department of Microbiology and [4]Department of Medicine, School of Medicine, University of Sevilla, Sevilla, Spain

ABSTRACT

The in-vivo activities of cefepime, imipenem and meropenem against the porin-deficient strain *Klebsiella pneumoniae* C2 and its derivative *K. pneumoniae* C2(pMG252) coding for the AmpC-type β-lactamase FOX-5 were determined. Bactericidal activities were determined with the kill-curve method. A pneumonia model in guinea-pigs was developed, and C_{max}, $t_{½}$ and ΔT/MIC were calculated for the three agents tested. Animals were treated for 72 h with sterile saline (control group) or with cefepime, imipenem or meropenem (240 mg/kg/day, intramuscularly, three times daily). Bacterial counts in lungs (log_{10} CFU/g tissue) were determined by serial dilution. MICs (mg/L) of cefepime, imipenem and meropenem against *K. pneumoniae* C2/*K. pneumoniae* C2(pMG252), determined by macrodilution, were: 0.5/4, 0.5/0.5 and 0.25/0.5, respectively. Bacterial counts in the lungs of animals infected with *K. pneumoniae* C2 and treated with antimicrobial agents were always lower than in the control group (cefepime, 4.4 ± 0.5; imipenem, 4.6 ± 0.4; meropenem, 4.7 ± 0.5; control group, 5.6 ± 0.8; $p < 0.01$). No significant differences were observed among the groups receiving therapy ($p > 0.05$). Bacterial lung clearance was higher in treated animals than in control animals following infection with *K. pneumoniae* C2(pMG252) (cefepime, 4.5 ± 0.4; imipenem, 4.0 ± 0.3; meropenem, 4.6 ± 0.4; control group, 6.1 ± 0.6; $p < 0.01$), with imipenem producing better clearance than either cefepime or meropenem ($p < 0.05$). Thus, in the guinea-pig pneumonia model, cefepime, imipenem and meropenem were each effective against the porin-deficient *K. pneumoniae* strain C2 and its derivative expressing the plasmid-mediated AmpC type β-lactamase FOX-5.

Keywords Carbapenems, cefepime, FOX-5, guinea-pig model, *Klebsiella pneumoniae*, pneumonia

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INTRODUCTION

Infections caused by *Klebsiella pneumoniae* isolates that produce plasmid-mediated enzymes related to the AmpC-type chromosomal β-lactamases of Gram-negative bacteria (PACBLs) are becoming a serious therapeutic problem [1]. Most organisms producing PACBLs behave like derepressed mutants of organisms encoding AmpC-type chromosomal β-lactamases, and are commonly resistant to penicillins, first- and second-generation cephalosporins, cephamycins, oxyimino-cephalosporins, and combinations of β-lactams and classical β-lactamase inhibitors [1]. Zwitterionic cephalosporins and carbapenems, which penetrate very efficiently through the outer-membrane of Gram-negative bacteria [2] and are poor substrates for PACBLs, are frequently active *in vitro* against organisms producing high levels of AmpC-type β-lactamases [1,3,4]. PACBLs have been found worldwide in *K. pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Salmonella* spp., *Proteus mirabilis* and other enterobacteria [1,5,6], and the results of recent epidemiological studies with

Corresponding author and reprint requests: L. Martínez-Martínez, Service of Microbiology, University Hospital Marqués de Valdecilla, Avda Valdecilla s/n, 39008, Santander, Spain
E-mail: lmartinez@humv.es

clinical isolates indicate that microorganisms with this resistance mechanism are being isolated with increasing frequency [5,6].

Treatment of infections caused by *K. pneumoniae* strains producing PACBLs may be difficult because of the presence of additional mechanisms of resistance; for example, the organism may also express an extended-spectrum β-lactamase, which precludes the use of zwitterionic cephalosporins [5]. Loss of porins may cause decreased susceptibility or resistance of PACBL-producing *K. pneumoniae* to both zwitterionic cephalosporins and carbapenems [4]. Plasmids coding for extended-spectrum β-lactamases or PACBLs can also encode resistance to aminoglycosides, co-trimoxazole, tetracycline or fluoroquinolones, or resistance determinants could be present on additional plasmids [1,7]. Other chromosomal mutations may also be present in genes causing topoisomerase changes or involved in active efflux [8].

It has been shown previously [4] that, with a standard inoculum of 10^5 CFU/mL, the porin-deficient strain *K. pneumoniae* C2 containing plasmid pMG252 (which codes for the PACBL FOX-5) [9] is resistant to ampicillin, cephalothin, cefoxitin, ceftazidime and cefotaxime, either alone or combined with clavulanic acid, but is susceptible to cefepime, imipenem and meropenem. The organism became resistant to cefepime, and less susceptible to imipenem and meropenem, when an inoculum of 10^7 CFU/mL was used. In order to evaluate the in-vivo significance of these findings, the present study tested cefepime, imipenem and meropenem in an experimental guinea-pig pneumonia model, with infection caused by *K. pneumoniae* C2 or a derivative expressing FOX-5.

MATERIALS AND METHODS

Bacterial strains

K. pneumoniae C2 is a ceftazidime-susceptible strain derived *in vitro* from the clinical isolate *K. pneumoniae* NEDH-1 (deficient in porins OmpK35 and OmpK36, and producing SHV-2) [4]. Plasmid pMG252 was obtained from a clinical isolate of *K. pneumoniae* [10] and codes for bla_{FOX-5}, the quinolone-resistance determinant *qnr*, and additional genes involved in resistance to chloramphenicol, kanamycin, gentamicin, streptomycin, sulphamethoxazole, tobramycin, trimethoprim and mercuric chloride [9]. Plasmid pMG252 was introduced into *K. pneumoniae* C2 by conjugation [11], and was found to be stable following at least ten subcultures in antibiotic-free medium (data not shown).

Antibiotics

Imipenem plus cilastatin was obtained from Merck, Sharp and Dohme (Madrid, Spain), meropenem from AstraZeneca (Madrid, Spain) and cefepime from Bristol-Myers Squibb (Madrid, Spain).

Susceptibility tests and time-kill curve experiments

MICs of cefepime, imipenem and meropenem against *K. pneumoniae* strains C2 and C2(pMG252) were determined by macrodilution according to NCCLS guidelines [12], but with three different inocula (10^5, 10^6 and 10^7 CFU/mL). MICs were also determined by Etests (AB Biodisk, Solna, Sweden), used according to the manufacturer's instructions.

Minimum bactericidal concentrations (MBCs) were determined by subculturing 100-μL aliquots from inoculated tubes containing antimicrobial concentrations higher than or equal to the MIC of each agent on to antibiotic-free Mueller–Hinton agar. Plates were incubated at 35°C for 48 h, and colonies were counted. The MBC was defined as the concentration that killed ≥99.9% of the initial inoculum.

The bactericidal activities of cefepime, imipenem and meropenem against the two tested strains were determined by the kill-curve method, in which their killing activities were determined over time (0 h, 3 h, 6 h and 24 h) with a starting inoculum of *c.* 5–6 × 10^5 CFU/mL and concentrations of the antimicrobial agents equivalent to 1× MIC and 4× MIC.

Determination of β-lactamase activity

β-Lactamase activity was determined spectrophotometrically as described previously [13], with crude supernatants of sonicated cells as a source of enzyme, and cephaloridine (0.1 mM; Sigma, Madrid, Spain) as the substrate. One unit of enzyme was defined as the amount of enzyme that hydrolysed 1 μmol/min of substrate at 30°C, as determined at 295 nm.

Animals

Hartley strain guinea-pigs (weight, 350–400 g) were obtained from B & K Universal (Barcelona, Spain). The animals had a specific pathogen-free sanitary status. Animals were housed in regulation cages and were given free access to food and water.

Drug pharmacokinetics

The levels of cefepime, imipenem and meropenem were determined in plasma after the administration of single intramuscular doses (60 mg/kg) of each antimicrobial agent. Blood was extracted by cardiac puncture after 10, 15, 30, 60, 90, 120 and 150 min from anaesthetised non-infected guinea-pigs, in groups of three at each time-point. The drug concentrations in plasma were measured with a bioassay method, with *Micrococcus luteus* ATCC 9341 as the indicator strain. The maximum plasma concentration (C_{max} in mg/L) and the terminal half-life ($t_{1/2}$ in h) were calculated with the PKCALC program [14]. The time during which the plasma concentration remained above the MIC (ΔT/MIC, h) was estimated by extrapolation from the regression line for plasma elimination [15], with use of the MIC obtained with an inoculum of 10^5 CFU/mL.

Experimental pneumonia model in guinea-pigs

Pennington's model [16–19] was used for the production of pneumonia. The guinea-pigs were anaesthetised by intraperitoneal injection of ketamine hydrochloride (Parke-Davis, Barcelona, Spain) 100 mg/kg, plus, locally, with 0.2 mL of lidocaine 2% w/v (Braun Medical, Jaén, Spain). A midline aseptic incision was made in the neck, the trachea was exposed, and a 25 G needle was inserted between the tracheal rings. Then, 0.25 mL of an inoculum containing *c.* 10^9 CFU/mL was instilled into the trachea. The needle was removed inmediately after inoculation, the neck was sutured, and the animals were placed upright for 10–15 min to facilitate distal migration of the bacteria by gravity.

K. pneumoniae strains C2 and C2(pMG252) were used to induce pneumonia in guinea-pigs. The inoculum was prepared from an 18–20-h culture of *K. pneumoniae* in trypticase-soy broth (TSB; Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C. To obtain the final inoculum, the bacterial suspension was mixed with the same volume of porcine mucin (M-2378; Sigma, St Louis, MO, USA) diluted to 10% v/v in saline. The size of the inoculum was measured for each experiment. Histological studies were performed on lungs from animals infected with *K. pneumoniae* C2 or C2(pMG252) and not treated with antimicrobial agents (see below), in order to evaluate inflammatory changes.

Antimicrobial treatment

To evaluate the effectiveness of the different treatment regimens, 49 guinea-pigs were inoculated with the C2 strain and 46 guinea-pigs with C2(pMG252), and then divided into four groups. The control groups received sterile saline intramuscularly for 72 h (n = 17 and n = 11 for C2 and C2(pMG252), respectively), while the other three groups received one of the following treatments for 72 h: (1) imipenem 240 mg/kg/day intramuscularly (n = 10 and n = 12 for C2 and C2(pMG252), respectively); (2) meropenem 240 mg/kg/day intramuscularly (n = 12 and n = 12 for C2 and C2(pMG252), respectively); or (3) cefepime 240 mg/kg/day intramuscularly (n = 10 and n = 11 for C2 and C2(pMG252), respectively). The first dose of every antibiotic was administered 4 h after inoculation, with the following dosing schedule: 60 mg/kg at 08:00 and 14:00 h, and 120 mg/kg at 20:00 h. In order to confirm that the drugs were not directly toxic to the animals, each antibiotic was given to groups of ten non-infected guinea-pigs for 72 h.

All the animals were observed during treatment for 72 h. The surviving guinea-pigs were killed humanely 4 h after the last dose by intraperitoneal administration of sodium thiopental 5% w/v (Braun Medical, Barcelona, Spain). Following death, thoracotomy was performed; the heart and lungs were extracted together, and the lungs were later separated on a sterile Petri dish and weighed. The lungs were homogenised (Stomacher 80; Tekmar, Cincinnati, OH, USA) in 10 mL of sterile saline solution, after which viable counts were determined by plating 100-μL aliquots of ten-fold dilutions on Columbia sheep blood agar plates and incubating for 24 h at 37°C. The results were expressed as mean ± SD of the $\log_{10}$ CFU/g of lung.

Selection and characterisation of antibiotic-resistant mutants

Selection of mutants derived from either *K. pneumoniae* C2 or C2(pMG252) was performed by inoculation of lung homogenates either on media containing 4× MIC of cefepime, imipenem or meropenem against the tested strain, or on media containing a gradient (0–16× MIC) of each antibiotic. The latter approach was used in an attempt to obtain mutants with different levels of resistance to the selecting agent. Lungs from three animals treated with cefepime, imipenem or meropenem were used in these assays. Colonies were counted after incubation at 35°C for 48 h.

In a parallel experiment, mutants were also selected under the same conditions with use of a suspension of bacteria grown in TSB (35°C for 20 h). The frequency of mutation was expressed as the ratio between the number of colonies on plates containing 4× MIC of each agent and the size of the original inoculum. The results from gradient plates were evaluated qualitatively by counting the number of colonies growing on zones of the plates corresponding to 0×, 4×, 8× and 16× MIC.

Up to four different isolated colonies on every single- or gradient-concentration plate were selected for further studies. Bacteria were subcultured twice on antibiotic-free Mueller–Hinton agar. The MIC of the agent used for selection was determined for every original colony by microdilution, as described above. True mutants were defined as those for which the MIC of the selecting agent increased at least four-fold. β-Lactamase activity in mutants and the corresponding parent strains were determined as described above.

Statistical analysis

Numbers of surviving animals, numbers of sterile lung tissue specimens, and β-lactamase activity in parental strains and derived mutants were evaluated with Fisher's exact test. A p value < 0.05 was considered significant. CFU/g of lung tissue were analysed with ANOVA and post-hoc tests (Tukey–Kramer and Dunnet tests). The SPSS 8.0 statistical package (SPSS Inc., Chicago, IL, USA) was used.

RESULTS

In-vitro tests

MICs of cefepime, imipenem and meropenem against *K. pneumoniae* C2 and C2(pMG252) are shown in Table 1. MIC results obtained with the macrodilution method with an inoculum of 10^5 CFU/mL were within one dilution step of those obtained by Etest and those obtained previously by microdilution. The results of time-kill curve experiments with cefepime are presented in Fig. 1. Cefepime at 1× or 4× MIC produced a > 2 $\log_{10}$ reduction in the number of viable bacteria in comparison with the untreated control for both *K. pneumoniae* C2 and C2(pMG252) at 3 h or 6 h. This killing activity was maintained at 24 h against the parental strain C2. In the transconjugant expressing FOX-5, bacterial regrowth was observed at 24 h (particularly at 1× MIC), although the reduction in the number of viable bacteria remained at > 2 $\log_{10}$ at that time. Imipenem and

Table 1. MICs and MBCs (mg/L) of cefepime, imipenem and meropenem against two strains of *Klebsiella pneumoniae* at three different inocula

		Cefepime inoculum (CFU/mL)			Imipenem inoculum (CFU/mL)			Meropenem inoculum (CFU/mL)		
Strain		10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
C2	MIC	0.5	1	1	0.5	0.5	2	0.25	0.5	1
	MBC	0.5	1	1	0.5	1	2	0.25	1	2
C2 (pMG252)	MIC	4	32	> 64	0.5	1	2	0.5	2	4
	MBC	32	> 64	> 64	0.5	1	4	0.5	2	4

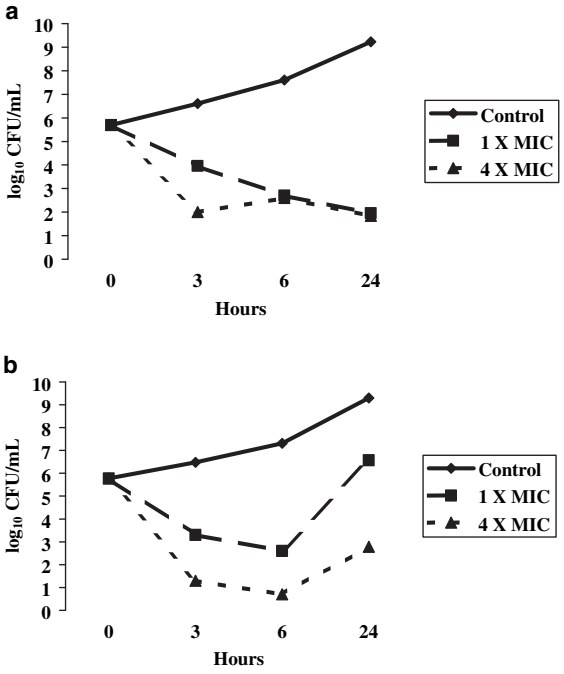

Fig. 1. Viable bacterial counts in time-kill curve assays with cefepime against (a) *Klebsiella pneumoniae* C2, and (b) *K. pneumoniae* C2 (pMG252) at 1× MIC or 4× MIC, in comparison with a cefepime-free culture (control).

meropenem were both bactericidal against the two tested strains after 3 h or 6 h (> 2 $\log_{10}$ reduction in viable bacteria compared to the control). No bacterial regrowth of *K. pneumoniae* C2 or C2(pMG252) was observed at 24 h with either imipenem or meropenem (data not shown).

Pharmacokinetic and histological studies

The pharmacokinetic/pharmacodynamic parameters (C_{max}, $t_{1/2}$, and ΔT/MIC) for each antimicrobial agent are shown in Table 2.

Lungs from untreated animals showed acute pneumonia, predominantly central, and atelectasis. The lungs appeared haemorrhagic and had gross inflammatory lesions. Microscopic examination revealed perivascular inflammatory cells (data not shown).

Therapeutic efficacy in experimental pneumonia

Survival

The low mortality rates in control groups precluded the use of mortality rate as an indicator of therapeutic efficacy (Tables 3 and 4).

Bacterial clearance from lung

There were no sterile lungs in either the control group or the treatment groups. The bacterial counts found in the lungs of the different groups

Table 2. C_{max} and $t_{1/2}$ after a single dose (60 mg/kg) of cefepime, imipenem or meropenem in guinea-pigs, and ΔT/MIC of the three agents for two strains of *Klebsiella pneumoniae*

Treatment	C_{max} (mg/L)	$t_{½}$ (h)	*K. pneumoniae* C2 ΔT/MIC (h)	*K. pneumoniae* C2(pMG252) ΔT/MIC (h)
Cefepime	176.5	0.64	2.46	2.41
Imipenem	21.6	0.66	2.32	2.32
Meropenem	43.9	0.47	2.26	2.25

Table 3. Effect of antibiotic therapy on the mortality and the clearance of *Klebsiella pneumoniae* C2 from the lungs in guinea-pigs

Treatment	Dosage	*n*	Mortality (%)	Log_{10} CFU/g of lung
Control	Saline	17	0	5.65 ± 0.82
Cefepime	240 mg/kg/day	10	0	4.41 ± 0.50[a]
Imipenem	240 mg/kg/day	10	20	4.65 ± 0.39[a]
Meropenem	240 mg/kg/day	12	8.3	4.72 ± 0.55[a]

n, number of animals tested.
[a]$p < 0.01$ compared to the control group.

Table 4. Effect of antibiotic therapy on mortality and the clearance of *Klebsiella pneumoniae* C2(pMG252) from the lungs in guinea-pigs

Treatment	Dosage	*n*	Mortality (%)	Log_{10} CFU/g of lung
Control	Saline	11	18	6.13 ± 0.58
Cefepime	240 mg/kg/day	11	0	4.54 ± 0.45[a]
Imipenem	240 mg/kg/day	12	0	4.04 ± 0.33[a,b]
Meropenem	240 mg/kg/day	12	0	4.68 ± 0.36[a]

n, number of animals tested.
[a]$p < 0.01$ compared to the control group; [b]$p < 0.05$ compared to cefepime or meropenem.

are shown in Tables 3 and 4. When animals infected with the parental strain C2 were considered, all the groups receiving treatment showed greater lung clearance of *K. pneumoniae* than did the control group ($p < 0.01$), but no significant differences were observed between the three antimicrobial agents. When guinea-pigs were infected with the transconjugant producing FOX-5, significantly higher lung clearance of *K. pneumoniae* was seen in animals treated with cefepime, imipenem or meropenem than in the control group ($p < 0.01$), and imipenem produced a greater reduction ($p < 0.05$) in the number of viable bacteria in the lungs than did either cefepime or meropenem.

Selection of resistant mutants

When the lung homogenates from three guinea-pigs infected with *K. pneumoniae* C2 and treated with cefepime were used to select resistant mutants, bacteria were recovered from plates containing 4× MIC of cefepime (at a frequency of *c*. 10^{-3}) and from gradient plates in the zone up to 4× MIC from two of the animals. MICs of cefepime against these mutants were in the range 4–16 mg/L (compared to an MIC of 0.5 mg/L for the parent strain). The β-lactamase activities (mU/mg of protein in crude sonicate) of the mutants evaluated were 313, 303, 233 and 219 mU/mg, compared to 5 mU/mg for *K. pneumoniae* C2 ($p < 0.01$). No resistant mutants derived from *K. pneumoniae* C2 were obtained from lung homogenates of animals treated with imipenem or meropenem. Similarly, no resistant mutants were obtained from any of the lung homogenates of the three animals treated with cefepime, imipenem or meropenem and infected with *K. pneumoniae* C2 (pMG252), either on plates containing 4× MIC of antibiotic or on gradient plates.

When TSB-grown bacteria were used for selection of resistant mutants, $> 10^3$ colonies grew from *K. pneumoniae* C2(pMG252) on plates containing 4× MIC of cefepime. Four independent colonies were selected from individual plates with bacterial growth, but in each case the MICs of the selecting agents were within one dilution step of the MIC for the corresponding parent strain. This indicated that none of the chosen colonies contained true (as defined for the purposes of this investigation) antibiotic-resistant mutants. However, no bacterial growth was obtained from the parent C2 strain, and no growth was obtained with either *K. pneumoniae* C2 or C2 (pMG252) when bacteria grown *in vitro* were used in an attempt to select imipenem- or meropenem-resistant mutants on plates containing 4× MIC of the corresponding agent. Similarly, no colonies were recovered from the gradient plates of any of the three β-lactams beyond the zone corresponding to 4× MIC of the corresponding agent; however, isolated colonies were observed from the MIC limit up to 4× MIC of cefepime.

DISCUSSION

PACBLs have been found in a variety of enterobacteria, but they seem to be particularly frequent among *K. pneumoniae* isolates from the hospital setting [1,5,6]. The spectrum of resistance observed among *K. pneumoniae* isolates expressing plasmid-mediated PACBLs is often similar to that of bacteria producing derepressed chromosomal AmpC enzymes; i.e., in-vitro susceptibility to zwitterionic cephalosporins and carbapenems unless other resistance mechanisms are present. Loss of both OmpK35 and OmpK36 porins, in combination with the presence of PACBLs, has resulted in decreased susceptibility to cefepime and resistance to both imipenem and meropenem [4]. In other cases, resistance to cefepime in *K. pneumoniae* producing PACBLs is the consequence of production by the same organisms of an extended-spectrum β-lactamase [7].

A previous study [4] showed that PACBL production in porin-deficient *K. pneumoniae* might cause in-vitro resistance to carbapenems. Cefepime (and in some cases cefpirome) was still active, unless a high inoculum was used. FOX-5 was unable to confer in-vitro resistance to carbapenems, but it increased the MIC of cefepime from 0.25 to 4 mg/L with an inoculum of 10^5 CFU/mL, and to > 64 mg/L with an inoculum of 10^7 CFU/mL. This was confirmed in the present study, where resistance to cefepime was also found with an inoculum of 10^6 CFU/mL.

A guinea-pig pneumonia model was chosen for the present study. This experimental model was described initially for the evaluation of different antimicrobial treatments and for investigating the pathogenesis of pneumonia caused by *Pseudomonas aeruginosa* [16–19]. The main advantages of this model are the easy handling of the animals in

the laboratory, the similarity between the immune systems of guinea-pigs and humans, and the absence of endemic colonisation of the respiratory tract, which is frequent with other laboratory animals such as rats [20]. Additionally, the guinea-pig model of experimental pneumonia allows meropenem to be evaluated, since, in contrast to what happens in the mouse experimental model, meropenem is not degraded by the renal dehydropeptidase I in guinea-pigs [21]. The pathological findings in the model confirmed that the animals developed pneumonia and that, independent of FOX-5 expression, bacterial counts in control animals ($0.45–1.35 \times 10^6$ CFU/g lung) were significantly higher than those in treated animals. The main limitations of the model are the absence of mortality and the difficulty in establishing a high bacterial inoculum when Gram-negative microorganisms other than *P. aeruginosa* are used in immunocompetent guinea-pigs.

The in-vivo activities of cefepime and carbapenems have also been evaluated in a non-lethal model of pneumonia caused by *Enterobacter cloacae* in immunocompetent rats [22,23]. It was proved that both cefepime and imipenem were effective against strains either producing a basal level of AmpC or hyperproducing AmpC, while ceftazidime was not active against the hyperproducing AmpC strain [22]. In another study by the same group [23], strains producing a basal level of both AmpC and the NmcA carbapenemase, and a derivative that overproduced both enzymes, were evaluated. It was observed that carbapenems were not useful, that ceftazidime only caused a significant decrease in bacterial counts in the lungs for strains producing basal levels of enzymes, and that only cefepime was effective against strains with both basal levels and overproduction of AmpC and NmcA β-lactamases.

The in-vitro data obtained on the activities of cefepime, imipenem and meropenem confirm and expand previous observations [4]. MIC values indicated good bacteriostatic and bactericidal activity for both imipenem and meropenem against *K. pneumoniae* C2, and this effect was not dependent on FOX-5 expression. In contrast, cefepime had high bacteriostatic and bactericidal activity against strain C2, but reduced activity (particularly with a high inoculum) against the transconjugant producing FOX-5. Time-kill studies supported those findings, in that bacterial regrowth of the FOX-5-producing strain was observed with cefepime at 24 h, but not with the two carbapenems. The clinical significance of the bacterial regrowth observed is unclear, but this observation reinforces the importance of an adequate cefepime administration schedule for the maintenance of concentrations above the MIC between dosage intervals [24].

An additional risk of therapy failure would result from the emergence of resistant mutants [25,26]. Two different approaches were used for mutant selection and, in general, similar results were obtained with both methods. Neither imipenem nor meropenem selected mutants from *K. pneumoniae* C2 or its FOX-5-producing derivative, with bacteria grown either *in vitro* or *in vivo*. Although in-vitro growth of *K. pneumoniae* C2(pMG252) was noted on plates containing 4× MIC of cefepime, these organisms did not seem to be actual mutants, and perhaps represented organisms able to survive the bactericidal activity of cefepime by unknown mechanisms related to the phenomenon of tolerance [27]. Additional studies with an in-vivo model in which a high lung inoculum is achieved would be needed to evaluate the clinical significance of these organisms. The only true stable mutants were selected by cefepime from *K. pneumoniae* C2 grown *in vivo*. MICs for these mutants increased 16–32-fold, and approached the recognised breakpoint for clinical resistance to cefepime. These mutants have not yet been characterised fully, but preliminary results indicate that they are β-lactamase overproducers. Strain C2 was derived originally from a clinical isolate producing two β-lactamases of pI 5.4 (compatible with TEM-1) and pI 7.6 (SHV-2). *K. pneumoniae* C2 has lost SHV-2, but still expresses the pI 5.4 enzyme [4]. The fact that similar mutants were not obtained from C2(pMG252) on plates containing 4× MIC could be explained by the much higher absolute concentration of cefepime (16 mg/L) involved.

All three β-lactams tested were shown to have in-vivo activity against *K. pneumoniae* C2, whether or not FOX-5 was produced, as inferred from the significantly lower bacterial counts in treated animals than in control animals. Doses of 240 mg/kg/day were chosen because they achieved similar ΔT/MIC of the three antimicrobial agents with both strains (37.5% and 41% of the dosing interval; Table 2) and pharmacodynamic parameters correlating with the antimicro-

bial activity of β-lactams in animal models [28]. Cefepime, imipenem and meropenem reduced the bacterial counts in lung tissues of animals infected by *K. pneumoniae* C2 by *c.* 1 $\log_{10}$. The reduction was even higher (*c.* 1.5–2 $\log_{10}$) for the strain producing FOX-5; in this case, the greatest reduction was noted with imipenem. This agent was also superior to cefepime in reducing the mortality rate caused by SHV-5-producing *K. pneumoniae* in a septic model in mice [29], in which the MICs of cefepime were 1 and >256 mg/L with inocula of 10^5 and 10^7 CFU/mL, compared with 0.125 and 0.5 mg/L for imipenem [29]. It is clear that FOX-5 expression is critical in decreasing the in-vitro activity of cefepime, but the reduction in the number of viable bacteria in lungs suggests that expression of this β-lactamase does not imply that cefepime would not be clinically useful, provided that pharmacodynamic parameters are maintained adequately.

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