Cefepime and amikacin synergy \textit{in vitro} and \textit{in vivo} against a ceftazidime-resistant strain of \textit{Enterobacter cloacae}

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The activities of cefepime and amikacin alone or in combination against an isogenic pair of \textit{Enterobacter cloacae} strains (wild type and stably derepressed, ceftazidime-resistant mutant) were compared using an experimental model of pneumonia in non-leucopenic rats. Animals were infected by administering $8.4 \log_{10}$ cfu of \textit{E. cloacae} intratracheally, and therapy was initiated 12 h later. At that time, the animals' lungs showed bilateral pneumonia and contained more than $7 \log_{10}$ \textit{E. cloacae} cfu/g tissue. Because rats eliminate amikacin and cefepime much more rapidly than humans, renal impairment was induced in all animals to simulate the pharmacokinetic parameters of humans. In-vitro susceptibilities showed an inoculum effect with cefepime proportional to the bacterial titre against the two strains, but more pronounced with the stably derepressed mutant strain, whereas with bacterial concentrations of up to $7 \log_{10}$ cfu/mL, no inoculum effect was observed with amikacin. In-vitro killing indicated that antibiotic combinations were synergistic only at intermediate concentrations. At peak concentrations, the combination was merely as effective as amikacin alone. At trough concentrations, a non-significant trend towards the superiority of the combination over each antibiotic alone was noted. Moreover, cefepime was either bacteriostatic or permitted regrowth of the organisms in the range of antibiotic concentrations tested. Although each antibiotic alone failed to decrease bacterial counts in the lungs, regardless of the susceptibility of the strain used, the combination of both antibiotics was synergistic and induced a significant decrease in the lung bacterial count 24 h after starting therapy when compared with tissue bacterial numbers in untreated animals or animals treated with either antibiotic alone. No resistant clones emerged during therapy with any of the antibiotic regimens studied.

Introduction

The incidence of infections caused by \textit{Enterobacter} spp. is increasing in clinical practice. A recent analysis of the National Nosocomial Infection Study data collected between 1986 and 1989 showed that these bacteria are now among the five most common nosocomial pathogens isolated in American hospitals, being responsible for about 9% of the infections in intensive care unit patients and 11% of lower respiratory tract infections. In Europe, the incrimination of \textit{Enterobacter} spp. in nosocomial infections is also increasing, accounting for about 8% of all nosocomial infections. These bacteria are also becoming more resistant. Their chromosomally encoded, inducible $\beta$-lactamase renders them intrinsically resistant to aminopenicillins and early cephalosporins. The greater enzyme stabilities and lower inducer activities of newer cephalosporins and the monobactams, however, made these drugs highly active against \textit{Enterobacter} spp. The organisms responded to these newer agents essentially by further increasing their enzyme levels through chromosomal mutations which led to the constitutive expression of very large amounts of enzymes of chromosomal origin. The organisms spontaneously mutate at a frequency of $10^{-6}$ to $10^{-8}$ within the susceptible bacterial populations. The stably derepressed

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mutants produce enough β-lactamase to resist all currently available β-lactams except carbapenems. A iterations of outer membrane permeability, by favouring hydrolysis of third-generation cephalosporins is and thus diminishing the amount of antibiotic in contact with the enzyme,⁸ are also associated with bacterial resistance.

Cefepime is a new fourth-generation cephalosporin that exhibits a broader spectrum of antimicrobial activity than the other new members of this drug family. It appears to have low in-vitro affinity for major chromosomally derived β-lactamases and good stability against enzymatic hydrolysis.⁴ A gain in comparison with older cephalosporins, cefepime was shown to cross the outer membrane more quickly.⁵ Overall, in a given time, more cefepime molecules penetrate into the periplasmic space, avoid β-lactamase attack and obtain access to the target molecules for which cefepime does not show better affinity.⁶ Finally, when cefepime was used to treat infections due to Enterobacter cloacae resistance emerged significantly less frequently than with older cephalosporins.⁷ To date, there are few in-vivo data confirming the efficacy of cefepime alone or in combination against stably derepressed mutants of Enterobacter spp.

The purpose of the present study was to compare the bactericidal activities of human regimens of cefepime and amikacin alone or in combination against an isogenic pair of E. cloacae strains (wild type and stably derepressed mutant) using a previously developed model of pneumonia in non-neutropenic rats.⁸

Materials and methods

Bacterial strains

An isogenic pair of E. cloacae strains (wild type, strain 474S; stably derepressed mutant, strain 474R, resistant to ceftazidime) kindly provided by Dr J. Caillon (Bacteriology Laboratory, Hôpital Nord, Nantes, France) was used for these studies. Each strain was stored at −70°C in Mueller–Hinton broth (BioMérieux, Marcy-l’Etoile, France) supplemented with 10% glycerol; fresh inocula were prepared for each experiment from cultures grown for 24 h in 10 mL of trypticase soy broth (BioMérieux).

Antibiotics

Cefepime and amikacin were purchased from Bristol-Myers Squibb (Paris, France). All antibiotics were kept in powder form and were freshly diluted with saline before each experiment in accordance with the manufacturer’s instructions. Cefepime and amikacin laboratory standards were used for in-vitro studies.

In-vitro studies

The MIC of each antibiotic was determined in Mueller-Hinton broth by means of a macrotube dilution method using geometric two-fold serial dilutions and inocula of 5 log₁₀, 6 log₁₀, 7 log₁₀ and 8 log₁₀ cfu/mL. The mean of four separate determinations performed on different days was used to calculate the MIC of each antibiotic. In-vitro time–kill curves for each antibiotic alone and in combination were determined in duplicate using cultures grown in Mueller–Hinton broth at 37°C. Three sets of drug doses were chosen to simulate those obtained in the animals’ lungs 1, 6 and 24 h after starting therapy. Stationary-phase organisms were obtained from a culture grown for 24 h in 10 mL of trypticase soy broth; the bacterial suspension was diluted 1/10 to obtain a final concentration of 7.8 log₁₀ cfu/mL. A liquid (0.1 mL) were removed 0, 3, 6 and 24 h after adding antibiotic solutions and quantitatively cultured using a Spiral Système plater (Interscience, Saint-Nom-la-Bretêche, France) after serial dilutions (up to 1/10⁶) to minimize the effect of antibiotic carryover. In-vitro antibiotic synergy was defined as a >100-fold increase of bacterial killing by the antibiotic combination relative to that achieved by the most active single agent.

Antibiotic assay

The cefepime concentration was determined using a modified version of an HPLC assay described elsewhere.¹⁰ The lower detection limit of the assay was 1 mg/L and the inter-day coefficient of variation ranged from 7% at 1 mg/L to 6% at 50 mg/L. The amikacin concentration was determined using an immunoenzyme assay (Emit; Syva, Dardilly, France). The lower detection limit of the assay was 1 mg/L and the coefficients of variation were <8% over the entire range of measurement.

Pneumonia model

We used an animal model previously developed by us and described in detail elsewhere.⁸ Briefly, animals were rendered renally insufficient by administering 1 mg/kg of uranyl nitrate (Merck, Darmstadt, Germany) subcutaneously, so as to simulate the pharmacokinetics of cefepime and amikacin in humans (both t₁/₂β~ 2 h), and infected 72 h later by administering a bacterial suspension of 8.4 log₁₀ E. cloacae cfu intratracheally. Previous studies had shown that, 12 h after bacterial inoculation, all animals had developed bilateral pneumonia with similar bacterial densities (>7.5 log₁₀ cfu/g tissue) in both lungs and an intense inflammatory reaction (data not shown).

Treatment regimens

Each strain used to induce pneumonia was studied separately. Of the total 150 animals studied (75 rats in each group), 59 wild type and 55 stably derepressed mutant strain recipients were still alive 12 h after bacterial...
inoculation; at this time, six from each group were killed to document that pneumonia had been established. The remaining rats were randomly assigned to one control (i.e. no antibiotic) group and three treatment groups. Treatment groups received intraperitoneal injections of cefepime alone (50 mg/kg bd), amikacin alone (18 mg/kg od) or a combination of both antibiotics given at the same dosages for a total of 24 h. We chose these dosages in order to obtain serum concentrations close to those observed in humans.

E valuation of antibiotic treatment

Subgroups of four to eight animals from the control group and each treatment group were killed 6 h and 24 h after therapy started. Blood was obtained by aortic puncture and the serum was stored in three aliquots at −20°C for determination of antibiotic and creatinine concentrations. Serum creatinine levels were determined to document that renal impairment was well established. Right lungs were removed aseptically, weighed and placed in 15 mL of an ice-cold mixture of Mueller–Hinton broth and glycerol (50:50) and homogenized (Ultraturax, Staufen, Germany) to obtain a suspension. The homogenate was quantitatively cultured after serial dilution on Drigalski agar (Bio-Mérieux) using a Spiral Système plater. After overnight incubation at 37°C, viable bacteria were counted and expressed as log_{10} cfu/g lung (lower limit of bacterial detection was 2.5 log_{10} cfu/g lung). In-vivo antibiotic synergy was defined as a bactericidal effect of the drug combination statistically significantly more pronounced than the sum of the bactericidal effects of each agent alone in comparison with untreated animals.11 Emergence of resistance during therapy was examined by plating the lung homogenates on to agar containing either cefepime (32 mg/L) or amikacin (16 mg/L). After incubation in air for 24 h at 37°C, emergence of resistant strain(s) was defined as growth of at least two colonies of E. cloacae. Left lungs were gently blotted with absorbent paper to remove blood and immediately placed in preweighed containers to prevent evaporation of water, the containers were then reweighed and the contents frozen at −20°C for later determination of antibiotic levels. After thawing and homogenization in 1 mL of saline, each sample was centrifuged at approximately 1000g for 10 min at 3°C and the supernatant was then assayed. A ntibiotic measurements were not corrected for blood contamination as blood represents <6% of lung weight and neither antibiotic accumulates in red blood cells; therefore, the presence of blood does not lead to substantial errors in the evaluation of tissue cefepime or amikacin concentrations.12

D ata analysis

Results are expressed as medians and their ranges. A ntibiotic concentrations and lung bacterial counts in the control and treatment groups were compared using one way non-parametric analysis of variance (Kruskal–Wallis test); when the value of this test was statistically significant, each treatment group was compared with the control group and each of the other treatment groups using the Mann–Whitney U-test. For all tests, a P value of <0.05 was considered significant.

R esults

I n-vitro studies

The MICs of cefepime and amikacin at various initial bacterial concentrations are given in Figure 1. As expected, an inoculum effect proportional to the bacterial titre was observed with cefepime against the two strains, but more pronounced with the stably derepressed mutant strain, whereas with bacterial concentrations of up to 7 log_{10} cfu/mL, no inoculum effect occurred with amikacin. A mikacin bacterial killing was very fast only at peak concentration, whereas cefepime was either bacteriostatic or allowed regrowth of the organisms in the range of concentrations tested (Figure 2). Significantly increased killing by the combination was obtained only with the intermediate concentrations of antibiotics (cefepime 16 mg/L, amikacin 4 mg/L; at peak concentrations (cefepime 64 mg/L, amikacin 16 mg/L), the combination was merely

![Figure 1. MICs of cefepime (a) and amikacin (b) against an isogenic pair of E. cloacae (□, wild type; ■, stably derepressed mutant) using various initial bacterial concentrations. Breakpoints for determination of resistance are as follows: cefepime >32 mg/L, amikacin >16 mg/L.](image-url)
as effective as amikacin alone and at trough concentrations (cefepime 4 mg/L, amikacin 1 mg/L), an insignificant trend towards the superiority of the combination over each antibiotic alone was noted (Figure 2).

Pharmacokinetic analysis

Serum creatinine levels measured 6 h and 24 h after starting therapy were not statistically different between the four study groups, indicating that renal impairment was identical, regardless of the treatment given (data not shown). Lung and plasma concentrations of both antibiotics, 6 h and 24 h after beginning therapy, did not differ significantly when each antibiotic was administered alone or in combination. Results for each antibiotic used alone or in combination were then pooled to simplify the presentation (Table I).

Efficacy of therapy

The six animals from each study group killed at the start of therapy (0 h) had bilateral pneumonia, with mean E. cloacae counts of log_{10} 7.9 (range 7.5–8.3) and 8.2 (range 7.5–8.4) cfu/g lung for the wild type and the stably derepressed mutant strains, respectively (Table II). All untreated animals showed slight decreases in their numbers of bacteria 6 and 24 h after the theoretical start of therapy. All treatment groups had the same or slightly lower bacterial

<table>
<thead>
<tr>
<th>Time (h) after starting therapy</th>
<th>Cefepime</th>
<th>Aminoglycoside</th>
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<tbody>
<tr>
<td></td>
<td>plasma</td>
<td>lung tissue</td>
</tr>
<tr>
<td></td>
<td>n median range</td>
<td>n median range</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
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<tr>
<td>24</td>
<td>26</td>
<td>&lt;1-10</td>
</tr>
</tbody>
</table>

Figure 2. Time–kill curves for the isogenic pair of E. cloacae (top, wild type; bottom, stably derepressed mutant) with test antibiotics at three set drug concentrations. A total of 7.8 log_{10} cfu/mL were incubated for 24 h with no antibiotic (□), cefepime alone (○), amikacin alone (◇), and combined antibiotic therapy ( △) given at the following concentrations: (a) cefepime 64 mg/L and amikacin 16 mg/L; (b) cefepime 16 mg/L and amikacin 4 mg/L; (c) cefepime 4 mg/L and amikacin 1 mg/L.
Cefepime against ceftazidime-resistant
E. cloacae

counts than untreated animals. This decrease was sig-
nificant only at 6 and 24 h for animals receiving cefepime
and amikacin in combination. Significantly decreased
bacterial titres were also observed at 24 h for animals
receiving combined antibiotic therapy, compared with
those receiving each antibiotic alone. In-vivo synergy
between cefepime and amikacin was observed at the two
times studied. No clone resistant to cefepime or amikacin
was detected in any of the antibiotic-treated animals.

Discussion

We used our experimental model of E. cloacae pneumonia
developed in non-neutropenic rats to study the in-
vivo efficacy of cefepime alone or in combination with
amikacin against an isogenic pair of E. cloacae
strains (wild type and stably derepressed mutant). Because
cefepime and amikacin pharmacodynamics are highly
dependent upon residence time, renal impairment was in-
duced in all the animals so as to simulate conditions
observed in humans. This model has previously been
shown to confront the antibiotics with the most difficult
conditions: initial bacterial concentrations in the lungs are
high, and initiation of therapy is delayed after bacterial
inoculation, when a predominance of the bacterial popula-
tion is in the stationary phase of growth. Comparing the bactericidal activity of human regimens of cefepime,
amikacin and combined antibiotic therapy for a total period of 24 h, we found that, regardless of the strain
used, each antibiotic alone or in combination with another antibiotic, significantly decreased lung bacterial
counts more than untreated animals. This decrease was sig-
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was detected in any of the antibiotic-treated animals.

Table II. Bacterial counts observed in untreated animals and in those receiving either cefepime alone (50 mg/kg bd), amikacin alone (18 mg/kg od) or both antibiotics

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Lung titre (log₁₀ E. cloacae cfu/g tissue) at the indicated time after starting therapy</th>
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<tbody>
<tr>
<td></td>
<td>ceftazidime-susceptible strain</td>
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<tr>
<td></td>
<td>0 h</td>
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<td></td>
<td>n</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>Cefepime</td>
<td>6</td>
</tr>
<tr>
<td>Amikacin</td>
<td>6</td>
</tr>
<tr>
<td>Combination</td>
<td>6</td>
</tr>
</tbody>
</table>

Significantly lower bacterial titres than untreated animals.

Significantly lower bacterial titres than untreated animals or animals treated with each antibiotic alone.
the inoculum effect and low antibiotic concentration could explain the weak in-vivo reduction of the lung bacterial titre, even when both antibiotics were used in combination, and could be predicted by our time-kill curves (Figure 2). These two phenomena could be important factors in the management of infections involving high bacterial concentrations, suggesting that increasing the antibiotic dose and using antibiotic combinations could be advantageous when treating such infections.

To date, there are not enough data to propose cefepime as first-line therapy against stably derepressed mutant strains of *E. cloacae*. However, the bactericidal activity of cefepime alone or in combination with amikacin against the wild-type and stably derepressed mutant strains was comparable in our study, despite a lower in-vitro susceptibility of the resistant strain to cefepime. These encouraging results are supported by a recent clinical study, in which 15 of 17 infections due to *Enterobacter* spp. with low susceptibility or resistance to ceftazidime, but susceptible to cefepime, were successfully treated with cefepime; in particular, cefepime was successfully used in the management of chronic infections that had responded poorly to repeated therapy with imipenem, aminoglycosides or ciprofloxacin. Further larger studies are warranted to confirm these preliminary results before the use of cefepime alone or in combination with amikacin be proposed for the treatment of such infections.

Acknowledgement

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References


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