Levofloxacin/imipenem prevents the emergence of high-level resistance among *Pseudomonas aeruginosa* strains already lacking susceptibility to one or both drugs

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Received 11 October 2005; returned 6 December 2005; revised 10 February 2006; accepted 14 February 2006

**Objectives:** Previous studies have demonstrated that a combination of levofloxacin with imipenem could prevent the emergence of resistance during the treatment of susceptible *Pseudomonas aeruginosa* isolates in a two-compartment pharmacodynamic model of infection. In this study, the efficacy of levofloxacin/imipenem was further evaluated against a panel of characterized *P. aeruginosa* strains that lacked susceptibility to one or both drugs in the combination.

**Methods:** Five *P. aeruginosa* strains with characterized resistance mechanisms were evaluated. Log-phase cultures were inoculated into the peripheral compartment of the in vitro pharmacokinetic model and treated using simulated doses of 750 mg levofloxacin (dosed every 24 h) and 250 mg or 1 g doses of imipenem (dosed every 12 h). Peak levels were adjusted for protein binding. Pharmacodynamic interactions were evaluated by measuring the changes in viable counts over 30 h. To evaluate the emergence of resistance, samples removed at 30 h were plated onto agar containing the drug at 4·MIC, and potential mutants were evaluated for changes in susceptibility.

**Results:** Against strains overexpressing MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps, levofloxacin/imipenem prevented the emergence of resistance and achieved a 5 log total kill of one strain and eradication of two strains. Levofloxacin/imipenem also eradicated an imipenem-resistant strain lacking OprD. Although the combination initially killed 6–7 logs of a dual-resistant strain lacking OprD and overexpressing MexXY, it could not prevent the emergence of resistance when the 250 mg dose of imipenem was simulated in the combination. However, when the 1 g dose of imipenem was simulated with the combination, resistance was suppressed.

**Conclusions:** These data suggest that levofloxacin/imipenem may be an effective combination for preventing the emergence of resistance among *P. aeruginosa*, even with strains already lacking susceptibility to one or both drugs in the combination. Clinical evaluation of this combination is warranted.

Keywords: antibacterials, combinations, pharmacodynamics

**Introduction**

Data from a previous study suggested that a combination of levofloxacin with imipenem would be effective in preventing the emergence of resistance among susceptible *P. aeruginosa*. The rationale for this combination was that fluoroquinolone-selected resistance involves mutational changes within the fluoroquinolone targets or mutations leading to overexpression of multidrug efflux pumps or both. Target mutations do not affect the activity of imipenem. Likewise, mutants overexpressing MexAB-OprM, MexCD-OprJ and MexXY-OprM efflux pumps do not lose their susceptibility to imipenem. In comparison, imipenem-selected resistance involves mutations that specifically decrease the expression of the OprD porin in the outer membrane, a mechanism that does not alter susceptibility to fluoroquinolones.

In the first study, levofloxacin/imipenem prevented the emergence of resistance in three susceptible *P. aeruginosa* clinical isolates during treatment in a two-compartment *in vitro* pharmacokinetic model (IVPM). Surprisingly, levofloxacin/imipenem prevented the emergence of resistance even when mutant subpopulations overexpressing the MexEF-OprN efflux pump were
present. Although imipenem is not a substrate for this pump, mutants overexpressing MexEF-OprN exhibit a concurrent decrease in the production of OprD and a loss of susceptibility to both the fluoroquinolones and imipenem. Although these data were promising, they did not address the question of whether the combination would be effective against _P. aeruginosa_ strains already lacking susceptibility to one or both drugs in the combination. Therefore, a study was designed to evaluate the pharmacodynamics of levofloxacin/imipenem against _P. aeruginosa_ strains expressing characterized mechanisms of resistance.

**Materials and methods**

**Bacterial strains and culture conditions**

Five characterized _P. aeruginosa_ strains were evaluated (Table 1). The _P. aeruginosa_ strains selected for this study exhibited a broad range of resistance mechanisms expected to provide a therapeutic challenge for the combination. Since this was not an isogenic panel of mutants, it is possible that other factors influenced susceptibility. Therefore, data were not analysed to evaluate direct correlations between specific resistance mechanisms and pharmacodynamic interactions. Logarithmic-phase cultures were prepared by inoculating colonies from overnight agar cultures into 70 mL of Mueller–Hinton broth to equal an optical density at 540 nm of 0.1. Cultures were then incubated at 37°C with shaking until the optical density increased to 0.4. Logarithmic-phase cultures were diluted 10-fold into fresh 37°C broth to give a final inoculum between 5 × 10^7 and 1 × 10^8 cfu/mL.

**Antibiotics and susceptibility testing**

Levofloxacin was obtained from Ortho-McNeil Pharmaceutical Co. and imipenem was obtained from Merck Co. Susceptibility was evaluated using the agar dilution method.  

**Pharmacodynamic experiments**

The IVPM used in these studies has been described in detail. Logarithmic-phase cultures (5 × 10^7 to 1 × 10^8 cfu/mL) were introduced into the peripheral compartment of the IVPM and were treated with simulated human doses of 750 mg of levofloxacin (dosed every 24 h), 250 mg of imipenem (dosed every 12 h) and a combination of levofloxacin with imipenem. The 250 mg dose of imipenem was dosed every 12 h, as opposed to standard recommended dosing intervals, to promote the emergence of resistance and to provide a greater therapeutic challenge for the combination. In studies using _P. aeruginosa_ strain 289, which exhibited the lowest susceptibility to imipenem, the maximum 1 g dose of imipenem (dosed every 12 h) was also evaluated alone and in combination. Targeted peak concentrations were adjusted for protein binding (25% for levofloxacin and 20% for imipenem), and were 6.5 mg/L for levofloxacin and 15 mg/L for the 250 mg dose of imipenem and 58 mg/L for the 1 g dose of imipenem. Antibiotic concentrations were confirmed using bioassay.

Pharmacodynamic interactions were evaluated by removing 0.5 mL samples from the peripheral compartment at 0, 2, 4, 6, 12, 24 and 30 h and incubating the samples with Amberlite antibiotic-removal beads for 15 min to prevent antibiotic carryover. Total viable bacterial counts were measured by plating 1 mL of serial 10-fold dilutions into Mueller–Hinton agar (limit of detection 10 cfu/mL). In addition, samples taken at 30 h were plated into agar containing antibiotic (4× MIC) to detect mutants with significantly decreased susceptibility. Potential mutants were evaluated for changes in susceptibility using the agar dilution method.  

**Results and discussion**

**Pharmacodynamics against _P. aeruginosa_ K-1455 overexpressing MexAB-OprM**

_P. aeruginosa_ K-1455 is a MexAB-OprM-overexpressing laboratory-generated mutant, kindly provided by Keith Poole (Department of Microbiology and Immunology, Queen’s University, Kingston, Ontario, Canada). Strain K-1455 is intermediately resistant to levofloxacin and susceptible to imipenem (Table 1). Levofloxacin treatment provided a rapid 4 log kill by 6 h, but thereafter viable counts increased rapidly to >10^9 cfu/mL (Figure 1a). Rapid regrowth was associated with the selection of a fully resistant subpopulation (levofloxacin MIC of 16 mg/L). In contrast, both imipenem and levofloxacin/imipenem continued to decrease viable counts throughout the 30 h experimental period. Killing with imipenem reached 4.5 logs by 30 h, whereas levofloxacin/imipenem achieved total eradication. Although the extent of killing with levofloxacin/imipenem exceeded that of imipenem alone, the prevention of resistance by the combination appears to be related to the imipenem alone.

**Pharmacodynamics against _P. aeruginosa_ 164CD-921C overexpressing MexCD-OprJ**

_P. aeruginosa_ 164CD-921C is a MexCD-OprJ-overexpressing mutant selected with ciprofloxacin from strain 164CD (fully derepressed for AmpC production). 11 164CD-921C shows

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**Table 1. Characteristics and antibacterial susceptibility of _P. aeruginosa_ strains**

<table>
<thead>
<tr>
<th><em>P. aeruginosa</em> strain number</th>
<th>Characterized resistance mechanism</th>
<th>Levo (mg/L)</th>
<th>Imip (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-1455</td>
<td>MexAB-OprM overexpression</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>164CD-921C</td>
<td>MexCD-OprJ overexpression</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>PAO1-Tokai#1</td>
<td>MexEF-OprN overexpression and OprD down-regulation</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>244</td>
<td>OprD deficient</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>289</td>
<td>MexXY overexpression and OprD deficient</td>
<td>4</td>
<td>32</td>
</tr>
</tbody>
</table>

*a* Efflux pump overexpression was confirmed using RT–PCR analysis. OprD down-regulation and deficiency was confirmed using RT–PCR analysis and SDS–PAGE analysis of outer membrane proteins.  

*b* MICs were measured using the agar dilution method.  

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**Figure 1.** Pharmacodynamics of levofloxacin, imipenem and a combination of levofloxacin with imipenem against *P. aeruginosa* K-1455 (a), *P. aeruginosa* 164CD-921C (b), *P. aeruginosa* PAO1-Tokai#1 (c), *P. aeruginosa* 244 (d) and *P. aeruginosa* 289 (e). Each data point represents the mean number of viable bacterial counts for duplicate experiments. Error bars represent the standard deviation.
Resistance to levofloxacin through the overexpression of MexCD-OprJ, and the 1 mg/L imipenem MIC is 4-fold lower than the MIC for the parent strain 164CD. Both levofloxacin and imipenem rapidly decreased the viable counts almost 4 logs by 4 h (Figure 1b). With levofloxacin-treated cultures, viable counts increased rapidly between 4 and 24 h until they exceeded $10^9$ cfu/mL. Although there was a delay of regrowth in cultures treated with imipenem, viable counts exceeded $10^9$ cfu/mL by 30 h. Treatment with levofloxacin and imipenem alone was associated with the emergence of resistance. The MIC of levofloxacin against the levofloxacin-selected mutant was 32 mg/L, a 4-fold increase over the parent strain. The MIC of imipenem against the levofloxacin-selected mutant was 8 mg/L, an 8-fold increase over the parent strain. In contrast to each drug alone, the combination of levofloxacin with imipenem prevented the emergence of resistance and achieved eradication by 30 h.

**Pharmacodynamics against P. aeruginosa PAO1-Tokai#1 overexpressing MexEF-OprN**

P. aeruginosa PAO1-Tokai#1 is a MexEF-OprN-overexpressing laboratory-generated mutant from strain PAO1, kindly provided by Taiji Nakae (Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Japan). Strain PAO1-Tokai#1 is intermediately resistant to both levofloxacin (4 mg/L) and imipenem (8 mg/L), and displays one of the phenotypes hypothesized to be a problem for the combination of levofloxacin with imipenem (see Introduction). Levofloxacin rapidly decreased viable counts of P. aeruginosa PAO1-Tokai#1 4.5 logs by 4 h, but viable counts increased rapidly thereafter and exceeded $10^9$ cfu/mL by 24 h (Figure 1c). Imipenem decreased viable counts 3 logs by 2 h, and although there was a delay in regrowth, viable counts approached $10^9$ cfu/mL by 30 h. Emergence of full resistance (MIC of 32 mg/L) was associated with both levofloxacin and imipenem treatment. In contrast, the combination of levofloxacin with imipenem suppressed the emergence of mutational resistance throughout the 30 h experimental period. Although strain PAO1-Tokai#1 was not eradicated, over 5 logs of killing was observed within 4 h and viable counts changed very little thereafter. The decreased antibacterial activity observed between 12 and 30 h, despite additional doses of each drug, is most likely the result of adaptive resistance. Adaptive resistance is the reversible decrease in susceptibility of bacterial cells to an antibiotic upon repeated exposure.

The ability of levofloxacin/imipenem to suppress the emergence of full mutational resistance from this strain further supports previous data demonstrating the ability of the combination to prevent emergence of MexEF-OprN-associated resistance from fully susceptible isolates. Furthermore, these data suggest that this unique phenotype may not be a therapeutic problem for levofloxacin/imipenem.

**Pharmacodynamics against P. aeruginosa 244 deficient in OprD production**

P. aeruginosa 244 is a levofloxacin-susceptible clinical isolate expressing imipenem resistance through the lack of a functional OprD porin in its outer membrane. Surprisingly, the first dose of imipenem provided 4.5 logs of killing by 6 h (Figure 1d). However, from that point onward there was a rapid increase in viable counts to $>10^9$ cfu/mL and very little change in viable counts with the second dose. Inoculum regrowth was not due to the selection of mutants expressing higher levels of resistance. Levofloxacin rapidly decreased viable counts $>6$ logs within 4 h, but by 6 h it appeared as if the cultures had been eradicated. However, between 12 and 24 h there was a rapid increase in viable counts, and this rapid regrowth was due to the emergence of a mutant subpopulation exhibiting an 8-fold decrease in susceptibility to levofloxacin. The pharmacodynamics of levofloxacin/imipenem were very similar to that of levofloxacin alone for 12 h. However, in contrast to levofloxacin alone, total eradication was achieved with levofloxacin/imipenem. These data suggest that the combination of levofloxacin with imipenem may be effective in preventing the emergence of resistance in levofloxacin-susceptible P. aeruginosa, even when the strains express imipenem resistance through loss of OprD from the outer membrane.

**Pharmacodynamics against P. aeruginosa 289 overexpressing MexXY and deficient in OprD production**

P. aeruginosa 289 was the most challenging strain for the combination. This strain is a clinical isolate expressing intermediate resistance to levofloxacin through overexpression of MexXY and resistance to imipenem through loss of OprD from its outer membrane.

The 250 mg dose of imipenem alone exhibited very little antibacterial activity against this OprD-deficient isolate, whereas the 1 g dose of imipenem provided 4.5 logs of killing over the
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Initial 6 h, before regrowth brought numbers up to the range of the initial inoculum (Figure 1e). Levofoxacin provided almost 6 logs of killing by 12 h. Thereafter, rapid regrowth was observed due to the outgrowth of a resistant subpopulation. The levofoxacin MIC against this resistant subpopulation was 64 mg/L, a 16-fold increase compared with the parent strain. In contrast to other strains in this study, the combination of levofoxacin with imipenem (250 mg imipenem) was unable to suppress the emergence of this highly resistant subpopulation and the pharmacodynamics of the combination were similar to that of levofoxacin alone. However, when the 1 g dose of imipenem was simulated with the combination, regrowth decreased substantially, no resistant subpopulations were detected on 30 h drug-selection plates and viable counts were maintained almost 5 logs below the initial inoculum.

In summary, levofoxacin with imipenem appears to be an effective combination for preventing the emergence of antibacterial resistance during therapy against P. aeruginosa. Not only is the combination effective against fully susceptible isolates, but data from this study also demonstrate the effectiveness of levofoxacin with imipenem against strains already lacking susceptibility to one or both drugs in the combination. The greatest challenge for the combination appears to be strains exhibiting high-level resistance to one drug in the combination while lacking susceptibility to the other drug. Nevertheless, clinical evaluation of levofoxacin/imipenem is warranted, and further experiments evaluating the ability of levofoxacin/imipenem to prevent emergence of resistance for longer than 30 h are required. Furthermore, the effectiveness of ciprofloxacin in combination with imipenem should be evaluated due to its antipseudomonal potency.

Acknowledgements

We would like to thank Keith Poole and Taiji Nakae for their kind gifts of strains used in this study. In addition, the authors would like to thank Jennifer Black for her technical assistance with susceptibility testing assays. This study was funded by a grant from Ortho-McNeil Pharmaceutical Company.

Transparency declarations

The corresponding author has received research grant support from both Merck and Ortho-McNeil, and also honoraria for lectures and symposia sponsored by Ortho-McNeil.

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