Selectivity of ertapenem for Pseudomonas aeruginosa mutants cross-resistant to other carbapenems

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Objectives: Ertapenem and other carbapenems will be used increasingly, as extended-spectrum β-lactamases become more prevalent even among community-acquired pathogens. There is, however, concern that this use will select for resistances to imipenem and meropenem in nosocomial pathogens, notably Pseudomonas aeruginosa, and we investigated the validity of this concern.

Methods: Single-step selection experiments were performed by plating P. aeruginosa cultures on to agar containing doubling dilutions of ertapenem. MIC patterns, outer membrane protein profiles and the effects of efflux inhibitors were examined for selected mutants.

Results: At 2–8 × MIC, ertapenem selected (i) for OprD- mutants of P. aeruginosa, with cross-resistance only to carbapenems, (ii) for putative efflux types with broader cross-resistance, and also (iii) for various less familiar phenotypes. Efflux mutants were predominantly, but not exclusively, selected from carbenicillin-hypersusceptible strains and OprD- mutants largely from strains with normal levels of carbenicillin susceptibility. Whilst these data indicate potential cross-selectivity, they must be set against the observation that 20% serum raised the ertapenem MICs, and the drug concentrations needed for mutant selection, by over four-fold, reflecting the compound’s strong protein binding. Since, following a 1 g intravenous dose the free ertapenem concentration in the serum falls below 4 mg/L—corresponding to the lower of two MIC<sub>50</sub> estimates—within 4 h (17% of the dosage interval) selectivity in vivo should be minimized.

Conclusions: Whilst ertapenem can select for P. aeruginosa mutants with cross-resistance to imipenem and ertapenem in vitro, this selectivity should be minimal under clinical conditions.

Keywords: OprD, efflux, mutations

Introduction

Ertapenem is a new carbapenem, differing from imipenem and meropenem in having only weak activity against Pseudomonas and Acinetobacter spp. and in requiring only once-daily administration. It is marketed for use in severe community-acquired infections, where non-fermenters are unlikely, and is licensed for intra-abdominal infections, community-acquired pneumonia and acute pelvic infection. In addition, except in the European Union, it is licensed for skin and soft tissue infections and for complicated urinary infections. Ertapenem use seems likely to be driven by the changing epidemiology of extended-spectrum β-lactamases (ESBLs), which are now disseminating outside hospitals. In particular, Escherichia coli with CTX-M ESBLs are being reported in the gut flora and as agents of urinary infection in community patients in Europe, the Middle East, East Asia and North America. Many E. coli isolates with CTX-M β-lactamases are susceptible only to carbapenems, nitrofurantoin and fosfomycin.

Ertapenem is clinically effective, and might be used empirically in infections and locales where ESBL producers are likely. Nevertheless, there is concern that extensive first-line use will select cross-resistance to imipenem and meropenem, which are the last good defences against many nosocomial infections caused by multiresistant pathogens. Particular concern is expressed about selection of carbapenem resistance in Pseudomonas aeruginosa, a species with a well-known propensity to develop resistance to imipenem and reduced susceptibility to meropenem via loss of porin OprD. P. aeruginosa also can develop reduced susceptibility to meropenem, along with

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resistance to fluoroquinolones and most β-lactams except imipenem, via up-regulation of MexAB-OprM-mediated efflux. We examined the selectivity of ertapenem for these and other mutant types at clinically relevant concentrations.

Materials and methods

P. aeruginosa strains with graded intrinsic resistance

Isolates (n = 45) were from our strain collection, assembled via previous surveys and referrals, and were chosen on the basis of their graded level of ‘intrinsic’ resistance to carbenicillin. These organisms lacked acquired β-lactamases (or, in one case, had TEM-2 enzyme), and were not derepressed for AmpC. Representatives were previously shown to vary in efflux function, but not in penicillin-binding protein profile or affinity.

Isolates with carbenicillin MICs of ≤ 16 mg/L and with unusually low MICs of other β-lactams were graded as ‘hypersusceptible’; those with carbenicillin MICs of 32–256 mg/L as ‘normal’; and those with MICs of ≥ 512 mg/L as ‘highly resistant’. Strain R58, with carbenicillin resistance owing to TEM-2 β-lactamase, was included with the hypersusceptible group on the grounds of exceptionally low MICs of non-substrate β-lactams.

Minimum inhibitory concentrations

MICs were determined by the BSAC agar dilution method using Iso-Sensitest agar (Oxoid, Basingstoke, UK) with ertapenem and imipenem (Merck, Hoddesdon, UK), meropenem (AstraZeneca, Macclesfield, UK), carbenicillin, ceftazidime, nalidixic acid and tetracycline (Sigma, Poole, UK), piperacillin plus 4 mg/L tazobactam (Wyeth, Taplow, UK), and aztreonam and cefepime (Bristol-Myers Squibb, Hounslow, UK). In experiments to investigate the effects of serum binding, the agar was prepared at 125% of recommended strength and diluted with water or with heat-inactivated donor horse serum (TCS, Buckingham, UK) immediately before the plates were poured. In experiments to test the role of efflux, Iso-Sensitest agar was supplemented with phenylalanine–arginine–β-naphthylamide (Sigma) at 32 mg/L.

Selection of resistant mutants with ertapenem

Single-step mutants were selected by plating 100 μL volumes of overnight broth cultures (∼5 × 10^8 cfu) onto Iso-Sensitest agar containing ertapenem at doubling concentrations from 2–16 MIC. The resulting collection was supplemented with mutants selected previously by similar methodology but on Mueller–Hinton agar. Mutation frequencies were calculated based on the number of colonies that grew on the selective agar, with duplicate plate counts on antibiotic-free agar as a denominator. In some experiments, selection was performed in the presence of 20% heat-inactivated horse serum, with the medium prepared as for MIC determinations above, and with the selective concentrations re-based on the MICs in the presence of serum. In other experiments, the selective concentrations used were based on the serum drug levels, calculated or published, to remain 8–12 h post-dose.

Outer membrane protein profiles

Outer membranes were extracted as Sarkosyl-insoluble fractions from logarithmic phase cells; their preparation and electrophoresis were as described previously.

Results

Effect of serum on carbapenem MICs for P. aeruginosa

MICs were determined for 45 P. aeruginosa strains varying in intrinsic (i.e. efflux-determined) resistance in the presence and absence of 20% serum. Ertapenem and meropenem MICs were higher in the presence of the serum, whereas those of imipenem were unaffected (Figure 1). For ertapenem, the mode MIC rose from 4 mg/L without serum to 32 mg/L in its presence, while the geometric mean MIC rose from 6.4 to 27.9 mg/L; corresponding rises for meropenem were from 0.25 to 0.5 mg/L for the mode and from 0.5 to 1.3 mg/L for the geometric mean; the mode for imipenem was constant at 1 mg/L and the geometric mean fell from 0.9 to 0.8 mg/L with serum. The magnitude of the serum effect for individual strains was independent of their starting level of intrinsic resistance. As reported previously, MICs of ertapenem and meropenem were raised for the strains with increased intrinsic resistance to carbenicillin, whereas those of imipenem were little affected.

Selection of mutants at MIC multiples

Mutants were obtained from 12 of the 14 strains studied (Table 1) with one of the exceptions being strain H, which
Table 1. Frequency and phenotypes of ertapenem-selected mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Categorya</th>
<th>MIC (mg/L)</th>
<th>Mutation frequency with ertapenem at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Erta- penem</td>
<td>Imi- penem</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>hs mucoid</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>J (AHP)</td>
<td>hs</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td>K (R58)</td>
<td>hs</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>L (799wt)</td>
<td>n</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>n</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>n</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>n</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>n</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>n mucoid</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>n mucoid</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>n OprD−</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>M (PAO1)</td>
<td>n</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>N (M1251)</td>
<td>hr</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>O (M76)</td>
<td>hr</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
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</tbody>
</table>

*aIn relation to carbenicillin MIC: hs, hypersusceptible (MIC ≤ 16 mg/L); n, normal (MIC 32–256 mg/L); hr, highly resistant (≥ 512 mg/L).
bMICs increased for carabapenems only; OprD absent.
cMICs raised for all β-lactams tested except imipenem.
began as OprD deficient and imipenem resistant. Mutation frequencies to resistance with selective concentrations of two to four times the starting MIC of ertapenem were generally between $10^{-6}$ and $10^{-8}$, or lower, although higher rates (up to $1.8 \times 10^{-5}$) were seen for strain R58, the otherwise hypersusceptible strain with TEM-2 β-lactamase. Mutants were obtained, at diminishing frequencies, at up to eight times the starting MIC of ertapenem, but not at 16 times the starting MIC. When 20% inactivated horse serum was included in the medium, the selective concentrations had to be raised commensurately, typically by four-fold. MIC testing and membrane profiling showed no difference between the mutant phenotypes selected with and without serum and these groups were pooled for further analysis.

A total of 133 selected mutants were retained from the present and previous studies. These were passaged through antibiotic-free medium and subjected to susceptibility testing. One hundred and fifteen were confirmed to be at least two-fold more resistant to ertapenem than their parent strains; MICs for the remaining 18 mutants had reverted to the parental values, and these organisms were discarded. The hypersusceptible strains largely yielded mutants with cross-resistance, or reduced susceptibility, to cephalosporins, meropenem and carbencillin but not imipenem. In contrast, most of the mutants selected from the strains with normal susceptibility and high-level resistance to carbencillin were cross-resistant only to imipenem and meropenem, not to non-carbapenems. These trends were not, however, absolute and exceptions were seen. In particular, some of the mutants of ‘normal’ strains (especially strain 779wt) had the broader cross-resistance, sparing imipenem. In addition, whereas one of the two highly resistant strains, M1251, yielded mutants resistant only to carbapenems, the other (M76) consistently gave those with ≥ 4-fold increased MICs of both carbapenem and non-carbapenem β-lactams.

To understand better the mechanisms present in these various mutant phenotypes we profiled outer membrane proteins for 41 of them (Table 1), particularly seeking the presence or absence of OprD (D2), the ‘carbapenem-specific’ porin. Mutants with the broad-spectrum resistance were mostly found to retain OprD, as illustrated for variants of strain G in Figure 2. Such behaviour is consistent with a general increase in efflux. This is probably mediated by up-regulation of MexAB-OprM, although we cannot exclude involvement of other systems. Mutants resistant only to carbapenems, on the other hand, were mostly found to have lost OprD, as is apparent for most mutants of strain F (Figure 2), but other patterns were also seen. Thus (i) one mutant of strain F (Figure 2, track j) retained OprD and had substantial (eight- to 16-fold) MIC rises for ertapenem and meropenem, but minimal shifts (two-fold or less) for imipenem and non-carbapenem β-lactams, and (ii) mutants of strain M76 (Figure 2, tracks p–s) with approx. four-fold increased resistance to all β-lactams, retained OprD but also expressed a rather larger outer membrane protein (~54 kDa), which may or may not be the exit portal for an efflux system. These latter mutants also had increased expression of a 27 kDa protein, putatively G, a component that is normally rather variable in its expression in the species. Attempts to confirm efflux-based mechanisms further by comparing MICs of carbencillin and ertapenem in the presence and absence of phenylalanine–arginine–β-naphthylamide were

![Figure 2](https://academic.oup.com/jac/article-abstract/55/3/306/758347/2)
unconvincing. Two strains were susceptible to this inhibitor at the concentration used (32 mg/L) and none of the organisms had resistance reduced to the baseline levels seen for efflux-deficient mutants. Moreover, we could not exclude non-specific effects, independent of inhibition of MexAB-OprM; in particular it is plausible that efflux would work more efficiently in a strain with reduced impermeability (just as a β-lactamase does), distorting the degree of synergy.

Selectivity at clinical concentrations

Based on a 1 g intravenous dose of ertapenem, with total and free serum peaks of 156 and 13 mg/L, respectively, together with a $t_{1/2}$ of 3.8 h, a first-order equation predicts total and free levels of ertapenem of 19 mg/L and 1 mg/L, respectively, at the mid-point (12 h) between doses. Measured levels are lower: thus Majumdar et al. and Nix et al. both found total drug levels of 9–10 mg/L at 12 h post-dose, and the latter authors show the free drug level falling below 4 mg/L at 4 h post-dose, below 1 mg/L at 8 h and down to 0.5 mg/L by 12 h.

On this basis, selection experiments were performed with 1 mg/L ertapenem, using the six strains AHP and R58 (MICs, 1 mg/L), 799wt and PAO1 (MICs, 8 mg/L) and M1251 and M76 (MICs, 16 mg/L). Unsurprisingly, since the ertapenem MICs for these strains were high compared with the selective concentration, there was confluent overgrowth, not selection of mutant colonies. Selection studies with the same six strains were also conducted with imipenem at 5 mg/L and meropenem at 3.5 mg/L, estimated as mid-interdose serum levels. Strain M1251 (meropenem MIC 8 mg/L) overgrew during meropenem selection, and strain M76 (MIC, 4 mg/L) had an apparent mutation frequency of $>10^{-5}$. These two strains also yielded imipenem-resistant mutants, at frequencies of $5.6 \times 10^{-7}$ and $5 \times 10^{-9}$, respectively; otherwise no imipenem or meropenem resistant mutants were selected, although mutants were selected with similar concentrations from other strains in a previous experimental series.

Discussion

Ertapenem is clinically effective, and the rapidly growing distribution of CTX-M β-lactamas in E. coli from community-acquired infections seems likely to drive its wider use. Nevertheless there is anxiety that, as a carbapenem, ertapenem may select cross-resistance to imipenem and meropenem, which are the last ‘good’ drugs in many infections caused by multi-resistant Gram-negative bacilli. This concern relates partly to the slow emergence of Enterobacteriaceae with IMP, VIM and KPC carbapenemases and partly to the potential for selection of imipenem- and meropenem-resistant mutants of P. aeruginosa. We examined the validity of this latter fear, both in laboratory experiments with MIC multiples and at the concentrations likely to apply in the patient.

At two- to eight-fold its starting MIC, ertapenem—like imipenem, meropenem and doripenem—selected for P. aeruginosa mutants lacking the ‘carbapenem-specific’ porin OprD (D2). This observation confirms that ertapenem can use this porin for uptake, and supports earlier data showing that imipenem-selected OprD$^+$ mutants were eight- to 16-fold more resistant to ertapenem than their parent strains. In addition, ertapenem—like meropenem and doripenem, but not imipenem—was selective for mutants with broad-spectrum resistance to β-lactams, sparing only imipenem. Although a precise mechanism was not proven, this profile corresponds to that typically associated with up-regulation of efflux systems, principally MexAB-OprM. Ertapenem also selected phenotypically diverse mutants that did not convincingly fit with either of these profiles, including (i) those with raised MICs only for ertapenem and meropenem (e.g. mutant of strain F in track j of Figure 2) and (ii) those from the highly carbencillin-resistant strain M76, (Figure 2 tracks p–s) with resistance to both carbapenem and non-carbapenem β-lactams and with an additional outer membrane protein.

The diversity of mutant types selected by ertapenem accords with data presented by Kohler et al., who also found that ertapenem selected for both OprD$^+$ and efflux mutants, and for a phenotype, dubbed MK-X, with resistance to ertapenem and meropenem only. This latter phenotype superficially resembles the mutant of strain F in track j of Figure 2; however, Kohler et al. associated their mechanism with the appearance of an additional outer membrane protein, larger than OprD, and no such band is visible in track j. A protein with this mobility is evident in the mutants of strain O (Figure 2, tracks p–s), but these had a wider cross-resistance. These discrepancies imply that, besides loss of OprD and up-regulation of efflux, other mechanisms affect the MICs of ertapenem for P. aeruginosa.

Virtually all of the mutants selected from the three carbencillin-hypersusceptible strains had phenotypes implying up-regulated efflux, supporting earlier data published with just one of these organisms. By contrast, OprD$^+$ mutants were the predominant, but not the exclusive type selected from strains with normal and raised levels of carbencillin resistance. The reasons for this association are unclear, but it should be noted that the hypersusceptible phenotype is believed to reflect deficiency in MexAB-OprM-mediated efflux and that this may predispose to reversionary or compensatory mutations.

These mutant studies show that ertapenem can select resistance affecting other carbapenems in P. aeruginosa, and beg the question of whether it will do so at clinically achievable concentrations. Answering this question is complicated by the fact that ertapenem is strongly protein-bound, with this extent of binding being concentration-dependent. Even with 20% serum we found ertapenem MICs for P. aeruginosa were raised by four- to eight-fold, and the effect seems likely to be greater in neat serum. A curious peripheral observation was that serum also raised the MICs of meropenem, an agent not usually considered to be protein bound; this phenomenon deserves further investigation. Whether or not the MICs of protein-bound antimicrobials should be reviewed against total or free-drug concentrations is an old and contentious issue, but recent data for ceftiraxone indicate that the critical parameter for efficacy is the duration for which the free-drug concentration exceeds the MIC. It seems reasonable, therefore, to anticipate that free rather than the total drug levels will also critically define selectivity for resistance and that, as for efficacy, selectivity will need a free-drug concentration above the MIC for 30%–40% of the dosage interval. Calculation and published data suggest that the free ertapenem level will only rather briefly exceed the MICs for most P. aeruginosa strains (2–32 mg/L, with an MIC$_{50}$ of 4–8 mg/L).

Taking the estimate of Nix et al. of 4 mg/L free ertapenem in the serum by 4 h post-dose (17% of the dosage interval), 1 mg/L by 8 h (33%) and 0.5 mg/L by 12 h (mid-point between doses), significant selectivity seems unlikely, as any resistant mutants
would be advantaged only briefly. The only exceptions may arise for the most ertapenem-susceptible \textit{P. aeruginosa} strains with MICs $\leq 1\, \text{mg/L}$, and these are clinically rare.\textsuperscript{23} When selection experiments were performed with ertapenem at 1 mg/L (the free-drug level 8–12 h post-dose) there was overgrowth of the parent strains, not selection, even for the hypersusceptible organisms (AHP and RS8).

In conclusion, whilst ertapenem can select for various carbapenem-resistance types in \textit{P. aeruginosa}, selective concentrations pertain only very briefly \textit{in vivo}, militating against selection in the patient. This is analogous to ceftriaxone, which can be used in the laboratory to select for AmpC derepressed mutants of \textit{P. aeruginosa} with cross-resistance to ceftazidime, but which has not been blamed for doing so in the clinic, doubtless again because selective concentrations pertain only briefly. For ertapenem, this pharmacodynamic conclusion accords well with the OASIS-1 (Optimizing Abdominal Surgery with Invan Study) trial, where no patients in the ertapenem arm became rectally colonized with imipenem-resistant \textit{P. aeruginosa} during treatment.\textsuperscript{23}

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References