Carbapenem Activities against *Pseudomonas aeruginosa*: Respective Contributions of OprD and Efflux Systems

THILO KÖHLER,* MEHRI MICHEA-HAMZEPOUR, SIMONE F. EPP, AND JEAN-CLAUDE PECHERE

Department of Genetics and Microbiology, Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland

Received 12 June 1998/Returned for modification 2 October 1998/Accepted 24 November 1998

While meropenem MICs were strongly influenced by the presence or absence of the MexAB-OprM efflux pump in both OprD-proficient and -deficient strain backgrounds, MICs of imipenem and of ER-35786 remained unchanged, demonstrating that meropenem is a substrate of MexAB-OprM but not imipenem and ER-35786. In vitro, all three carbapenems selected loss of OprD as a first mechanism of resistance. However, in an OprD-deficient background, meropenem was able to select MexAB-OprM overproducers as a secondary resistance mechanism, while ER-35786 selected a mutant cross-resistant to sparfloxacin and cefpirome.

In *Pseudomonas aeruginosa*, the potency of β-lactam molecules is limited by several barriers. First, the bacterium’s rather impermeable outer membrane (1, 24) significantly decreases the access of the mostly hydrophilic β-lactams to their targets, the penicillin-binding proteins. Second, chromosomal and plasmid-carried β-lactamases (2, 15) enzymatically hydrolyze β-lactam molecules in the periplasmic space. Finally, active efflux systems extrude β-lactams (16, 17). Indeed, the constitutively expressed MexAB-OprM efflux system (9, 10) includes most β-lactams in its broad-substrate spectrum, while the MexCD-OprJ system (19), when derepressed, extrudes only cephalosporins (4, 11). The MexEF-OprN system (7) does not contribute to β-lactam efflux; however, its overexpression indirectly affects the efficacy of carbapenems through a concomitant reduction (7, 12) of the carbapenem-specific OprD porin.

Masuda and Ohya (12) showed that mutants overexpressing MexAB-OprM are more resistant to meropenem but not to imipenem or panipenem compared to wild type. This finding led to the suggestion (9) that meropenem behaves as a substrate of this pump because of the presence of a hydrophobic side chain at position 2, whereas imipenem or panipenem, containing strongly charged, hydrophilic side-chains, cannot become a substrate. However, the correlation between resistance and efflux may not be simple, because the influx of carbapenems is affected by the levels of OprD.

In the present study, we therefore examined the activity of the three carbapenems, imipenem, meropenem, and ER-35786 (Fig. 1), in the presence and absence of OprD, and determined the mechanisms of resistance selected in vitro by the three antibiotics.

**Activity of carbapenems against mutants with well-defined resistance mechanisms.** Derivatives of PAO1 with all possible combinations of OprD (influx) and MexAB-OprM (efflux) expression were constructed (Table 1). The oprD::ΩTc knockout mutant PASE1 (2a) was transduced with phage E79n2 (5) grown on the oprM::ΩHg mutant K613 (20) to generate the defined oprD-oprM double mutant PA1425. A nalB-type derivative of strain PASE1, called PA1424 and overexpressing the mexAB-oprM operon, was obtained by plating PASE1 on Luria-Bertani (LB) agar containing carbenicillin (SmithKline Beecham Pharmaceuticals, Worthing, Great Britain) at a concentration of 100 μg/ml. Western blot analysis with a rabbit anti-OprD antibody (2a) confirmed the absence of OprD in strains PASE1, PA1425, and PA1426. By using an anti-OprM antibody (25), OprM was determined to be undetectable in PAO1T and PA1425 but was overexpressed in PA1423 and PA1426 (data not shown). None of the strains produced detectable β-lactamase activities under noninducing conditions, thereby excluding fortuitous derepression of β-lactamases during the construction of the strains.

Susceptibility to antimicrobial agents was assayed by the microdilution method with Mueller-Hinton broth (6). In an OprD-sufficient background, the OprM-deficient strain PAO1T was hypersusceptible to all antibiotics tested except imipenem (Merck-Scharp and Dohme-Chibret, Zurich, Switzerland) and ER-35786 (Eisai Co., Ltd., Tsukuba, Japan) (Table 2). By contrast, strain PA1423, overexpressing the MexAB-OprM system, showed increased resistance to all the antibiotics tested, again with the exception of imipenem and ER-35786. Parallel MIC changes were also observed in an OprD-negative background, where both MexAB-OprM deficiency (PA1425) and MexAB-OprM overexpression (PA1426) altered the MICs of meropenem (Imperial Chemical Industries, Macclesfield, Great Britain), without changing those of imipenem and ER-35786. These results strongly suggest that meropenem is a substrate of the MexAB-OprM system, while imipenem and ER-35786 are not.

One possible explanation for the differential behavior of imipenem and meropenem regarding the MexAB-OprM system is their different permeation rates. Based on liposome swelling assays, imipenem (736 nm/s) penetrates about 10 times more rapidly through OprD than meropenem (73 nm/s), while the penetration coefficients for imipenem (6 nm/s) and meropenem (5.5 nm/s) are comparable in OprD-deficient strains (23). Therefore, the rapid imipenem influx through OprD could saturate the efflux pump such that increased MexAB-OprM expression would not affect imipenem MICs. However, since in the OprD-deficient mutant imipenem activity is not influenced by the levels of MexAB-OprM expression, efflux pump saturation is not a valid explanation.

Alternatively, the physicochemical properties of the three carbapenems could be responsible. Each side chain attached at position 2 of the molecule contains nitrogen atoms which can be protonated (Fig. 1). While imipenem and meropenem contain basic groups with measured pKₐ values of 9.91 (21) and 7.4 (22), respectively, ER-35786 contains 2 basic centers in the
pyrrolidine rings with pK_a values of >10. This means that at physiological pH, >99% of the C2 side chains of imipenem and ER-35786 are positively charged, against 50% of the meropenem side chains. If membrane insertion, as suggested previously (9, 17), is a prerequisite for subsequent extrusion by an efflux mechanism, then the difference in "amphiphilicity" could explain the differential behavior of the three carbapenems with respect to efflux systems. Interestingly, panipenem, which has a similar imine radical as imipenem at the C2-substituent, also remains unaffected in its activity by efflux systems (13).

![Chemical structures of the three carbapenems studied. Arrowheads indicate nitrogen atoms which can be charged positively.](image)

**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th><em>P. aeruginosa</em> strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Wild type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>PA1423</td>
<td>Overexpressing MexAB-OprM</td>
<td>8</td>
</tr>
<tr>
<td>PAO1T oprM::ΩHg</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>PASE1 oprD::ΩTc</td>
<td></td>
<td>S. F. Epp et al.</td>
</tr>
<tr>
<td>PA1425 oprD::ΩTc oprM::ΩHg</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>PA1426 oprD::ΩTc, overexpressing MexAB-OprM, selected on carbenicillin</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>PA1433 oprD::ΩTc, decreased expression of 55-kDa protein, selected on meropenem</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>PA1434 oprD::ΩTc, overexpressing MexAB-OprM, selected on meropenem</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>PA1436 oprD::ΩTc, decreased expression of 55-kDa protein, selected on ER-35786</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

![Western blots of total cell lysates obtained from the same strains as in panel A. The blots were revealed with anti-OprM antibody by using a chemiluminescent detection kit.](image)
In vitro selection of carbapenem resistance. Another way to look at the contribution of efflux and OprD to carbapenem activity is to analyze the mechanisms of resistance after carbapenem exposure. For this purpose, $10^9$ to $10^{10}$ CFUs of wild-type PAO1 were exposed on LB agar plates containing carbapenem concentrations ranging from 1 to 16 times the MIC. Spontaneous resistant colonies appeared after two to four times the MICs, frequencies varied from 3 to 10$^{-8}$ to 10$^{-7}$ and were less than 10$^{-9}$ at 8 times the MIC. All colonies tested (eight from each selection) displayed increased resistance only to the three carbapenems at concentrations of two, three, four, or eight times the MIC. Selection of outer membrane fractions (14) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed decreased expression of a protein band of approximately 55 kDa in mutants PA1433 and PA1436 (Fig. 2A, lanes 3 and 4) compared to the parental strain PASE1 (Fig. 2A, lane 2). This protein could represent a new porin protein. Indeed, alternative pathways of entry have been proposed for meropenem (18) and for the synthetic carbapenem BMS-181139 (3). Interestingly, analysis of preliminary sequence data from the P. aeruginosa genome (http://www.pseudomonas.com/) suggests the existence of 14 open reading frames sharing significant homology with either OprD or OprE porins (http://www.interchg.ubc.ca/bobh/).

In conclusion, a second step of carbapenem resistance is possible in OprD-deficient strains, affecting this time not only carbapenems but also non-carbapenem antibiotics such as cephalosporins and quinolones. The fact that this selection occurred with meropenem and ER-35786 but not after in vitro exposure to imipenem may have some clinical significance.

We are grateful to I. Zicha-Zarifi and P. Plesiat (University Hospital Center, Besançon, France) for providing the OprM antisera and to O. Hillaire and C. Beaufort (Analytik Jena, France) for performing the pKa analysis of synthetic carbapenem BMS-181139.

This work was supported by the Fonds National Suisse pour la Recherche Scientifique.

REFERENCES
Committee for Clinical Laboratory Standards, Villanova, Pa.


