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

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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 cephems (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 protein.

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 E79*tv2* (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 PA1426 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-Sharp 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, Macclesfild, 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 pK_a values of 9.91 (21) and 7.4 (22), respectively, ER-35786 contains 2 basic centers in the

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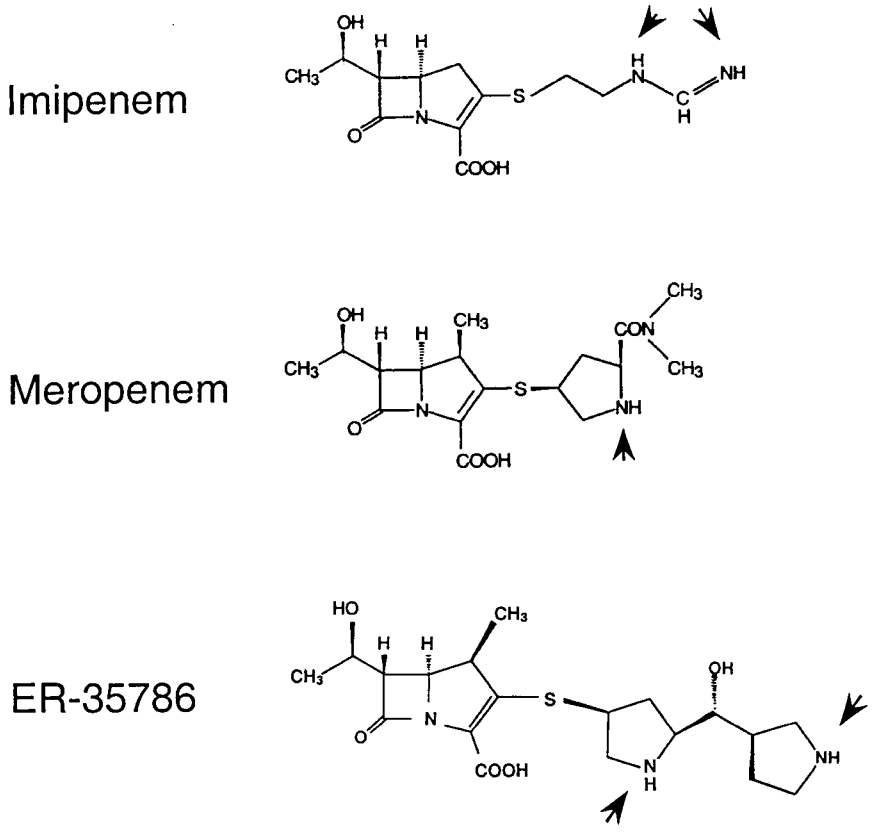

FIG. 1. Chemical structures of the three carbapenems studied. Arrowheads indicate nitrogen atoms which can be charged positively.

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).

TABLE 1. Bacterial strains

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

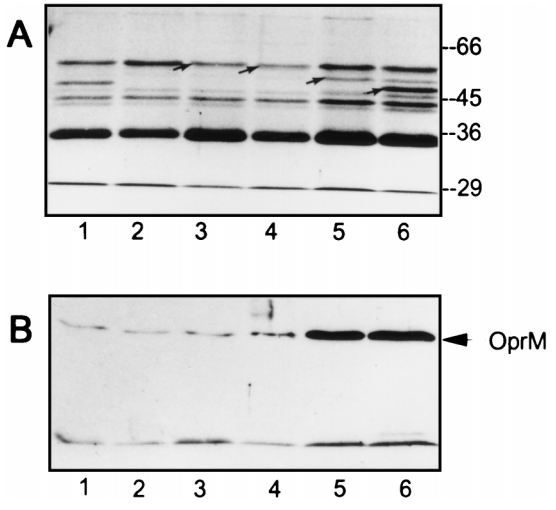

FIG. 2. (A) Outer membrane preparations of defined and spontaneous PAO1 derived mutants. Lane 1, PAO1; lane 2, PASE1; lane 3, PA1433; lane 4, PA1436; lane 5, PA1434; lane 6, PA1423. Arrows indicate reduced expression of 55-kDa protein (lanes 3, 4) and presence of OprM (lane 5) and OprD (lane 6). (B) 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.

TABLE 2. Effects of OprM and OprD expression levels on susceptibilities of carbapenems and other antimicrobial agents

Antibiotic	MICs (μg/ml) for strains with indicated phenotype[a]					
	PAO1 OprM+ OprD+	PAO1T OprM− OprD+	PA1423 OprM++ OprD+	PASE1 OprM+ OprD−	PA1425 OprM− OprD−	PA1426 OprM++ OprD−
Imipenem	1	1	1	16	16	16
Meropenem	0.5	0.125	4	4	0.5	16
ER-35786	0.25	0.25	0.25	1	0.5	0.5
Carbenicillin	64	1	256	64	2	256
Cefpirome	2	0.25	4	2	0.25	8
Sparfloxacin	0.5	0.125	2	0.5	0.125	2

[a] +, wild-type expression level; −, knockout mutation; ++, overexpression.

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 carbapenems at concentrations of two, three, four, or eight times the MIC. Spontaneous resistant colonies appeared after incubation at 37°C for 24 to 48 h with similar frequencies for the three compounds. At two to four times the MICs, frequencies varied from 3×10^{-8} to 1×10^{-9} 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. Total lysates of five spontaneous mutants obtained on each carbapenem were analysed by Western blotting for the presence of OprD. None of them showed OprD reactivity, demonstrating loss of OprD as the first mechanism of resistance.

Interesting differences between the three carbapenems were seen when the selection was performed with the OprD-deficient strain PASE1. Again 10^9 to 10^{10} CFUs were spread on agar plates containing carbapenem concentrations ranging from 3 to 16 times the MICs. After 48 h of incubation, no colonies grew on imipenem (48 μg/ml) containing agar. At 16 μg of meropenem/ml (4 × MIC) and at 8 μg of ER-35786/ml (8 × MIC) resistant colonies were obtained at a frequency of 2×10^{-8}. Analysis of the antibiotic resistance profile of seven colonies obtained on meropenem revealed that six were *nalB*-type mutants. Indeed, one representative clone, called PA1434, showed a strong outer membrane protein band hybridizing with anti-OprM antibody after Western blotting, confirming overexpression of the MexAB-OprM system (Fig. 2B). Compared to the parental strain PASE1, the remaining mutant, termed PA1433, showed slightly increased MICs of meropenem, ER-35786, and sparfloxacin (Rhône-Poulenc, Paris, France) but not of imipenem (Table 3) and expressed wild-type levels of OprM (Fig. 2B). Spontaneous mutants obtained on ER-35786 were found to have very similar antibiotic resistance profiles. One representative mutant, called PA1436, was characterized by increases in the MICs of ER-35786 (eightfold), sparfloxacin (fourfold), and cefpirome (twofold) but showed unchanged imipenem MICs (Table 3). Careful examination 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 ports 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/).

TABLE 3. Resistance phenotypes obtained with OprD-deficient mutant PASE1 after exposure to carbapenems

Antibiotic	MICs (μg/ml)				
	PAO1	PASE1	PA1433	PA1434	PA1436
Imipenem	1	16	16	16	16
Meropenem	0.5	4	8	8	2
ER-35786	0.25	1	4	1	8
Carbenicillin	64	64	64	256	32
Cefpirome	2	2	2	4	4
Gentamicin	0.5	0.5	0.5	0.5	0.5
Chloramphenicol	64	64	64	128	32
Sparfloxacin	0.5	0.5	1	1	2

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 cefpirome 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.

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REFERENCES

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. Kropinski, and R. E. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21:**299–309.
2. **Chen, H. Y., M. Yuan, and D. M. Livermore.** 1995. Mechanisms of resistance to beta-lactam antibiotics amongst *Pseudomonas aeruginosa* isolates collected in the UK in 1993. J. Med. Microbiol. **43:**300–309.

2a. **Epp, S. F.** Unpublished data.

3. **Fung-Tomc, J. C., E. Gradelski, B. Kolek, B. Minassian, M. Pucci, R. E. Kessler, and D. P. Bonner.** 1995. Activity of carbapenem BMS-181139 against *Pseudomonas aeruginosa* is not dependent on porin protein D2. Antimicrob. Agents Chemother. **39:**386–393.
4. **Gotoh, N., H. Tsujimoto, M. Tsuda, K. Okamoto, A. Nomura, T. Wada, M. Nakahashi, and T. Nishino.** 1998. Characterization of the MexC-MexD-OprJ multidrug efflux system in Δ*mexA-mexB-oprM* mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **42:**1938–1943.
5. **Haas, D., B. W. Holloway, A. Schamböck, and T. Leisinger.** 1977. The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **154:**7–22.
6. **Jorgensen, J. H., M. J. Ferraro, W. A. Craig, G. V. Doern, S. M. Finegold, J. Fung-Tomc, S. L. Hansen, J. Hindler, L. B. Reller, J. M. Swenson, F. C. Tenover, R. T. Testa, and M. A. Wikler.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. The National

Commitee for Clinical Laboratory Standards, Villanova, Pa.
7. **Köhler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J. C. Pechère.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
8. **Köhler, T., M. Michéa-Hamzehpour, P. Plésiat, A. Kahr, and J. C. Pechère.** 1997. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2540–2543.
9. **Li, X.-Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
10. **Li, X.-Z., H. Nikaido, and K. Poole.** 1995. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1948–1953.
11. **Masuda, N., N. Gotoh, S. Ohya, and T. Nishino.** 1996. Quantitative correlation between susceptibility and OprJ production in NfxB mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40:**909–913.
12. **Masuda, N., and S. Ohya.** 1992. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1847–1851.
13. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
14. **Michéa-Hamzehpour, M., J.-C. Pechère, P. Plésiat, and T. Köhler.** 1995. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**2392–2396.
15. **Minami, S., M. Akama, H. Araki, Y. Watanabe, H. Narita, S. Iyobe, and S. Mitsuhashi.** 1996. Imipenem and cephem resistant *Pseudomonas aeruginosa* carrying plasmids coding for class B beta-lactamase. J. Antimicrob. Chemother. **37:**433–444.
16. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barrier and active efflux. Science **264:**382–388.
17. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
18. **Perez, F. J., C. Gimeno, D. Navarro, and J. Garcia-de-Lomas.** 1996. Meropenem permeation through the outer membrane of *Pseudomonas aeruginosa* can involve pathways other than the OprD porin channel. Chemotherapy **42:**210–214.
19. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, X. Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21:**713–724.
20. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
21. **Smith, G. B., and E. F. Schoenewaldt.** 1981. Stability of N-formimidoylthienamycin in aqueous solution. J. Pharm. Sci. **70:**272–276.
22. **Takeuchi, Y., M. Sunagawa, Y. Isobe, Y. Hamazume, and T. Noguchi.** 1995. Stability of a 1 beta-methylcarbapenem antibiotic, meropenem (SM-7338) in aqueous solution. Chem. Pharm. Bull. (Tokyo) **43:**689–692.
23. **Trias, J., and H. Nikaido.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**52–57.
24. **Yoshimura, F., and H. Nikaido.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152:**636–642.
25. **Ziha-Zarifi, I., C. Llanes, T. Köhler, J. C. Pechere, and P. Plesiat.** In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob. Agents Chemother., in press.