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NOTE

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Estimation of outer membrane permeability of carbapenem antibiotics to *Pseudomonas aeruginosa*

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Abstract
The outer membrane permeability of carbapenems (imipenem [IPM], panipenem [PAPM], meropenem [MEPM], and biapenem [BIPM]) and ceftazidime (CAZ) to *Pseudomonas aeruginosa* was determined by the Zimmermann and Rosselet method. The permeability coefficients of β-lactams tested at 50 μM concentration of substrates ranged from $(0.40 \pm 0.10) \times 10^{-6}$ cm/s for CAZ to $(2.33 \pm 0.33) \times 10^{-6}$ cm/s for IPM, indicating that the outer membrane permeability of carbapenems to *P. aeruginosa* was high in comparison with that of CAZ. In particular, IPM and BIPM showed a higher rate of penetration than MEPM and PAPM.

Key words Outer membrane permeability · *Pseudomonas aeruginosa* · Carbapenem

Introduction

Pseudomonas aeruginosa shows intrinsic resistance to many categories of antibiotics, including β-lactams,[1,2] and this resistance has often been attributed to the low permeability of its outer membrane.[3,4] A method to measure the permeability of β-lactams to intact cells has been developed by Zimmermann and Rosselet[5] and Sawai et al.[6] This method is already used for several species of gram-negative bacteria;[7,8] however, the method is useful only for β-lactams which are hydrolyzed by β-lactamases. In a previous study,[9] we reported a plasmid, pMS350, which encoded a novel β-lactamase classified as a metalloenzyme (carbapenemase).[10] In this study, we determined the permeability rate of various β-lactams through the outer membrane of *P. aeruginosa* by the Zimmermann and Rosselet method.

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Materials and methods

The antimicrobial agents IPM, PAPM, MEPM, BIPM, and CAZ were obtained from Banyu Pharmaceuticals, Sankyo, Sumitomo Pharmaceuticals, Lederle (Japan), and Nippon Glaxo, respectively (all in Tokyo, Japan). Antibiotic susceptibility tests were carried out against *P. aeruginosa* PAO 4141 (β-lactamase-deficient mutant of PAO1) and PAO 4141 harboring pMS354,[11] which was used for the assay of outer membrane permeability.

The Michaelis constant (Km) of the β-lactamase was determined spectrophotometrically using purified enzyme, while the rates of hydrolysis of β-lactams were measured spectrophotometrically, using intact cells or sonic extract cells. Enzyme activity was determined at 30°C in 50 mM 3-morpholinopropanesulfonate (MOPS)-NaOH buffer (pH 7.0) containing 5 mM $MgCl_2$. The rate of hydrolysis by the sonic extract was used to calculate the maximum velocity (Vmax) of the β-lactamase, and Vmax was calculated from the modified Michaelis equation as follows:

$$\mathrm{V\,max} = \left(1 + \mathrm{Km/Co}\right) \times \mathrm{Vd} \quad (1)$$

where Vd is the rate of hydrolysis of β-lactams by the sonic extract of cells and Co denotes the β-lactam concentration outside. The β-lactam concentration inside (Ci) was calculated from the formula of Zimmermann and Rosselet[5] as follows:

$$\mathrm{Ci} = \left(\mathrm{Vi} \times \mathrm{Km}\right)/\left(\mathrm{Vmax} - \mathrm{Vi}\right) \quad (2)$$

where Vi is the rate of hydrolysis of β-lactams by the intact cells. The permeability coefficient, Pz, was calculated ac-

cording to the equation of Zimmermann and Rosselet[5] or according to Nikaido et al.:[12]

$$Pz \times A = Ci \times Vmax/(Ci + Km) \times (Co - Ci) \quad (3)$$

where A denotes the area of the membrane. According to the method of Nikaido et al.,[12] the approximate surface/weight ratio, 132 cm^2/mg (dry weight), was used.

Results

The minimum inhibitory concentrations (MICs) of carbapenems and CAZ against PAO 4141 and PAO 4141 harboring pMS354 are shown in Table 1. The plasmid pMS354 conferred resistance to IPM, PAPM, MEPM, and BIPM, and higher resistance to CAZ.

The permeability coefficients of *P. aeruginosa* PAO 4141 outer membrane harboring pMS354 to β-lactam antibiotics are shown in Table 2. The permeability coefficients Pz, was calculated by the formula of Zimmermann and Rosselet (formula 3). The order of penetrability for these β-lactams at 50 μM concentration of substrates was:

IPM = BIPM > MEPM = PAPM > CAZ

that is, IPM and BIPM showed higher rates of penetration than MEPM, PAPM, and CAZ (*t*-test; $P < 0.05$).

Table 1. Antibacterial activity of carbapenems and ceftazidime against *P. aeruginosa* PAO4141 strains

Antibiotic	MIC (μg/ml)	
	PAO4141	pMS354/PAO4141
Imipenem	0.20	3.13
Panipenem	0.20	12.5
Meropenem	0.20	12.5
Biapenem	0.10	6.25
Ceftazidime	0.78	200

MIC, Minimum inhibitory concentration

Table 2. Permeation rates of carbapenem antibiotics and ceftazidime through outer membrane of *P. aeruginosa* PAO4141 harboring pMS354

Antibiotic	Mol wt[a]	Permeability coefficient Pz ($\times 10^{-6}$ cm/s)[b] at 50 μM	No. of experiments[c]
Imipenem	299	2.33 ± 0.33	4
Panipenem	339	0.81 ± 0.20	3
Meropenem	384	0.85 ± 0.21	3
Biapenem	350	2.03 ± 0.45	3
Ceftazidime	547	0.40 ± 0.10	3

[a] Molecular weights of compounds in free form
[b] The permeability coefficients of antibiotics measured at 50 μM of the substrates were calculated from formula 3 (Materials and Methods), and the mean ± SD values of the coefficients of independent experiments were calculated
[c] Number of independent experiments

Discussion

The outer membrane barrier of gram-negative bacteria contributes to the degree of resistance to β-lactams, as the influx of the antibiotics is slow via the barrier and the drugs are hydrolyzed by β-lactamase.[3,4] We determined the permeability coefficients of β-lactams to *P. aeruginosa* by the Zimmermann and Rosselet method. Leakout of β-lactamase was a serious problem in the assay of outer membrane permeability of β-lactams to *P. aeruginosa* . We kept intact cells at 20°C in MOPS-NaOH buffer containing 5 mM $MgCl_2$ buffer during experiments, and cell lysis was reduced to an undetectable order in the assay system. In an earlier report,[13] changes in permeability coefficients due to the concentration of substrate were pointed out in the Zimmermann-Rosselet assay. When pMS354-mediated enzyme was used for the assay, the permeability coefficient, Pz, for IPM was not significantly different between the concentrations of 50 and 100 μM (data not shown). Moreover, the adequacy of this experiment was confirmed by the finding that our results of pilot experiments for cephaloridine, cefepime, and cefpirome were close to the results found with the other assay system, of Nikaido et al.[14] (data not shown). In this study, the permeability coefficients of carbapenems were higher than those of CAZ.

The permeablity coefficient of carbapenems in *P. aeruginosa* is considered to be the value which would be influenced by deficiency of the porin protein, OprD, or overproduction of MexAB-OprM efflux pump proteins.[15]

From our results, it is suggested that both the higher rates of penetration of carbapanems, especially IPM and BIPM, and the high stabilities to chromosomal β-lactamase[11] may contribute to the in potent antibacterial activities against *P. aeruginosa* strains, except for carbapenemase-producing strain. We are convinced that this finding will increasingly spur further research and the development of new carbapenem derivatives.

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