OUTER MEMBRANE PERMEABILITY OF IMIPENEM IN COMPARISON WITH OTHER β-LACTAM ANTIBIOTICS

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Imipenem (formerly MK-0787, *N*-formimidoylthienamycin) is a derivative of thienamycin[1] and one of the first carbapenem antibiotics with a broad antibacterial spectrum[2,3]. In addition to potent antibacterial activity, it possesses inhibitory activity towards various types of β-lactamases.

In order to elucidate the mechanism of the antibacterial activity of this novel β-lactam, several studies have been conducted, for instance, the binding affinity for target proteins (penicillin-binding proteins) in bacteria[4,5] and the mechanism of inhibition of β-lactamases[6,7]. One important aspect of the mechanism of action requires investigation of the outer membrane permeability of imipenem in Gram-negative bacteria. Previous work on the estimation of permeability of β-lactam antibiotics through the bacterial outer membrane, known to be a barrier against penetration to the target proteins, was reported by SAWAI *et al.*[8], and ZIMMERMANN and ROSSELET[9]. The procedure used was based on a kinetic treatment of the hydrolysis of β-lactam antibiotic by the β-lactamase located in the periplasm of intact cells, however, this method is inapplicable to β-lactamase-stable β-lactams such as imipenem.

In this study, we have estimated the permeability of imipenem through the outer membrane of intact cells of *Escherichia coli*, *Enterobacter cloacae* and *Citrobacter freundii*, compared to thienamycin, latamoxef, cefoxitin and cloxacillin, which are β-lactamase-stable β-lactams also.

The relative permeability of the β-lactamase-stable β-lactams through the outer membrane of the three bacterial species was estimated on the basis of the fact that the β-lactams are competitive inhibitors for the chromosomally mediated β-lactamases (cephalosporinases) of the three bacterial species. Intact cells and sonically disrupted cells were prepared in phosphate buffered saline, pH 7.0, and β-lactamase activity was measured by microiodometric assay[8]. Cephaloridine was used as a cephalosporinases substrate; this β-lactam is known to have high permeability through the outer membrane of Gram-negative bacteria[10], and is a good substrate for the cephalosporinases in this study. The inhibitory activities of β-lactamase-stable β-lactams against β-lactamases were measured under two sets of conditions, *i.e.*, intact cells and disrupted cells.

The relative permeability of the β-lactamase-resistant β-lactams through the outer membrane was expressed as the ratio of the inhibitor concentration required for 50% inhibition (I_{50}) against the β-lactamase activity in the two methods.

An additional measure of outer membrane permeability of the β-lactams was performed by minimum inhibitory concentration (MIC) determination in the presence of ethylenediaminetetraacetic acid (EDTA) which is known to reduce the barrier effect of the outer membrane[11]. The MIC of each antibiotic was determined in Trypticase soy broth (BBL) in the presence of subinhibitory concentration (1/2 MIC) of EDTA and in its absence; β-lactamase defective mutants were used so that the influence of β-lactamase production on the antibacteral activity is excluded. We assume that the reduction of the MIC value in the presence of EDTA will be greatest in those cases where the outer membrane permeability of the β-lactam is low.

Imipenem and thienamycin were provided by Merck Sharp and Dohme Research Laboratories, Rahway N.J., U.S.A. Cephaloridine, latamoxef, and cefoxitin were commercial products of Torii & Co., Ltd., Tokyo, Japan; Shionogi & Co., Ltd., Osaka, Japan and Banyu Pharmaceutical Co., Ltd., Tokyo, Japan, respectively. Cloxacillin is obtained from Sigma Chemicals Co., St. Louis, U.S.A. Bacterial strains used in this study were *E. coli* 255, *E. cloacae* 363, and *C. freundii* GN346. These strains are constitutive producers of species-specific cephalosporin-

Table 1. Concentrations required for 50% inhibition of cephalosporinase activity located in the periplasm or in disrupted cell suspension.

Source of cephalosporinase	Inhibitor	$I_{50\cdot intact}$ (A) (μM)	$I_{50\cdot disrupted}$ (B) (μM)	(A)/(B) ratio
E. coli 255	Imipenem	8.1	0.91	8.9
	Thienamycin	3.2	0.58	5.5
	Latamoxef	1.4	9.8×10^{-2}	14.3
	Cefoxitin	19.0	0.40	47.5
	Cloxacillin	>500	1.5×10^{-3}	$>3.3\times10^{5}$
E. cloacae 363	Imipenem	10.2	0.86	11.9
	Thienamycin	2.1	0.30	7.0
	Latamoxef	1.5	2.5×10^{-2}	60
	Cefoxitin	314	0.38	826
	Cloxacillin	539	2.7×10^{-3}	2.0×10^{5}
C. freundii GN346	Imipenem	12.0	0.29	41.4
	Thienamycin	10.2	0.22	46.4
	Latamoxef	2.5	1.5×10^{-2}	167
	Cefoxitin	322	0.51	631
	Cloxacillin	526	6.6×10^{-4}	8.0×10^{5}

One ml of the reaction mixture containing 50 μM cephaloridine as substrate, enzyme preparation, and various concentration of inhibitor in phosphate buffered saline (pH 7.0) was incubated for 10 minutes at 30°C, and the hydrolyzed substate was assayed.

Enzyme inhibition was expressed as the percentage of the enzyme activity measured in the absence of inhibitor, and the inhibitor concentration required for 50% inhibition of the activity was estimated from a semi-logarithmic plot of the enzyme activity against the inhibitor concentration.

Table 2. Effect of EDTA on antibacterial activity of five β-lactam antibiotics.

Organism	EDTA[a] (mM)	MIC (μg/ml)[b]				
		Imipenem	Thienamycin	Latamoxef	Cefoxitin	Cloxacillin
E. coli 255/L-7	0	0.1 (1)	0.2 (2)	0.2 (1)	3.1 (1)	400 (8)
	1.25	0.1	0.1	0.2	3.1	50
E. cloacae 363/1	0	0.1 (1)	0.2 (1)	0.05 (1)	3.1 (1)	400 (8)
	2.5	0.1	0.2	0.05	3.1	50
C. freundii GN346/16	0	0.1 (1)	0.2 (2)	0.4 (2)	6.3 (4)	400 (16)
	2.5	0.1	0.1	0.2	1.6	25

[a] Subinhibitory concentration (1/2 MIC of EDTA) was added in the medium.

[b] Determined by broth dilution method at the inoculum size of 10^5 cells/ml in Trypticase soy broth (BBL). The number in the parentheses indicates the magnitude of reduction of MIC.

ase[12], enzymes are located in the periplasmic space. β-Lactamase-defective mutants, *E. coli* 255/L-7, *E. cloacae* 363/1 and *C. freundii* GN346/16, derived from the above parent strains[13], were also used.

The β-lactamase-stable β-lactams used in this study inhibited the activities of the three cephalosporinases as shown in Table 1. These enzymes were highly susceptible to the inhibitors, especially to latamoxef and cloxacillin, when cell extracts were employed as the enzyme. The I_{50} values of the inhibitors of the released enzymes [$I_{50\cdot disrupted}$(B)] were lower than those of the enzymes in intact cells [$I_{50\cdot intact}$(A)]. Marked differences between the values of $I_{50\cdot disrupted}$ and $I_{50\cdot intact}$ were found for inhibition of the three cephalosporinases by cloxacillin. This result is consistent with the suggestion that the low Gram-negative activity of cloxacillin is due to poor permeability of the outer membrane.

Imipenem and thienamycin exhibited smaller (A)/(B) ratios, suggesting that these two carbapenems pass through the outer membranes of the three bacterial species more effectively than two cephalosporins, latamoxef and cefoxitin. The introduction of the *N*-formimidoyl group at C-2 of thienamycin had little effect, although the antibacterial activity of imipenem is somewhat enhanced[8] by this modification (Table 2). The (A)/(B) ratios listed in Table 1 may suggest that *C. freundii* presents the strongest barrier against the β-lactams tested. However, it should be noted that the difference in the ratio we measure is not necessary indicative of the difference in the permeability between different species; the rates will be influenced by the nature of the respective cephalosporinase and the concentration of cephaloridine achieved in periplasmic space.

Table 2 shows the susceptibility of β-lactamase-defective mutants to the five β-lactams in the presence or absence of EDTA. Cloxacillin required extremely high concentrations to exhibit antibacterial activity against all the strains tested, and the addition of EDTA to the medium caused a marked reduction in the MIC levels. On the contrary, the other four β-lactams were only slightly affected by the addition of EDTA, suggesting that they possess good permeability through the outer membranes (except for the case of cefoxitin to *C. freundii*). Although this experiment (Table 2) does not offer a quantitative evaluation to the outer membrane permeability of antibiotics, the results support the conclusion of the inhibition experiment (Table 1).

YOSHIMURA and NIKAIDO have reported diffusion rates of various β-lactams including imipenem, cefoxitin and latamoxef through the Omp F channel of *E. coli*, measured by using reconstituted membrane vesicles and the liposome swelling method[14]. Their data indicated that the diffusion rate of imipenem is about 5 to 6 times higher than those of cefoxitin and latamoxef. On the basis of the observations in this study and those by YOSHIMURA and NIKAIDO[14] we assume that imipenem possesses high outer membrane permeability *via* the porin pores in the *Enterobacteriaceae* species, and such a characteristic of imipenem contributes toward its potent antibacterial activity and broad antibacterial spectrum, including *Pseudomonas*. On the other hand, the data shown in Table 1 indicated a higher outer membrane permeability for latamoxef compared with cefoxitin, though there was no significant difference between the two β-lactams in the diffusion rates through the Omp F channel[14]. It is not obvious whether such a difference is due to differences in the membranes employed, *i.e.*, the reconstituted membrane and the native outer membrane, or to the methods employed for the estimation of the permeability.

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