Affinities of BO-2727 for Bacterial Penicillin-binding Proteins and Morphological Change of Gram-negative Rods

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Affinities of BO-2727, a new carbapenem, for penicillin-binding proteins (PBPs) of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. were studied. BO-2727 showed preferential affinity for PBP-2 of *E. coli*, and induced swollen and ovoid cells, when the organism was exposed to the MIC. In contrast, BO-2727 bound to both PBPs-2 and -3 of *P. aeruginosa* to a similar extent, and induced short filament cells with bulge. As compared with imipenem and meropenem, there was an observed difference in the affinity, especially for PBP-3, in both organisms; BO-2727 displayed intermediate affinity for PBP-3 between meropenem and imipenem. Studies on the affinity for PBPs of methicillin-susceptible *S. aureus* showed that the IC_{50} values for PBP-1 was roughly correlated with the MIC values of carbapenems tested, but those for the PBPs-2 and -3 appeared to be greater than the MIC values. In further studies on the affinity for PBP-2′ of methicillin-resistant *S. aureus*, BO-2727 displayed better binding kinetics than imipenem, which reflected the better activity of BO-2727 than that of imipenem.

Penicillin-binding proteins (PBPs) catalyze peptidoglycan synthesis in bacterial cell wall of Gram-positive and Gram-negative bacteria, and are the primary target of β-lactam antibiotics. In the past, studies on the roles of individual PBPs in *Escherichia coli* have revealed the essential nature of the high-molecular-weight PBPs for cell viability and morphology[1,2]. The PBPs of other bacteria such as *Pseudomonas aeruginosa* are presumed to have similar roles[3]. In *Staphylococcus aureus*, PBP-1[4] in addition to the PBPs-2 and -3[5] out of the four PBPs are essential for cell viability, while the low-molecular-weight PBP-4 is dispensable. However, the level of production of PBP-4 affects the susceptibility to β-lactam antibiotics[6]. It is also known that methicillin-resistant *S. aureus* (MRSA) has an extra PBP, PBP-2′, having low affinity for the ordinary β-lactam antibiotics, besides normal PBPs[7,8].

We have previously reported the antibacterial profile of BO-2727, a new carbapenem antibiotic, showing a potent activity against Gram-positive and Gram-negative bacteria including *P. aeruginosa*[9]. In this paper, we studied affinities of BO-2727 for PBPs of *E. coli* and *P. aeruginosa* and *S. aureus*. Furthermore, in order to elucidate the mechanism of the improved anti-MRSA activity of BO-2727, binding kinetics of BO-2727 for the MRSA PBP-2′ was investigated.

Materials and Methods

Bacterial Strains and Growth of Cells

E. coli K12 strain JE1011, *P. aeruginosa* PAO2142, *S. aureus* FDA 209P were used in assays of binding with the PBPs. MRSA strain BB6294, a clinical isolate showing β-lactamase-negative and homogeneously resistant, was used to investigate the binding to PBP-2′. *E. coli* K12 strain JE1011 and *P. aeruginosa* PAO2142 were generous gifts from MICHIO MATSUHASHI and HIDEKI MATSUMOTO, respectively. The strains were maintained as stock cultures in our laboratory, and were cultured at 30°C in broth containing 10 g of Polypeptone, 5 g of yeast extract, 5 g of sodium chloride, 1 g of glucose per liter, adjusted to pH 7.2, and were harvested in the log phase. In the case of *E. coli* JE1011, 20 mg of thymine per liter was added in the broth[10].

Antibiotics

BO-2727 and meropenem were synthesized at the Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan. Imipenem was also the product of Banyu Pharmaceutical Co., Ltd., Tokyo, Japan.

MIC Determination

MICs were determined by an agar dilution method using Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.). The culture grown overnight at 37°C for 20 hours was diluted to 3×10^6 cfu/ml, and about 10^4 cfu was spotted onto the agar plates containing serial two-fold dilutions of antibiotics with a replicating device (Microplanter; Sakuma Seisakusyo, Tokyo, Japan). The plates were incubated at 37°C for 20 hours. The MIC

was defined as the lowest concentration of antibiotics which prevented visible growth. In case of MRSA, 2% NaCl was supplemented to the medium, and MICs were read after the incubation for 48 hours at 35°C[7].

Assay for Binding Affinity for PBPs

The binding affinity of carbapenems for the penicillin-binding proteins (PBPs) was determined by the competition method with [^{14}C]benzylpenicillin, as described previously[1,8,10]. The membrane fraction was prepared by differential centrifugation of the sonicated cells, and was preincubated with the carbapenem solutions at 30°C for 10 minutes. The remaining enzymes were labelled by incubating with [^{14}C]benzylpenicillin (59 Ci/mol, Radiochemical Center, Amersham, England) at 30°C for 10 minutes. The labelled-proteins of *E. coli* and *P. aeruginosa* were separated by SDS/polyacrylamide gel electrophoresis[10]. For the better separation of the *S. aureus* PBPs, SDS/polyacrylamide gel electrophoresis was conducted according to the method by UTSUI and YOKOTA[8]. Binding affinity was expressed as the concentration (IC_{50}) of carbapenem necessary to inhibit the binding of [^{14}C]benzylpenicillin by 50%, which was determined by Bio-Imaging Analyzer (BAS2000, Fuji Photo Film Co., Ltd.) after exposure of dried gel film to the imaging plate.

Microscopic Observation

Overnight culture of the test strains was diluted with Mueller-Hinton broth (Difco) to 10^6 cfu/ml, and inoculated on the thin layer of antibiotic-containing Mueller-Hinton agar mounted on the glass slide. After incubation at 37°C for 2 hours, morphological change was photographed by phase-contrast microscope.

Results and Discussion

Affinity for PBPs of *E. coli* and *P. aeruginosa*

Table 1 shows the IC_{50} values of BO-2727 and imipenem tested for the PBPs of *E. coli* and *P. aeruginosa*. High-molecular-mass PBPs-1 to -3 in *E. coli* and *P. aeruginosa* were essential for bacterial growth[1~3]. BO-2727 preferentially bound to the PBP-2, and next PBPs-1A and -1Bs of *E. coli*, as did imipenem. A difference was observed in the affinity for the lethal target, PBP-3, for which BO-2727 had intermediate affinity between imipenem and meropenem.

As previously described[11], meropenem bound PBP-3 rather than PBP-2 in *P. aeruginosa*, while imipenem preferentially bound to PBP-2. Interestingly, in our studies, BO-2727 was found to bind to both PBP-2 and PBP-3 to a similar extent.

The higher affinity for PBP-3 might contribute to the better activity of carbapenems tested against both Gram-negative rods, although the outer membrane permeability and stability to β-lactamase must be taken into consideration for the mode of action of antibacterial activity. As a result, there was a difference among the carbapenems tested in the affinity especially for PBP-3 in both organisms; BO-2727 displayed intermediate affinity for PBP-3 between meropenem and imipenem.

Morphological Change of *E. coli* and *P. aeruginosa*

Morphological changes in two Gram-negative rods, *E. coli* and *P. aeruginosa*, were studied by microscopic observation after exposing the cells to the MIC of BO-2727, meropenem and imipenem (Fig. 1). *E. coli* cells became swollen and rounded, when the cells were exposed to the MIC of BO-2727 (Fig. 1, b), presumably by the inhibition of initial elongation due to preferential binding to PBP-2 in *E. coli*, as observed in imipenem (Fig. 1, d). In contrast, meropenem induced swollen and spindle shape (Fig. 1, c).

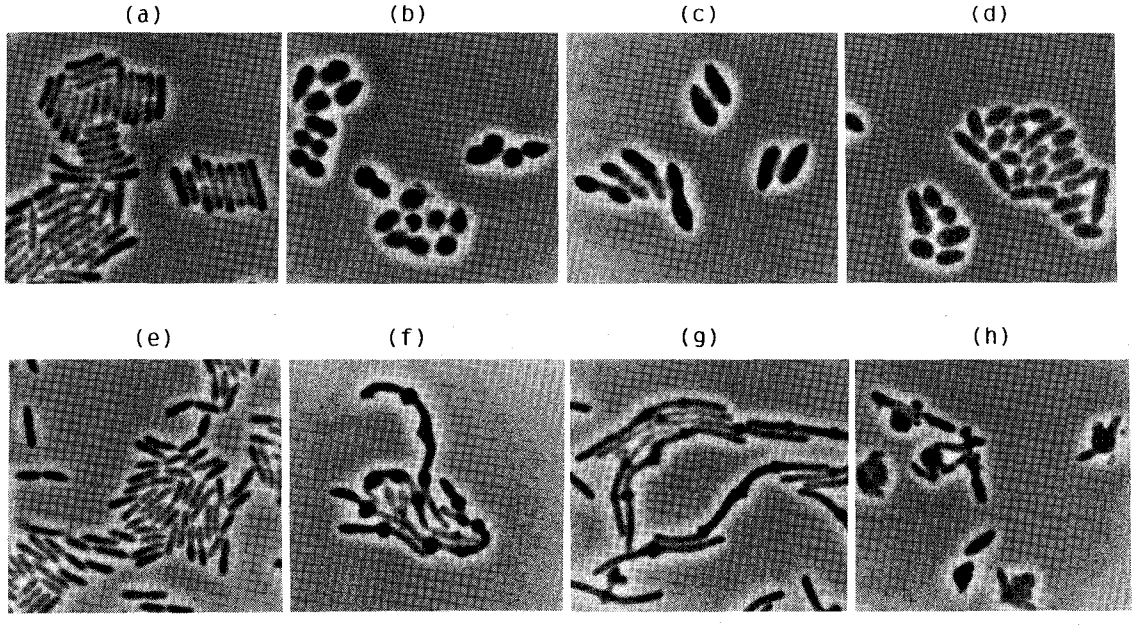
In *P. aeruginosa*, short filament cells with bulge were induced by BO-2727 (Fig. 1, f), which reflected the binding to both of PBP-2 and -3 at similar concentrations in *P. aeruginosa*. Meropenem induced filamentous form (Fig. 1, g), which is the evidence of the inhibition of bacterial septation due to the preferential affinity for PBP-3, and is consistent with the observation by SUMITA

Table 1. Binding affinity of carbapenems for the PBPs of *Escherichia coli* and *Pseudomonas aeruginosa*.

Strain	Antibiotic	IC_{50} (μg/ml)[a] for PBP							MIC (μg/ml)
		1A	1B(s)	2	3	4	5	6	
E. coli JE1011	BO-2727	0.24	0.37	0.011	9.2	0.14	4.6	>12.5	0.1
	Meropenem	0.36	0.56	0.024	0.23	0.036	0.74	22	0.05
	Imipenem	0.56	1.02	0.018	>12.5	<0.02	0.16	0.92	0.2
P. aeruginosa PAO2142	BO-2727	0.80	0.34	0.25	0.21	<0.02	>12.5	—	0.2
	Meropenem	0.78	0.34	0.13	0.051	<0.02	>12.5	—	0.39
	Imipenem	0.28	0.33	0.16	0.58	<0.02	1.3	—	0.78

[a] Concentration necessary to inhibit the binding of [^{14}C]benzylpenicillin by 50%.

Fig. 1. Phase-contrast micrographs of *Escherichia coli* JE1011 (a~d) and *Pseudomonas aeruginosa* PAO2142 (e~h).



Cells were nontreated (a and e), or exposed to the MICs of BO-2727 (b and f), meropenem (c and g), imipenem (d and h), respectively. The MICs of carbapenems were shown in Table 1.

Table 2. Binding affinity of carbapenems for the PBPs of *Staphylococcus aureus*.

Strain	Antibiotic	IC_{50} (μg/ml)[a] for PBP				MIC (μg/ml)
		1	2	3	4	
S. aureus FDA209P	BO-2727	0.033	0.47	15	0.030	0.025
	Meropenem	0.072	0.26	>12.5	0.038	0.05
	Imipenem	0.051	0.076	0.12	<0.02	0.012

[a] Concentration necessary to inhibit the binding of [^{14}C]benzylpenicillin by 50%.

et al.[11]. The *P. aeruginosa* cells exposed to meropenem look more elongated in length than those exposed to BO-2727, whereas imipenem induced spheroplast without filamentation (Fig. 1, h).

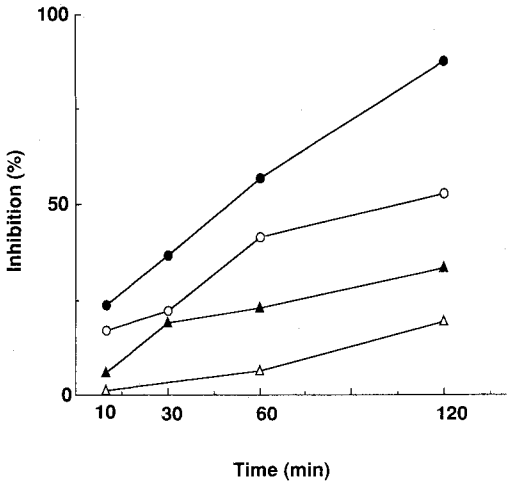
These morphological changes in two Gram-negative rods especially reflected the difference in the binding affinity for the PBP-2 and -3.

Affinity for PBPs of Methicillin-susceptible and -resistant *S. aureus*

Affinities for methicillin-susceptible *S. aureus* PBPs are shown in Table 2. The IC_{50} values of carbapenems tested for possible lethal targets, PBP-2 and -3[5], appeared to be greater than the MIC values. This phenomenon might be due to reversible binding to the PBPs-2 and -3, which was previously reported in the cases of imipenem[12] and meropenem[13]. Although the PBPs-2 and -3 of *S. aureus* had been reported to be essential for viability, the importance of PBP-1 in cell growth was also emphasized[4]. The IC_{50} values for PBP-1 seemed to be roughly correlated in the MICs of BO-2727 and meropenem. Imipenem showed slightly greater IC_{50} (0.051 μg/ml) than the MIC (0.012 μg/ml). The potent anti-staphylococcal activity of imipenem might be ascribed to stronger affinity for the low-molecular-weight PBP-4 as well as for the PBP-1 to PBP-3.

MRSA produces an extra PBP-2′, which displays low affinity for ordinary β-lactam antibiotics. In susceptibility testing against highly resistant homogeneous MRSA, BO-2727 showed better activity (MIC of 25 μg/ml) than imipenem (MIC of 100 μg/ml), this prompted us to investigate the affinity of BO-2727 for the MRSA PBP-2′. As shown in Fig. 2, BO-2727 displayed better kinetics in the binding to PBP-2′ than imipenem. In our study, BO-2727 inhibited the [^{14}C]benzylpenicillin binding by 50% after incubation for 120 minutes at the

Fig. 2. Binding kinetics of BO-2727 and imipenem to PBP-2′ of methicillin-resistant *Staphylococcus aureus* BB6294.



Membrane preparation was mixed with 25 (○, △) and 125 (●, ▲) μg/ml of BO-2727 (circles) and imipenem (triangles), respectively. After incubation for times indicated at 30°C, [^{14}C]benzylpenicillin was added to the reaction mixture and the mixture was further incubated for 10 minutes at 30°C. Inhibition of the [^{14}C]benzylpenicillin binding to PBP-2′ by carbapenems was expressed as the relative radioactivity in the PBP-2′ of the carbapenem-nontreated control.

concentration of 25 μg/ml, similar to the MIC. The binding kinetics of BO-2727 for PBP-2′ was slower than expected from the MIC. It could be explicable by the fact that the simultaneous inhibition of the PBPs-2 and -4 increased the susceptibility of MRSA to β-lactam antibiotics, even though the MRSA possessed intact PBP-2′[14].

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