In Vitro and *In Vivo* Evaluation of BO-2727 against Imipenem- and/or Meropenem-resistant *Pseudomonas aeruginosa*

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The *in vitro* and *in vivo* activity of BO-2727, a carbapenem antibiotic, against resistant clinical isolates of *Pseudomonas aeruginosa* was studied. The geometric mean MICs against three groups of clinical isolates resistant to imipenem, meropenem and both carbapenems were 4.28, 4.08 and 5.44 μg/ml, respectively. BO-2727 also inhibited multiply antibiotic resistant isolates and laboratory mutants including a *nalB*-type mutant, which showed resistance to antibiotics such as imipenem, meropenem, ceftazidime, and/or ciprofloxacin, at less than 1.56 μg/ml. Overall, BO-2727 was 4-fold more active than biapenem, meropenem, panipenem and imipenem with an MIC_{90} of less than 6.25 μg/ml.

The presence of basic amino acids in minimal medium less affected the antipseudomonal activity to a minimal extent, suggesting that BO-2727 has diverse penetration routes through the outer membrane other than OprD channel, which facilitates the diffusion of basic amino acids and carbapenems. The *in vitro* activity of BO-2727 reflected well in its therapeutic efficacy in experimental systemic infection in mice. These results suggest a possibility for the development of antipseudomonal carbapenems having activity against imipenem- and/or meropenem-resistant *P. aeruginosa* as well as a broad spectrum encompassing Gram-positive and -negative bacteria.

Since the discovery of thienamycin[1], carbapenem antibiotics are known to have a broad spectrum encompassing both Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa*. However, the recent problems are the emergence of carbapenem-resistant *P. aeruginosa*, in which the mechanisms of resistance are a) deficiency or decreased amount of OprD of the outer membrane, which facilitates the diffusion of carbapenem and basic amino acids[2~5], b) *nalB* mutation responsible for multiple resistance to cephems, quinolones, tetracyclines as well as meropenem[5] and c) the evolution of a class B metallo-β-lactamase capable of hydrolyzing carbapenems[6].

BO-2727 is a 1-β-methyl-2-(5-substituted pyrrolidin-3-ylthio)carbapenem carrying an (*R*)-1-hydroxy-3-*N*-methylaminopropyl group as a substituent. We have reported that BO-2727 has a well-balanced, potent, antibacterial spectrum with advantages over meropenem in its antibacterial activity, especially against Gram-positive bacteria and *P. aeruginosa*[7,8]. BO-2727 showed better activity against OprD deficient mutants, as previously described[9].

Our primary concern is directed toward the *in vitro* and *in vivo* antipseudomonal activity of BO-2727, especially against imipenem- and/or meropenem-resistant *P. aeruginosa*. We report that BO-2727 has a high potency against the *P. aeruginosa* isolates resistant to known carbapenems.

Materials and Methods

Antibiotics

BO-2727, biapenem, meropenem and panipenem were synthesized at the Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan. Imipenem and cilastatin were also the products of Banyu Pharmaceutical Co., Ltd., Tokyo, Japan. Ceftazidime was purchased from Nippon Glaxo Co., Ltd., Tokyo, Japan.

Organisms

The clinical isolates were from our stock cultures which have been collected from various districts in Japan. Spontaneous mutants with imipenem-resistance were isolated by spreading an overnight culture of imipenem-susceptible parent onto Mueller-Hinton medium agar plate (Difco Laboratories, Detroit, Mich.) containing 3.13 to 25 μg/ml of imipenem. Colonies on the agar plate containing the highest concentration of imipenem were purified on the same concentration of imipenem. The OprD deficiency in the outer membrane proteins of the resistant mutants was confirmed by SDS-polyacrylamide gel electrophoresis[2].

Five strains showing meropenem-resistant, but para-

doxically imipenem-susceptible were screened from our collections of clinical isolates. The *nalB* mutant was isolated by spreading *P. aeruginosa* PAO1 onto an agar plate containing ciprofloxacin and cefsulodin (1 μg/ml each)[5].

MIC Determination

MICs were determined by agar dilution using Mueller-Hinton medium. The culture grown at 37°C for 20 hours was diluted to 3×10^6 CFU/ml, and about 10^4 CFU/ml were spotted onto agar plates containing serial two-fold dilutions of antibiotics with a replicating device (Microplanter; Sakuma Seisakusho, 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.

Systemic Infection

CD-1 male mice, 4 weeks old, were intraperitoneally infected with two imipenem-resistant *P. aeruginosa*, strain BB6265 and BB6268, which were suspended in 5% gastric mucin. Antibiotics were dissolved in saline, and subcutaneously administered to the mice once at 2 hour after infection. The therapeutic efficacy (ED_{50}) was calculated by probit method from the survival rate on the day 4 after treatment. Cilastatin (CS, DHP-I inhibitor) was co-administered in cases of treatment with meropenem and imipenem due to the instability to the DHP-I[10].

Results and Discussion

Susceptibility of Clinical Isolates of *P. aeruginosa* to Antibiotics

BO-2727 inhibited all the *P. aeruginosa* tested including imipenem-, meropenem- and/or ceftazidime-resistant *in vitro* mutants as well as imipenem susceptible isolates at less than 6.25 μg/ml (Table 1). BO-2727 had an MIC_{90} of 1.56 μg/ml against imipenem-susceptible *P. aeruginosa*. BO-2727 was 4-fold more active in terms of MIC_{90} than biapenem, meropenem, panipenem and imipenem against imipenem- and/or meropenem-resistant isolates. As shown in the Table 1, the imipenem-resistant isolates had reduced susceptibility to all the carbapenems tested, but retrieved appreciable susceptibility to BO-2727 with an MIC_{90} of 6.25 μg/ml. The imipenem-resistant isolates exhibited cross-resistance to

Table 1. Comparative activity against clinical isolates of *P. aeruginosa*.

Isolates (No.)	Antibiotic	MIC (μg/ml)			
		Range	G-Mean[a]	50%	90%
Imipenem-susceptible (128)	BO-2727	0.1~6.25	0.54	0.39	1.56
	Biapenem	0.2~3.13	0.80	0.78	1.56
	Meropenem	0.1~25	0.54	0.39	3.13
	Panipenem	0.39~25	5.86	6.25	12.5
	Imipenem	0.2~6.25	1.63	1.56	3.13
	Ceftazidime	0.39~>100	3.74	3.13	25
Imipenem-resistant (22)	BO-2727	1.56~6.25	4.28	3.13	6.25
	Biapenem	6.25~25	14.6	12.5	25
	Meropenem	1.56~50	7.09	6.25	25
	Panipenem	6.25~50	24.5	25	50
	Imipenem	12.5~50	16.6	12.5	25
	Ceftazidime	0.39~50	9.72	12.5	50
Meropenem-resistant (13)	BO-2727	0.78~6.25	4.08	6.25	6.25
	Biapenem	0.78~25	11.2	12.5	25
	Meropenem	12.5~50	17.2	12.5	25
	Panipenem	12.5~50	23.7	25	25
	Imipenem	1.56~25	11.2	12.5	25
	Ceftazidime	3.13~>100	23.7	25	50
Imipenem- and meropenem-resistant (10)	BO-2727	3.13~6.25	5.44	6.25	6.25
	Biapenem	12.5~25	18.9	25	25
	Meropenem	12.5~50	17.7	12.5	25
	Panipenem	25~50	26.8	25	25
	Imipenem	12.5~25	16.5	12.5	25
	Ceftazidime	3.13~50	20.3	25	50
Ceftazidime-resistant (39)	BO-2727	0.2~6.25	1.43	1.56	6.25
	Biapenem	0.39~25	2.81	1.56	25
	Meropenem	0.2~50	2.48	3.13	25
	Panipenem	1.56~50	11.4	12.5	25
	Imipenem	0.78~50	4.01	3.13	25
	Ceftazidime	12.5~>100	31.5	25	100

[a] Geometric mean.

biapenem and panipenem. Interestingly, meropenem was active against some of the imipenem-resistant isolates, as shown by the susceptibility range of 1.56 to 50 μg/ml, although certain meropenem-resistant isolates showing cross-resistance to imipenem. Thus, *P. aeruginosa* isolates showing carbapenem-resistance were roughly grouped in the imipenem- and/or meropenem-resistance (Table 1). Susceptibility testing against the 13 isolates resistant to meropenem, and 10 isolates resistant to imipenem and meropenem (both MIC; ≥12.5 μg/ml) also displayed the high potency of BO-2727 with geometric mean MIC of 4.08 and 5.44 μg/ml, respectively. Furthermore, BO-2727 was more active than ceftazidime against ceftazidime-resistant isolates, which included imipenem- and/or meropenem-resistant isolates. As a result, BO-2727 inhibited all the *P. aeruginosa* resistant to imipenem, meropenem and/or ceftazidime at less than 6.25 μg/ml.

Susceptibility of Multiply Resistant *P. aeruginosa* to Antibiotics

Table 2 shows the susceptibility of meropenem-resistant, but paradoxically imipenem-susceptible, clinical isolates and of a *nalB* mutant derived from strain PAO1 which were associated with multiple resistance to ceftazidime and ciprofloxacin, and called *nalB*-type isolates[5]. The five *nalB*-type isolates and the laboratory mutant were more susceptible to BO-2727 than to the other carbapenems tested. The *nalB* mutation affected the susceptibility to meropenem, but not to other carbapenems.

Effect of Basic Amino Acids in Medium on the Antipseudomonal Activity

The decreased susceptibility of carbapenems in minimal medium with rich basic amino acids relates to increased competition for diffusion of carbapenems through OprD, as previously reported[3,4]. The geometric mean MICs of BO-2727, meropenem, panipenem, and imipenem against the imipenem-susceptible strains were 2.7-, 7.2-, 5.3-, and 3.5-fold increased by the presence of 50 mM L-lysine in the minimal medium, respectively. The activity of BO-2727 was less affected by basic amino acids than a positive agent like meropenem, suggesting a lesser degree of dependency on OprD for penetration through

Table 2. Comparative activity against meropenem-resistant *P. aeruginosa* including a *nalB* mutant.

Strain	MIC (μg/ml)						
	BO-2727	Biapenem	Meropenem	Panipenem	Imipenem	Ceftazidime	Ciprofloxacin
(A) Clinical isolates							
BB6176	0.78	0.78	12.5	12.5	1.56	12.5	6.25
BB6276	0.78	3.13	25	12.5	3.13	>100	3.13
BB6277	0.39	0.78	6.25	1.56	0.78	6.25	100
BB6285	1.56	3.13	12.5	25	6.25	25	0.78
BB6287	0.78	1.56	6.25	3.13	3.13	>100	3.13
(B) *nalB* mutant derived from strain PAO1							
PAO1 (parent)	1.56	1.56	0.78	6.25	3.13	0.39	0.05
BB6292 (mutant)[a]	1.56	1.56	3.13	6.25	3.13	3.13	0.2

[a] The mutant was isolated by spreading *P. aeruginosa* PAO1 onto an agar plate containing ciprofloxacin and cefsulodin (1 μg/ml each)[5].

Table 3. Effect of a basic amino acid on the antipseudomonal activity of BO-2727 and reference β-lactam antibiotics.

Strain (No.) Medium	Geometric mean MIC (μg/ml)					
	BO-2727	Biapenem	Meropenem	Panipenem	Imipenem	Ceftazidime
Imipenem-susceptible (12)						
Minimal medium[a]	0.22	0.25	0.082	0.74	0.44	1.97
Minimal medium with lysine	0.59 (2.7)[b]	0.78 (3.1)	0.59 (7.2)	3.94 (5.3)	1.56 (3.5)	1.97 (1.0)
Mueller-Hinton medium	0.52	0.66	0.93	4.96	1.47	7.43
Imipenem-resistant isogenic mutants (12)[c]						
Mueller-Hinton medium	3.72	13.2	9.36	22.3	16.7	7.43

[a] Modified Davis minimal medium supplemented with 0.4% sodium gluconate and solidified with 1.5% agar[4].
[b] Ratio of MIC in minimal medium with 50 mM L-lysine to that in minimal medium alone for indicated antibiotics.
[c] *In vitro* imipenem-resistant mutants spontaneously isolated from imipenem-susceptible strains.

Table 4. Therapeutic efficacy against systemic infections in mice.

Strain (CFU/mouse)	Antibiotic[a]	MIC (μg/ml)	ED_{50} (95% confidence limits)[b] (mg/kg)
P. aeruginosa BB6265	BO-2727	1.56	2.09 (0.79~5.73)
2.9×10^6 ($4.4 \times LD_{50}$)	Biapenem	6.25	3.00 (1.49~5.71)
	Meropenem/CS[c]	3.13	6.06 (2.41~15.6)
	Imipenem/CS	12.5	7.62 (1.15~19.3)
	Ceftazidime	0.78	7.15 (2.38~16.8)
P. aeruginosa BB6268	BO-2727	1.56	3.33 (1.28~7.29)
9.5×10^5 ($37 \times LD_{50}$)	Biapenem	3.13	8.05 (3.94~17.8)
	Meropenem/CS	6.25	17.4 (7.99~40.3)
	Imipenem/CS	12.5	12.0 (6.33~21.3)
	Ceftazidime	3.13	26.6 (9.89~79.1)

[a] Administered subcutaneously at 2 hours after i.p. infection.
[b] Calculated by the probit method.
[c] Cilastatin (CS) was coadministered[10].

the outer membrane in *P. aeruginosa*.

In Vivo Efficacy against Experimental Pseudomonal Infections

BO-2727 exhibited good efficacy in systemic infection with imipenem-resistant *P. aeruginosa*, which reflected the *in vitro* activity (Table 4). Ceftazidime showed weaker *in vivo* activity than could be expected from its *in vitro* activity (the MIC; 3.13 μg/ml) in spite of lack of cross-resistance between imipenem and ceftazidime based on their different mechanisms of resistance. The results suggested a possibility for the antipseudomonal agent having the activity against imipenem- and/or meropenem-resistant *P. aeruginosa* as well as broad spectrum encompassing Gram-positive and Gram-negative bacteria[11,12].

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