VOL. 52, NO. 2, FEB. 1999 THE JOURNAL OF ANTIBIOTICS pp. 142 — 149

Structure-activity Relationships of Carbapenems to the Antagonism of the Antipseudomonal Activity of Other β-Lactam Agents and to the β-Lactamase Inducibility in *Pseudomonas aeruginosa*: Effects of 1β-Methyl Group and C-2 Side Chain

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(Received for publication October 20, 1998)

The antagonism of the antipseudomonal activity of ceftazidime by meropenem (**1a**) was much less than those by imipenem (**2a**) and panipenem (**2b**). To reveal the major structural features of carbapenem compounds responsible for the antagonism, we investigated the structure-activity relationships of carbapenems to their antagonism of the antipseudomonal activity of ceftazidime and to their β-lactamase-inducibility in *P. aeruginosa*. The antagonistic effect of **1a** was less than that of desmethyl-meropenem (**1b**). Two other meropenem-analogues (**3**, **4**), with the highly basic C-2 side chain, showed greater antagonistic effects than that of **1a**, which has a weakly basic C-2 side chain. The β-lactamase-inducibility of **1a** in *P. aeruginosa* was lower than those of **2a**, **1b** and **4**. These results indicated that the antagonism of the antipseudomonal activity of ceftazidime by carbapenems was due to the induction of β-lactamase in *P. aeruginosa*. As a result of the study on the structure-activity relationships, we clarified that the introduction of a 1β-methyl group and/or the reduction of the basicity (cationic character) of the C-2 side chain in carbapenem skeleton decreased the antagonistic effect of carbapenems on the antipseudomonal activity of ceftazidime resulted mainly from the decreasing the β-lactamase inducibility.

Pseudomonas aeruginosa is an important pathogen that causes severe infections in immunocompromised hosts. This organism possesses an inducible β-lactamase which is chromosomally encoded.[1] In *P. aeruginosa* the induction of β-lactamase is one of the important factors in the development of resistance to β-lactam antibiotics.[2~4]

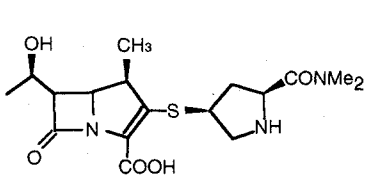
Imipenem (IPM), one of the representative carbapenem antibiotics with a potent antipseudomonal activity, is a good inducer of chromosomally-mediated β-lactamase in this organism. The addition of subinhibitory concentrations of IPM resulted in antagonism of the antipseudomonal activity of other β-lactam agents such as cephalosporins.[2] Much attention has been focused on this antagonism, because of its effect on the clinical efficacy of carbapenem antibiotics and other β-lactam agents.

Meropenem (MEPM) is the only 1β-methylcarbapenem antibiotic that has been accepted for clinical use. Against *P. aeruginosa*, MEPM shows higher antimicrobial activity and lower β-lactamase inducibility at subinhibitory concentrations than IPM does.[5] The antagonism by MEPM and panipenem (PAPM) of the antipseudomonal activity of other β-lactam agents has not been elucidated.

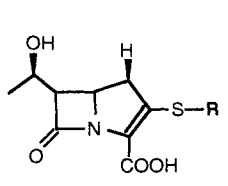
We previously investigated the structure-activity relationship (SAR) of carbapenems to their *in vitro* antimicrobial activity against several organisms, especially *P. aeruginosa*.[6~8] In this course of study, we discovered that the basicity of the C-2 side chain is indispensable for the antipseudomonal activity of carbapenems, and that the introduction of a 1β-methyl group variably affects the antipseudomonal activity, depending on the feature of the C-2 side chain.[8]

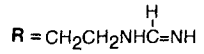
In this study, we further investigated the SARs of carbapenems to their antagonism of the antipseudomonal

Fig. 1. Chemical structures of MEPM, IPM, PAPM and CAZ.

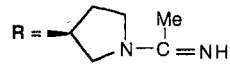


1a (MEPM)

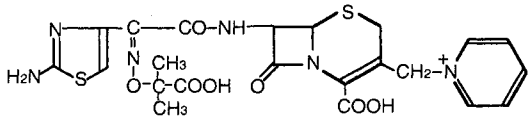




2a (IPM)



2b (PAPM)



CAZ

activity of ceftazidime (CAZ) and to their β-lactamase inducibility in *P. aeruginosa*, focusing on the basicity of the C-2 side chain and the introduction of a 1β-methyl group.

Materials and Methods

Antibiotics

IPM and PAPM were purified from imipenem/cilastatin (Banyu Pharmaceutical, Tokyo, Japan) and panipenem/betamipron (Sankyo, Tokyo), respectively, at the Sumitomo Pharmaceuticals Research Center (Osaka, Japan). The other carbapenem compounds used in this work were also prepared at the Sumitomo Pharmaceuticals Research Center according to reported procedures.[9,10] CAZ was obtained from a commercial source.

Bacterial Strains

P. aeruginosa PAO1 and IFO3451 were standard strains stored in our laboratories, and other strains were clinically isolated strains from various Japanese hospitals in recent years.

Determination of MICs

The minimum inhibitory concentrations (MICs) of β-lactams were determined by the twofold serial agar dilution method, with Mueller-Hinton agar (MHA, Difco, Detroit, MI). Cells of the tested strains were grown in Mueller-Hinton broth (MHB, Difco) at 37°C overnight, and diluted with phosphate buffered saline supplemented with 0.01% gelatin (BSG) to give a final concentration of approximately 10^6 CFU/ml. A portion (about 5 μl) of the dilution was plated onto a drug-containing agar surface with a Microplanter® (Sakuma Seisakusho, Tokyo). The plates were incubated at 37°C overnight. The MIC was defined as the lowest antibiotic concentration that completely prevented visible growth.

Determination of antagonism

The ability of carbapenems to antagonize the antipseudomonal activity of CAZ was determined by a comparison of the MIC of CAZ alone and those in combination with carbapenems. The MICs were determined by agar dilution method as described above.

In the comparison of three carbapenems (MEPM, IPM and PAPM) (Fig. 1), the MICs of CAZ alone and in combination with three subinhibitory concentrations (0.1, 0.2 and 0.39 μg/ml) of each carbapenem were examined.

In the examination of SARs to antagonism (Table 3, Fig. 3), the concentrations of antimicrobial agents were arranged in a checkerboard titration pattern with twofold serial dilutions. The values of MIC_{combi}/MIC_{alone} shown in Table 3 are the ratio of the maximum MIC of CAZ combined with carbapenems to the MIC of CAZ alone in the checkerboard titration assay.

Induction of β-Lactamase

To determine the induction of β-lactamase by tested compounds, an overnight culture was diluted 10-fold with 9 ml of fresh medium (Antibiotic Medium No. 3, Difco) and incubated at 37°C for 3 hours with shaking.

Fig. 2. Distribution of MICs of CAZ alone and in combination with a subinhibitory concentration (0.1 μg/ml) of IPM, PAPM and MEPM against 27 clinical isolates of *P. aeruginosa* shown in Table 1.

○, CAZ alone; ●, CAZ with MEPM; □, CAZ with IPM; △, CAZ with PAPM.

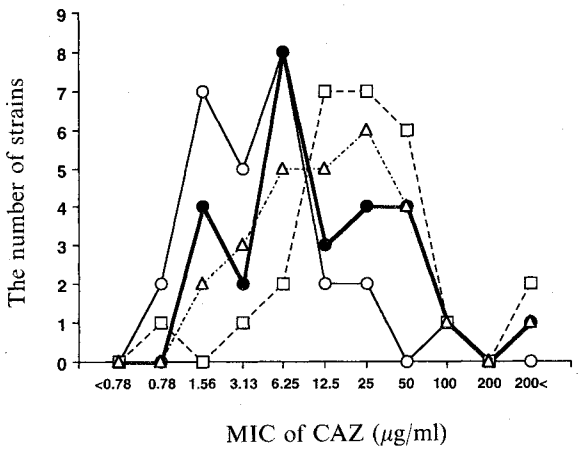


Table 1. Susceptibility of 27 clinical isolates of *P. aeruginosa* to carbapenems and CAZ[a].

Antibiotic[b]	MIC range	MIC_{50} (μg/ml)	MIC_{80}	MIC_{90}
MEPM	0.39~25	3.13	12.5	12.5
IPM	0.78~25	3.13	12.5	25
PAPM	0.78~50	12.5	25	50
CAZ	0.78~100	3.13	6.25	25

[a] MICs were determined by the twofold serial agar dilution method with MHA (inoculum size: 10^4 CFU/spot, incubated at 37°C overnight).
[b] MEPM: meropenem, IPM: imipenem, PAPM: panipenem, CAZ: ceftazidime.

The tested β-lactams were then added to the culture and incubated at 37°C for other 2 hours with shaking. The cells were harvested by centifugation (RPR20-2, Nissei Sangyo, Tokyo) at 10,000 rpm for 10 minutes at 4°C, and washed twice with cold 50 mM phosphate buffer (pH 7.0). The washed cells were suspended in 3 ml of 50 mM phosphate buffer (pH 7.0) containing 250 μg/ml each of DNase I (EC 3.1.32.1, Sigma Chemical, St. Louis, MO) and RNase A (EC 3.1.27.5). The cell suspension was passed three times through a French pressure cell (Ohtake Seisakusho, Tokyo) at 1,500 kg/cm^2. Cell debris and unbroken cells were removed by two sequential centrifugations (RPR18-3, Nissei Sangyo) at 15,000 rpm for 15 minutes at 4°C. The resulting supernatant was used as the crude enzyme for the β-lactamase assay. The concentration of protein in the enzyme sample was determined according to the method of LOWRY *et al.*[11] with bovine serum albumin as the standard.

β-Lactamase Assay

β-Lactamase activity was determined in 1 ml of the sample containing 50 μM PADAC® (chromogenic cephalosporin; Hoechst, Frankfurt, Germany) in 50 mM phosphate buffer (pH 7.0). The initial rates of hydrolysis were spectrophotometrically determined at 30°C by measuring the decrease in the A_{571} using a spectrophotometer (UV-2100; Shimadzu, Kyoto, Japan). Enzyme activity was defined as the A_{571} decreased under these conditions per milligram of protein per minute. The relative β-lactamase activity shown in the Figs. 4 and 5 is expressed as a multiple of the β-lactamase activity in uninduced cells.

Results and Discussion

Antagonism of the Antipseudomonal Activity of CAZ by IPM, PAPM and MEPM

Fig. 2 shows the distribution curves of the MICs of CAZ alone and in combination with a subinhibitory concentration (0.1 μg/ml) of IPM (**2a**), PAPM (**2b**) and MEPM (**1a**) against 27 clinical isolates of *P. aeruginosa*. These strains used in this examination were selected from recent clinical isolates in which **1a** and **2a** showed similar MIC, to enable an examination of the antagonistic effects of **1a** and **2a** at common concentrations. Therefore, the susceptibility of these strains to **1a** was relatively lower than that of typical isolates of *P. aeruginosa* reported by other research groups recently,[12,13] as shown in Table 1.

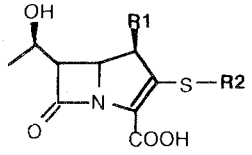
Similar distribution curves were also observed at the other two subinhibitory concentrations (0.2 and 0.39 μg/ml) of carbapenems (data not shown).

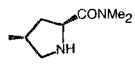
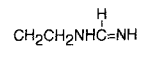
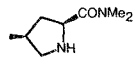
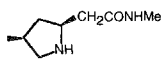
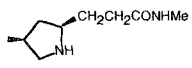
The addition of a subinhibitory concentration of **2a** caused a significant increase in the MICs of CAZ against the tested strains. PAPM (**2b**), which is structurally closely related to **2a** due to the presence of high basicity in the C-2 side chain and the absence of 1β-methyl group (Fig. 1), exhibited a similar antagonistic effect on the antipseudomonal activity of CAZ.

1a induced no or smaller CAZ-resistance compared with **2a** and **2b**.

These results prompted us to investigate the SARs of carbapenems to their antagonism of the antipseudomonal

Table 2. Susceptibility of 18 strains of *P. aeruginosa* to carbapenem antibiotics.



Strain		MIC (μg/ml)[a,b]				
	R_1:	Me	H	H	Me	Me
	R_2:					
		1a (MEPM)	**2a** (IPM)	**1b**	**3**	**4**
PAO1		0.78	1.56	1.56	1.56	1.56
IFO3451		0.78	0.78	0.78	1.56	0.78
BI-633		3.13	3.13	3.13	3.13	3.13
BI-634		0.78	1.56	1.56	0.78	0.78
BI-652		25	50	>50	25	25
BI-749		1.56	3.13	1.56	3.13	1.56
BI-758		3.13	1.56	3.13	3.13	3.13
BI-769		1.56	1.56	1.56	1.56	1.56
BI-782		1.56	0.78	1.56	1.56	0.78
BI-822		12.5	6.25	12.5	12.5	12.5
BI-845		0.78	0.78	0.78	0.78	0.78
BI-859		25	25	>50	25	25
BI-873		1.56	25	12.5	3.13	6.25
BI-915		25	12.5	50	12.5	25
BI-919		25	25	50	25	25
BI-972		25	25	50	12.5	25
BI-977		25	25	>50	25	25
BI-979		25	12.5	25	12.5	25

[a] MICs were determined by the twofold serial agar dilution method with MHA (inoculum size: 10^4 CFU/spot, incubated at 37°C ovemight).
[b] MEPM: meropenem, IPM: imipenem.

activity of CAZ in more detail.

Effects of 1β-Methyl Group and C-2 Side Chain on the Antagonism of the Antipseudomonal Activity of CAZ by Carbapenems

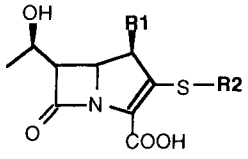
In a checkerboard titration assay, the antagonism of the antipseudomonal activity of CAZ by carbapenems was investigated using five carbapenem derivatives including IPM (**2a**) and MEPM (**1a**) as shown in Table 3. Table 2 presents the susceptibility data of the 18 strains used to the carbapenems.

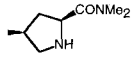
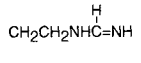
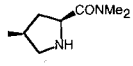
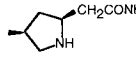
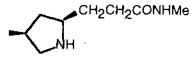
The antagonistic effect of **1a** was less than that of desmethyl-MEPM (**1b**) (Table 3). This result indicated that the introduction of a 1β-methyl moiety on the carbapenem skeleton decreases the antagonism of the antipseudomonal activity of CAZ by carbapenems due to the steric effect of the methyl group and/or the resulting conformational change of the C-2 side chain.[14]

The antagonistic effect of **2a** was greater than that of **1b** (Table 3). This result indicated that the antagonistic effect of the carbapenems was affected not only by the introduction of a 1β-methyl group but also by the structural features of the C-2 side chain. The basicity of the C-2 side chains of MEPM type **1a** and **1b** ($pK_a = 7.4$) is markedly different from that of **2a** ($pK_a = 9.9$).[15] Therefore, we focused on the effect of the basicity in the C-2 side chain of carbapenems, which is related to the cationic character in aqueous solution, on the antagonism of the antipseudomonal activity of CAZ.

Compounds **3** and **4** are structurally closely related to **1a**, but these have higher basicity ($pK_a = 8.9$ and 10.0, respectively) than that of **1a** in the C-2 side chain because of the presence of a methylene or ethylene spacer between the pyrrolidine ring and monomethylaminocarbonyl group.[15] Compound **4** exhibited the greatest antagonistic effect among compounds **1a**, **3** and **4**, and the antagonistic effect of **3** was greater than that of **1a** (Table 3).

Table 3. Effect of 1β-methyl group and C-2 side chain on the antagonism of the antipseudomonal activity of CAZ by carbapenems.



Strain	MIC of CAZ[a,b] (μg/ml)		MIC_{combi}/MIC_{alone}[c,d]				
		R_1:	Me	H	H	Me	Me
		R_2:					
			1a (MEPM)	**2a** (IPM)	**1b**	**3**	**4**
PAO1	1.56		4	16	8	8	16
IFO3451	0.78		2	8	2	4	4
BI-633	3.13		1	8	4	2	2
BI-634	3.13		2	8	4	4	4
BI-652	12.5		2	4	4	4	4
BI-749	3.13		2	8	4	8	16
BI-758	6.25		2	4	2	4	4
BI-769	12.5		1	2	2	2	4
BI-782	6.25		1	4	2	2	2
BI-822	6.25		2	4	2	4	8
BI-845	1.56		1	4	2	4	4
BI-859	6.25		4	8	16	8	16
BI-873	1.56		2	16	4	4	4
BI-915	6.25		4	8	4	4	4
BI-919	12.5		1	2	1	1	1
BI-972	3.13		4	8	4	4	4
BI-977	3.13		2	8	8	4	4
BI-979	3.13		4	8	4	4	8
Mean			2.3	7.1	4.3	4.2	6.1

[a] MICs were determined by the twofold serial agar dilution method with MHA (inoculum size: 10^4 CFU/spot, incubated at 37°C overnight).
[b] CAZ: ceftazidime.
[c] MIC_{combi}/MIC_{alone} is the ratio of the maximum MIC of CAZ combined with a carbapenem to the MIC of CAZ alone in checkerboard titration assay.
[d] MEPM: meropenem, IPM: imipenem.

These findings indicated that increasing the basicity of the C-2 side chain of carbapenem compounds increased their antagonistic effect, and the antagonistic effect of carbapenems which have a highly basic C-2 side chain was sufficiently large regardless of the presence or absence of a 1β-methyl group.

Based on these results, we suspected that the difference in the antagonistic effects of **1a** and **2a** was caused by the presence or absence of a 1β-methyl group and the different strengths of the basicity in the C-2 side chain.

The antagonistic effect of the carbapenems tested was not correlated to their antipseudomonal activity (Tables 2 and 3). This indicated that the antagonistic effect of the carbapenems was not affected by the antipseudomonal activity of the carbapenems.

Effect of 1β-Methyl Group and C-2 Side Chain on β-Lactamase Inducibility of Carbapenems in *P. aeruginosa*

The SARs of carbapenems to β-lactamase inducibility in *P. aeruginosa* was investigated, and the results are shown in Figs. 4 and 5. The strains BI-633 and BI-749, which showed similar susceptibility to five carbapenems tested (Table 2) and a marked difference in antagonism between **1a** and other carbapenems in the checkerboard titration assay (Table 3 and Fig. 3), were selected from the strains listed in Tables 2 and 3.

The β-lactamase inducibility of **2a** was markedly higher than that of **1a** at the concentration of 0.313 μg/ml (0.1 × MIC of **1a** and **2a**) in strain BI-633 (Fig. 4). This

Fig. 3. Antagonism of the antimicrobial activity of CAZ against *P. aeruginosa* BI-633 by MEPM and IPM in a checkerboard titration assay.

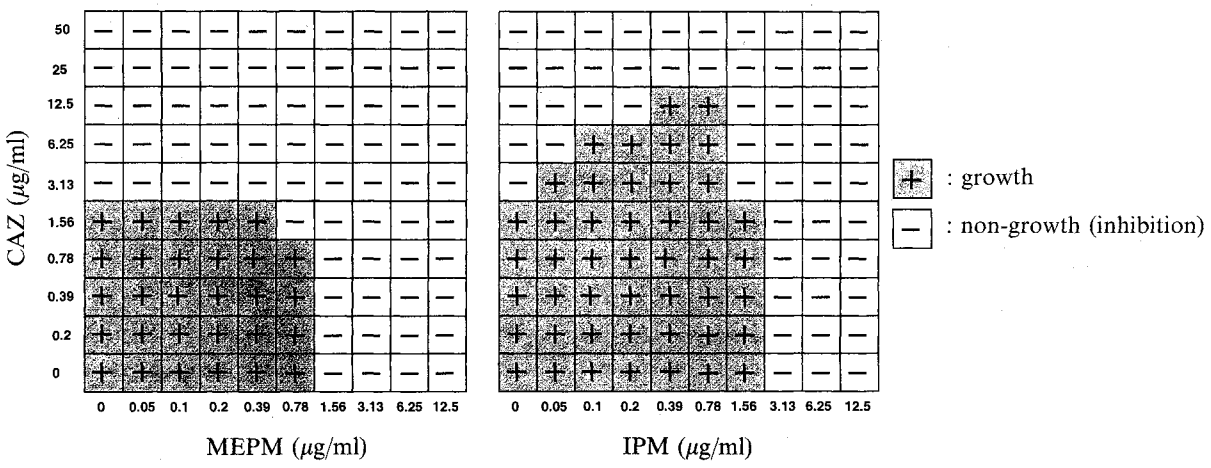
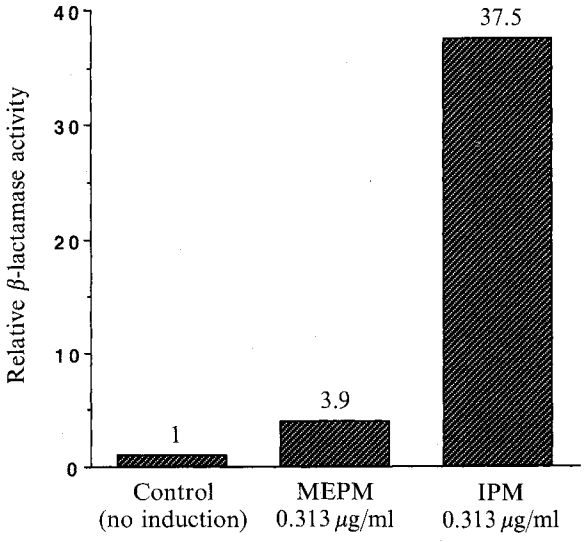


Fig. 4. β-Lactamase induction in *P. aeruginosa* BI-633 by MEPM and IPM.



The relative β-lactamase activity is expressed as a multiple of the basal level activity.

result is in agreement with a previous report,[5] and correlates with the results of the antagonism shown in Table 3 and Fig. 3.

We next investigated the SARs in detail, using four carbapenem compounds and strain BI-749 (Fig. 5). The same tendency of SARs was observed in β-lactamase inducibility as that of antagonism shown in Table 3, *i.e.*, the β-lactamase inducibility of desmethyl-MEPM (**1b**) and compound **4** were higher than that of **1a**, and that of **2a** was higher than that of **1b** at two subinhibitory concentrations, 0.39 μg/ml (1/8~1/4×MIC) and 0.78 μg/ml (1/4~1/2×MIC) (Fig. 5).

In this series of carbapenems, the β-lactamase inducibility of the carbapenems was decreased by the introduction of a 1β-methyl group and by the reduction of the basicity of the C-2 side chain.

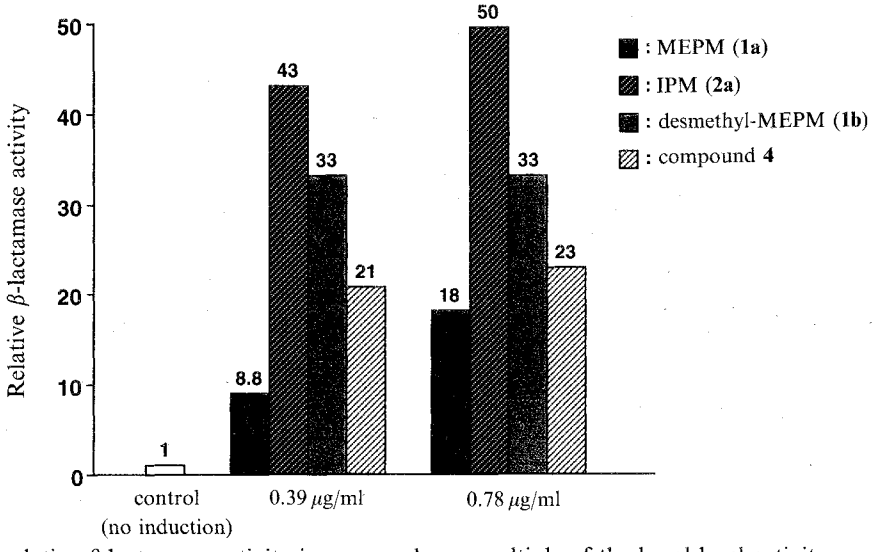
Relation of the Antagonism and β-Lactamase Inducibility

As described above, the structural features of carbapenems responsible for the antagonism of the antipseudomonal activity of CAZ were well consistent with those for the β-lactamase inducibility in *P. aeruginosa*. This indicated that the antagonism of the antipseudomonal activity of CAZ by carbapenems was caused mainly by the β-lactamase induction by carbapenems.

However, the β-lactamase inducibility of compound **4** was smaller than that of desmethyl-MEPM (**1b**) in BI-749 (Fig. 5), although the antagonistic effect of compound **4** was greater than that of **1b** (Table 3). This result suggested that the antagonistic effect of carbapenems was caused not only by the induction of β-lactamase but also by other unknown factors. Further study is needed to clarify the other factors.

In summary, we found that the 1β-methyl group in the carbapenem skeleton and the basicity of the C-2 side chain played important roles in the antagonism of the antipseudomonal activity of CAZ by carbapenems and in the β-lactamase inducibility of the carbapenems, as well as in their antipseudomonal activity as reported previously.[8] This study also clarified that the main factor

Fig. 5. β-Lactamase induction in *P. aeruginosa* BI-749 by MEPM, IPM, desmethyl-MEPM and compound **4**.



The relative β-lactamase activity is expressed as a multiple of the basal level activity.

in the antagonism of other β-lactams by carbapenems is the β-lactamase induction.

Although the clinical significance of this antagonism has not been established, it was reported that cefoxitin, which is a potent inducer of β-lactamase, antagonized the *in vivo* efficacy of other β-lactam agents in an animal infection model.[16] Therefore, the clinical implications of the antagonism should be elucidated in order to prevent treatment failure occurring in combination therapy (including continuous usage) for serious pseudomonal infections.

This information will be helpful for the selection of a suitable carbapenem in combination therapies with other antipseudomonal β-lactam antibiotics.

Acknowledgments

We wish to thank Ms. YASUKO HIRAI and Ms. YUKA KANENISHI for their excellent technical assistance and valuable discussions.

References

1) SYKES, R. B. & M. MATTHEW: The β-lactamases of Gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. 2: 115~157, 1976
2) TAUSK, F.; M. E. EVANS, L. S. PATTERSON, C. F. FEDERSPIER & C. W. STRATTON: Imipenem-induced resistance to antipseudomonal β-lactams in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 28: 41~45, 1985
3) BERTRAM, M. A. & L. S. YOUNG: Imipenem antagonism of the *in vitro* activity of the piperacillin against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 26: 272~274, 1984
4) SANDERS, C. C.; W. E. SANDERS, Jr. & R. V. GOERING: *In vitro* antagonism of beta-lactam antibiotics by cefoxitin. Antimicrob. Agents Chemother. 21: 968~975, 1982
5) NOUDA, H.; E. T. HARABE, Y. SUMITA, T. OKUDA & M. FUKASAWA: Beta-lactamase stability and inhibitory activity of meropenem combined with a potent antibacterial activity. Chemotherapy (Basel) 38: 218~224, 1992
6) SUMITA, Y.; Y. EGUCHI, M. FUKASAWA, T. OKUDA, H. YAMAGA, H. MATSUMURA & M. SUNAGAWA: The effect of 1β-methyl and imidoyl substituents on the anti-pseudomonal activity of carbapenems. J. Antibiotics 46: 1629~1632, 1993
7) SUMITA, Y.; H. YAMAGA & M. SUNAGAWA: The effect of 1β-methyl substituent and the basicity in C-2 side chain in carbapenem antibiotics on the activity against *Pseudomonas aeruginosa oprD2* and *nalB* mutants. J. Antibiotics 48: 89~91, 1995
8) SUNAGAWA, M.; H. MATSUMURA, A. SASAKI, H. YAMAGA, Y. SUMITA & H. NOUDA: Structure-activity relationship of 1β-methyl-carbapenems to its antibacterial activity: effect of the C-2 side chain and the 1β-methyl group. J. Antibiotics 49: 1175~1178, 1996
9) SUNAGAWA, M.; H. MATSUMURA, T. INOUE, M. FUKASAWA & M. KATO: A novel carbapenem antibiotic, SM-7338 structure-activity relationships. J. Antibiotics 43: 519~532, 1990
10) SUNAGAWA, M.; H. MATSUMURA, T. INOUE, M. FUKASAWA & M. KATO: A new series of carbapenem antibiotics with 5'-substituted pyrrolidinylthio group at C-2 position. J. Antibiotics 44: 459~462, 1991

11) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265～275, 1951
12) PITKIN, D. H.; W. SHEIKH & H. L. NADLER: Comparative *in vitro* activity of meropenem versus other extended-spectrum antimicrobials against randomly chosen and selected resistant clinical isolates tested in 26 north american centers. Clin. Infect. Dis. 24 (Suppl. 2): 238～248, 1997
13) BONFIGLIO, G.; V. CARCIOTTO, G. RUSSO, S. STEFANI, G. C. SCHITO, E. DEBBIA & G. NICOLETTI: Antibiotic resistance in *Pseudomonas aeruginosa*: an Italian survey. J. Antimicrob. Chemother. 41: 307～310, 1998
14) NISHIMURA, T.; J. IGARASHI, A. SASAKI & M. SUNAGAWA: Structural comparison of 1β-methylcarbapenem, carbapenem and penem: NMR studies and theoretical calculations. Bioorg. Med. Chem. 6: 367～375, 1998
15) NOUDA, H.; H. MATSUMURA, T. TANIO & M. SUNAGAWA: Structural features of carbapenem compounds for nephrotoxicity : effect of C-2 side chain. J. Antibiotics 49: 603～606, 1996
16) KUCK, N. A.; R. T. TESTA & M. FORBES: *In vitro* and *in vivo* antibacterial effects of combinations of beta-lactam antibiotics. Antimicrob. Agents Chemother. 19: 634～638, 1981