Structure-activity Relationships of Carbapenem Compounds to Anti-*Haemophilus influenzae* Activity and Affinity for Penicillin-binding Proteins

Effect of 1β-Methyl Group and C-2 Side Chain

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The anti-*H. influenzae* activity of meropenem (**1a**) was much higher than those of imipenem (**4**), panipenem (**2b**) and biapenem (**7**). To clarify the major structural features responsible for the anti-*H. influenzae* activity of carbapenem compounds, the structure-activity relationship to the anti-*H. influenzae* activity was investigated. The anti-*H. influenzae* activities of meropenem (**1a**) and 1β-methyl-panipenem (**2a**) were much higher than those of desmethyl-meropenem (**1b**) and panipenem (**2b**), respectively. Two carbapenems (**5**, **6**) and imipenem (**4**), that have a strong basic C-2 side chain, showed lower anti-*H. influenzae* activity than meropenem (**1a**) having a weakly basic C-2 side chain and *N*-acetyl thienamycin (**3**) having a neutral C-2 side chain, respectively. As a result, we found that the introduction of the 1β-methyl group or the reduction of the basicity (cationic character) of the C-2 side chain increased the antimicrobial activity and bactericidal activity of carbapenems against *H. influenzae* due to their increased affinity for PBP-4 and PBP-5.

Haemophilus influenzae is an important pathogen that causes purulent meningitis in infants and severe infections in immunocompromised adults[1]. Meropenem is a carbapenem antibiotic that has been accepted for clinical use, and its potency against *H. influenzae* is markedly higher than those of other carbapenems such as imipenem and panipenem[2~6]. The anti-*H. influenzae* activity of meropenem is equivalent to those of third-generation cephems[2,3,6], and sufficient for clinical use. Although imipenem-highly-resistant *H. influenzae* strains were isolated from clinical sources in recent years, meropenem has maintained sufficient activity against clinical isolates[3,5,6], and meropenem-resistant *H. influenzae* strains have never been isolated.

Previously, we investigated the relationship between the structure of carbapenems and their *in vitro* antimicrobial activity especially against *P. aeruginosa*[7,8], and discovered that the basicity of the C-2 side chain is indispensable for anti-pseudomonal activity and that the introduction of the 1β-methyl group variably affects the anti-pseudomonal activity depending on the feature of the C-2 side chain[8]. However, the structure-activity relationship of carbapenems to anti-*H. influenzae* activity and the mechanism of their anti-*H. influenzae* activity have not been investigated.

In the present study, we investigated the structure-activity relationships to their anti-*H. influenzae* activities, and their affinities for penicillin-binding proteins (PBPs) of *H. influenzae* to clarify the mechanism of their anti-*H. influenzae* activities, focusing on the introduction of a methyl group at the C-1 position and the cationic property in the C-2 side chain.

Materials and Methods

Antibiotics

Imipenem and panipenem were purified from imipenem/cilastatin (Banyu Pharmaceutical, Tokyo, Japan) and panipenem/betamipron (Sankyo, Tokyo, Japan), respectively, in Sumitomo Pharmaceuticals Research Center (Osaka, Japan). The other carbapenem compounds used in this work were prepared in Sumitomo Pharmaceuticals Research Center (Osaka, Japan) according to reported procedures[9~12]. The cephalosporins used in this report were obtained from commercial sources.

Bacterial Strains

Strains IID983 (ATCC9327), IID984 (ATCC9334) and IID1639 were β-lactam-susceptible *H. influenzae* strains obtained from Institute of Medical Science, University of Tokyo (Tokyo, Japan). Strain 54.24 (Rd) was a β-lactam-susceptible *H. influenzae* strain obtained from Institut Pasteur (Paris, France). Strains SP-11588 and SP-11590 were β-lactamase-negative, imipenem-resistant clinical isolates of *H. influenzae* obtained from Tokyo Clinical Research Center (Tokyo, Japan).

Determination of MICs and MBCs

MICs of β-lactams shown in Tables 1, 2, 3, 5 and 6

were determined by the twofold serial agar dilution method, with brain heart infusion agar (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 2 μg/ml-NAD and 10μg/ml-hemin (s-BHIA). Cells of the tested strains were grown on Chocolate-II Agar (Nippon Becton Dickinson, Tokyo, Japan) at 37°C in 5% CO_2 for 18 hours, and suspended in phosphate-buffered saline supplemented with 0.01% gelatin (BSG), and diluted with 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 Microplanter (Sakuma Seisakusyo, Tokyo, Japan). The plates were incubated at 37°C in 5% CO_2 for 24 or 48 hours. The MIC was defined as the lowest antibiotic concentration that completely prevented visible growth.

MICs and MBCs of β-lactams shown in Table 4 were determined by the macrobroth dilution method, with brain heart infusion broth (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 2 μg/ml-NAD and 10 μg/ml-hemin (s-BHIB). Cells of the tested strains were grown on Chocolate-II Agar at 37°C in 5% CO_2 for 18 hours, and suspended in BSG, and diluted with s-BHIB to give a final concentration of approximately 10^6 CFU/ml. The cell suspension (900 μl) added to 100 μl of a solution of the tested compound. The cultures were incubated at 37°C in 5% CO_2 for 24 hours without shaking. The MIC was the lowest concentration of the tested compound that demonstrated no visible growth. After the MIC was determined, 100-fold dilutions with BSG from each tube showing no turbidity were plated onto BHIA supplemented with 5% Fildes enrichment (Difco, Michigan, U.S.A.), and incubated at 37°C in 5% CO_2 for 24 hours. The MBC was the lowest concentration of the tested compound required to reduce viable cells by less than 0.01% of the initial inoculum.

In these experiments, the bacterial number of the inoculum was confirmed by the determination with Spiral System (Gunze Sangyo, Tokyo, Japan).

Culture of Cells and Preparation of Membrane Fraction for PBPs-affinity Assay

Cells of *H. influenzae* (IID984) were grown in s-BHIB at 37°C with shaking at 100 rpm, and harvested at mid-log phase ($OD_{600}=0.6$). The membrane fraction was prepared as previously described[13], and stored at −80°C.

PBPs-affinity Assay

Binding reactions and SDS-PAGE were carried out as previously described[13]. After electrophoresis, the gels were fixed with fixing solution-1 (50% ethanol, 10% acetic acid), and then fixed twice with fixing solution-2 (20% ethanol) for 1 hour. The fixed gels were dried on 3MM Chr paper (Whatman International, Maidstone, England) with Gel Slab Dryer Model 224 (BIO-RAD Laboratories, California, U.S.A.). A fluorogram was prepared by exposing the dried gel to Fuji Imaging Plate (IP) Type BAS-III (Fuji Photo Film, Tokyo, Japan) for 7 days. Quantification of the image density of the PBPs bound with [^{14}C]penicillin G was carried out by BAS-2000 (Fuji Photo Film). The binding affinities of β-lactam antibiotics for each of the PBPs were expressed in terms of IC_{50} values, which were the concentrations required to inhibit the binding of [^{14}C]penicillin G by 50%. We confirmed that the results obtained by the present method using IP and BAS-2000 were almost equivalent to the result obtained by the general method of fluorography using X-ray film[13].

Results and Discussion

Effect of 1β-Methyl Group on Anti-*H. influenzae* Activity

Table 1 shows the effect of the introduction of a 1β-methyl group on anti-*H. influenzae* activity. The anti-*H. influenzae* activities of meropenem (**1a**) and 1β-methyl-panipenem (**2a**) were markedly higher than those of desmethyl-meropenem (**1b**) and panipenem (**2b**), respectively. These findings, which were in agreement with the previous report[8], showed that the introduction of 1β-methyl group increases the anti-*H. influenzae* activity of carbapenem compounds due to the steric effect of the methyl group or the resulting conformational change of the side chain on C-2[14].

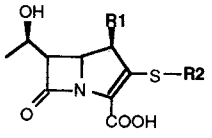
The compounds having 5′-dimethylaminocarbonyl pyrrolidin-3′-ylthio group as C-2 side chain (**1a**, **1b**) showed higher activity than the corresponding compounds having 1′-acetimidoylpyrrolidin-3′-ylthio group as C-2 side chain (**2a**, **2b**), respectively. This indicated that the anti-*H. influenzae* activity of carbapenem was not only affected by the introduction of a 1β-methyl group but also by the structural features of the C-2 side chain.

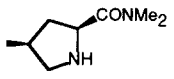
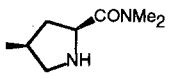
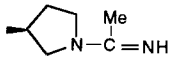
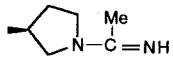
Effect of the Basicity of the C-2 Side Chain on Anti-*H. influenzae* Activity

The basicity of the C-2 side chain of meropenem (pKa = 7.4) is markedly different from that of panipenem (pKa = 10.9) and also that of imipenem (pKa = 9.9)[15]. Therefore, we focused on the influence of the basicity in the C-2 side chain of carbapenem, which is related to the cationic character in aqueous solution, on the anti-*H. influenzae* activity.

Table 2 shows the effect of the basicity in the C-2 side chain on anti-*H. influenzae* activity. *N*-acetylthienamycin (**3**) has a neutral C-2 side chain and the molecular weight is not very different from that of imipenem (**4**). The anti-*H. influenzae* activity of *N*-acetylthienamycin (**3**) was higher than that of imipenem (**4**). Compounds **5** and **6** are meropenem-analogues which have a highly basic C-2 side chain because of the presence of a methylene or

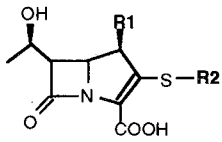
Table 1. Effect of 1β-methyl group of carbapenem compounds on anti-*H. influenzae* activity.

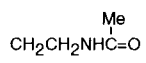
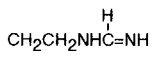
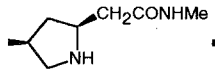
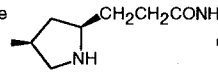
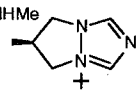


Strain		MIC (μg/ml)[a,b]			
	R_1:	Me	H	Me	H
	R_2:				
		1a (MEPM)	**1b**	**2a**	**2b** (PAPM)
IID983 (ATCC9327)		0.10	3.13	0.39	3.13
IID984 (ATCC9334)		0.10	0.78	0.20	1.56
IID1639		0.10	1.56	0.39	1.56
54.24 (Rd)		0.10	1.56	0.39	3.13
SP-11588		0.39	25	1.56	50
SP-11590		0.39	12.5	1.56	12.5

[a] Antimicrobial activity was measured by agar dilution method with s-BHIA (inoculumn size: 10^4 CFU/spot, incubated at 37°C in 5% CO_2 for 48 hours).
[b] MEPM: meropenem, PAPM: panipenem.

Table 2. Effect of C-2 side chain of carbapenem compounds on anti-*H. influenzae* activity.



Strain		MIC (μg/ml)[a,b]				
	R_1:	H	H	Me	Me	Me
	R_2:					
		3	**4** (IPM)	**5**	**6**	**7** (BIPM)
IID983 (ATCC9327)		3.13	6.25	0.39	0.78	1.56
IID984 (ATCC9334)		1.56	3.13	0.20	0.20	0.78
IID1639		1.56	6.25	0.39	0.39	1.56
54.24 (Rd)		1.56	6.25	0.39	0.39	12.5
SP-11588		25	100	1.56	3.13	50
SP-11590		6.25	12.5	1.56	3.13	25

[a] Antimicrobial activity was measured by agar dilution method with s-BHIA (inoculumn size: 10^4 CFU/spot, incubated at 37°C in 5% CO_2 for 48 hours).
[b] IPM: imipenem, BIPM: biapenem.

ethylene spacer between the pyrrolidine ring and methylaminocarbonyl group[15]. The anti-*H. influenzae* activities of these compounds (**5**, **6**) were lower than that of meropenem (**1a**). There was no significant difference among the anti-*H. influenzae* activities of compounds **2a**, **5** and **6** (Table 1, Table 2). These findings indicated that the 5'-substituent and the *N*-substituent on the pyrrolidinylthio group might not affect anti-*H. influenzae* activity, and that masking or reducing the basicity in the C-2 side chain of carbapenem compounds increased the anti-*H. influenzae* activity significantly.

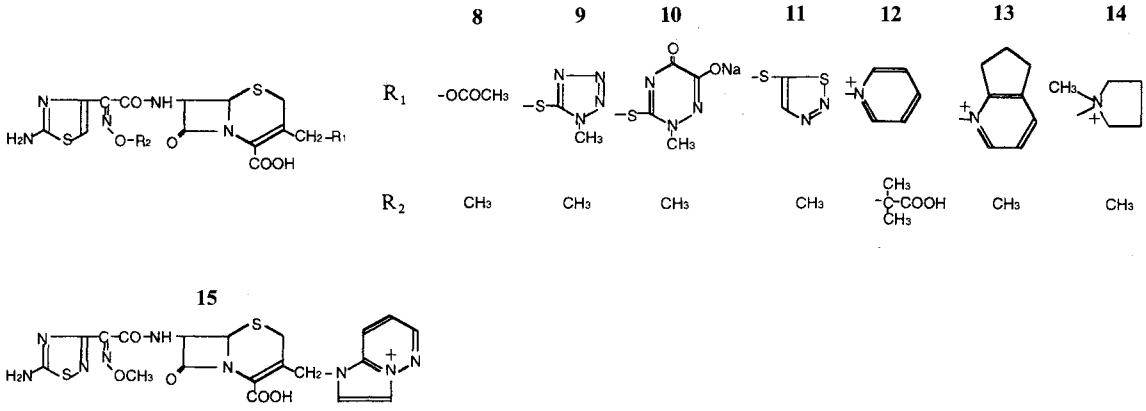
The anti-*H. influenzae* activity of biapenem (**7**) was lowest among the tested 1β-methyl carbapenem compounds, and almost equal to that of panipenem (**2b**)

Table 3. Anti-*H. influenzae* activity of third-generation cephalosporins and cefozopran.

Strain	MIC (μg/ml)[a,b]							
	CTX **8**	CMX **9**	CTRX **10**	CZON **11**	CAZ **12**	CPR **13**	CFPM **14**	CZOP **15**
IID983	0.025	≤0.013	≤0.013	≤0.013	0.10	0.05	0.10	0.10
IID984	0.050	≤0.013	≤0.013	0.025	0.10	0.10	0.10	0.39
IID1639	≤0.013	≤0.013	≤0.013	≤0.013	0.05	0.05	0.10	0.20
54.24	≤0.013	0.025	≤0.013	0.025	0.10	0.05	0.20	0.20
Cation in C-3 side chain	−	−	−	−	+	+	+	+

[a] Antimicrobial activity was measured by agar dilution method with s-BHIA (inoculumn size: 10^4 CFU/spot, incubated at 37°C in 5% CO_2 for 24 hours).

[b] CTX: cefotaxime, CMX: cefmenoxime, CTRX: ceftriaxone, CZON: cefuzonam, CAZ: ceftazidime, CPR: cefpirome, CFPM: cefepime, CZOP: cefozopran.



(Tables 1, 2), probably because of the cationic feature of the C-2 side chain.

A similar structure-activity correlation was also found in a series of cephalosporin antibiotics. Cephalosporins having no cation in their C-3 side chains (cefotaxime, cefmenoxime, ceftriaxon and cefuzonam) showed higher anti-*H. influenzae* activities compared with cephalosporins having a cationic C-3 side chain (ceftazidime, cefepime, cefpirome and cefozopran) (Table 3). Several previous reports[16~19] also support this correlation of the cephalosporin antibiotics. This suggests that the physico-chemical properties of the C-3 side chain of cephalospoins affects their anti-*H. influenzae* activity, like that of the C-2 side chain of carbapenems. It was also consistent with the result of a conformational analysis of carbapenems and cephalosporins reported previously, that is, the relative geometries of the C-2 side chain of carbapenems and the C-3 side chain of cephalosporins from each β-lactam ring and carboxylic acid group, in the most stable conformation could be well overlapped each other.[20]

Effect on Bactericidal Activity against *H. influenzae* IID984

Table 4 shows the effects of the introduction of a 1β-methyl group and the basicity of the C-2 side chain on bactericidal activity (MBC) against *H. influenzae* IID984. The bactericidal activity of the tested compound was increased by both the introduction of 1β-methyl group and the masking the basicity of the C-2 side chain, and correlated well with each antimicrobial activity (MIC). This indicated that the bactericidal activity paralleled the antimicrobial activity of carbapenems.

Table 4. Effects of 1β-methyl group and C-2 side chain of carbapenem compounds on bactericidal activity (MBC) against *H. influenzae* IID984.

Compound	MBC[a] (μg/ml)	(MIC)[b]
1a (Meropenem)	0.20	(0.10)
1b	1.56	(0.39)
5	0.39	(0.20)
3	3.13	(0.78)
4 (Imipenem)	6.25	(1.56)

[a] MBC values indicate concentrations of compounds required to reduce viable cells by less than 0.01% of initial inoculum (3×10^6 CFU/ml).

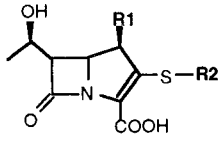
[b] Antimicrobial activity (MIC) was measured by macrobroth dilution method with s-BHIB (inoculum size: 3×10^6 CFU/ml, incubated at 37°C in 5% CO_2 for 24 hours).

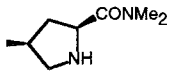
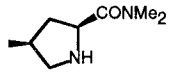
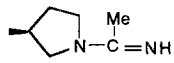
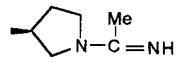
Affinities of β-Lactam Antibiotics for the PBPs of *H. influenzae* IID984

To clarify the structure-activity relationships of carbapenems to anti-*H. influenzae* activity in greater detail, we investigated the affinities of carbapenems for PBPs of *H. influenzae* which related to the mechanism of their anti-*H. influenzae* activity. The affinities (IC_{50}) of the tested carbapenems for PBPs of IID984 are shown in Tables 5 and 6. In addition, though the data are not shown, similar findings were obtained in experiments using the other strain, IID1639.

The affinities of the tested carbapenems for PBP-4 (data not shown, because the detected bands of PBP-4 were too weak to calculate the IC50 value exactly, but

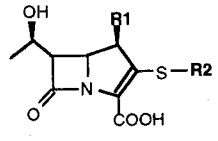
Table 5. Effect of 1β-methyl group of carbapenem compounds on affinity for PBPs of *H. influenzae* IID984.

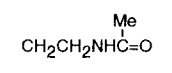
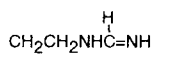
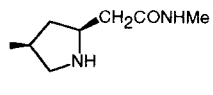
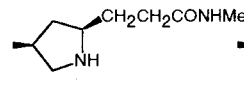
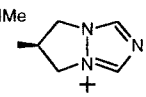


PBPs	IC_{50} (μg/ml)[a]			
R_1:	Me	H	Me	H
R_2:				
	1a (MEPM)	**1b**	**2a**	**2b** (PAPM)
PBP-1	>10.0	0.53	>10.0	0.36
PBP-2	0.046	0.061	0.031	0.018
PBP-3	0.019	0.072	0.018	0.016
PBP-5	0.033	0.15	0.083	0.58
PBP-6	0.023	0.012	0.018	0.078
PBP-7	0.48	0.098	1.0	0.095
MIC (μg/ml)[b]	0.10	0.78	0.20	1.56

[a] Values indicate concentrations of compounds required to reduce [^{14}C]-benzylpenicillin binding by 50%.
[b] Antimicrobial activity was measured by agar dilution method with s-BHIA (inoculum size: 10^4 CFU/spot, incubated at 37°C in 5% CO_2 for 48 hours).

Table 6. Effect of C-2 side chain of carbapenem compounds on affinity for PBPs of *H. influenzae* IID984.



PBPs	IC_{50} (μg/ml)[a]				
R_1:	H	H	Me	Me	Me
R_2:					
	3	**4** (IPM)	**5**	**6**	**7** (BIPM)
PBP-1	0.18	0.22	7.3	>10.0	3.9
PBP-2	0.053	0.061	0.022	0.018	0.029
PBP-3	0.064	0.046	0.010	0.0084	0.016
PBP-5	0.18	0.81	0.054	0.075	0.27
PBP-6	0.11	0.033	0.0060	0.0073	0.017
PBP-7	0.16	0.10	1.0	1.6	0.92
MIC (μg/ml)[b]	1.56	3.13	0.20	0.20	0.78

[a] Values indicate concentrations of compounds required to reduce [^{14}C]-benzylpenicillin binding by 50%.
[b] Antimicrobial activity was measured by agar dilution method with s-BHIA (inoculum size: 10^4 CFU/spot, incubated at 37°C in 5% CO_2 for 48 hours).

the affinities for the tested compounds were equivalent to those of PBP-5) and PBP-5 showed a good correlation with each antimicrobial activity. These results showed that the 1β-methyl group and the structural feature of the C-2 side chain affected to the anti-*H. influenzae* activity of carbapenems due to their affinity for PBP-4 and PBP-5 of *H. influenzae*.

It is known that PBP-4 and PBP-5 of *H. influenzae* are enzymes that have vital cell functions in cell wall incorporation and septum formation[21~23], and affinities of penicillins or cephems for these PBPs correlate with their anti-*H. influenzae* activities[24,25]. As the major factor of the mechanism of non-β-lactamase-mediated resistance to penicillins and cephems in *H. influenzae*, the low affinities of PBP-4 and PBP-5 for several β-lactam antibiotics have been reported[25~27].

On the other hand, the affinities of the 1β-methyl carbapenems (**1a**, **2a**, **5**, **6** and **7**) for PBP-1 and PBP-7 were obviously lower than those of the desmethyl carbapenems (**1b**, **2b**, **3** and **4**). These findings indicated that the introduction of 1β-methyl group decreased the affinities of carbapenems for PBP-1 and PBP-7. And there was no significant difference in the affinities for PBP-2, PBP-3 and PBP-6 among the tested compounds.

As a result, in this series of carbapenems, we considered that the affinities for PBP-4 and PBP-5 were important for their anti-*H. influenzae* activity, and the affinities for the other PBPs did not directly influence the anti-*H. influenzae* activity.

In summary, we found that the introduction of a 1β-methyl group or the reduction of the basicity of the C-2 side chain increased the antimicrobial activity and bactericidal activity of carbapenems against *H. influenzae* by increasing their affinity for PBP-4 and PBP-5. This is the first report describing the structure-activity relationships of carbapenems to anti-*H. influenzae* activity and affinity for PBPs, and this information will be helpful for designing new β-lactam antibiotics.

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