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The Mode of Action of 2-(Thiazol-2-ylthio)-1β-methylcarbapenems against *Pseudomonas aeruginosa*: The Impact of Outer Membrane Permeability and the Contribution of MexAB-OprM Efflux System

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Abstract The mode of action of a series of 2-(4-dihydropyrrolylthiazol-2-ylthio) and 2-(4-tetrahydropyridinylthiazol-2-ylthio)-1β-methylcarbapenem analogues against *Pseudomonas aeruginosa* was investigated with regard to contributions of the affinity for penicillin binding proteins (PBPs), the outer membrane permeability, and the effect of the MexAB-OprM efflux system. In this series of carbapenems, the introduction of a substituent in C-2 side chain with a change in physicochemical properties affected the antipseudomonal activity depending on the molecular weight. However, these structural modifications did not affect the affinity for pseudomonal PBPs significantly. It was confirmed that the affinity for PBPs was not an important determinant of the antipseudomonal activity of this series of carbapenems. OprD porin-deficiency did not affect antipseudomonal activity either. On the other hand, the MIC of these carbapenems against *P. aeruginosa* significantly decreased in the presence of outer membrane permeabilizer. This result strongly suggests that the cause of the relatively low antipseudomonal activity of these carbapanems is their low permeability through the outer membrane of *P. aeruginosa*. And also, in the presence of outer membrane permeabilizer, the MICs against MexAB-OprM deficient mutants remarkably decreased and were very close to the value of the IC_{50} for pseudomonal PBPs. From this result, it was clear that the effect of the MexAB-OprM efflux system was also an important determinant of antipseudomonal activity of these carbapenems. In conclusion, the major determinants of the antipseudomonal activity of the 2-(thiazol-2-ylthio)-1β-methylcarbapenems are the outer membrane permeability and the effect of the MexAB-OprM efflux system, not the affinity for pseudomonal PBPs.

Keywords 2-(thiazol-2-ylthio)-1β-methylcarbapenem, *Pseudomonas aeruginosa*, penicillin binding protein, outer membrane permeability, OprD, MexAB-OprM efflux system

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Introduction

The emergence of multiresistant Gram-positive cocci such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and penicillin-resistant *Streptococcus pneumoniae* (PRSP) has reduced the value of antibacterial chemotherapy in recent years [1, 2]. Therefore, there is an urgent need for new antibacterial agents effective against these resistant pathogens.

SMP-601 (formerly SM-216601) is a novel parenteral 1β-methylcarbapenem. SMP-601 exhibits antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, especially multiresistant Gram-positive pathogens, including MRSA, PRSP, and VRE. Moreover, this carbapenem showed favorable pharmacokinetics in mice, rats, dogs, and cynomolgus monkeys, suggesting a long-acting pharmacokinetic profile in humans, and exhibited decreased convulsant activity in mice. Thus, SMP-601 should be a promising candidate as a broad-spectrum β-lactam antibiotic [3].

We previously reported the structure-activity relationships of SMP-601 and its closely related derivatives, 2-(4-dihydropyrrolylthiazol-2-ylthio) and 2-(4-tetrahydropyridinylthiazol-2-ylthio)-1β-methylcarbapenems, with regard to their activities against Gram-positive and Gram-negative bacteria [4]. It was also suggested that the anti-Gram-negative bacterial activity, especially the antipseudomonal activity, was closely related to the ability to permeate the outer membrane of Gram-negative bacteria, because of the good correlation between the levels of activity and physicochemical properties, such as basicity (cp*K*a) and/or lipophilicity (clogP) [4, 5], although the affinity profile for bacterial penicillin binding proteins (PBPs) and other factors, which also affect antibacterial activity, could not be excluded.

In this paper, in order to clarify the contribution of the affinity for PBPs, outer membrane permeability, and the effect of the MexAB-OprM efflux system, to the antipseudomonal activity of 2-(thiazol-2-ylthio)-1β-methylcarbapenems, we examined the affinity for pseudomonal PBPs and antipseudomonal activities against the isogenic outer membrane or efflux pump mutants of *Pseudomonas aeruginosa* using eleven 2-(thiazol-2-ylthio)-1β-methylcarbapenem derivatives which have various C-2 side chain substituents and various physicochemical properties (molecular weight, cp*K*a, clogP).

Materials and Methods

Bacterial Strains

P. aeruginosa IFO3451 and NCTC10490 were used to assay the affinity for PBPs. *P. aeruginosa* PAO1 (wild-type), IMP3 and KG2603 (OprD-deficient mutant of PAO1), OCR1 and KG2212 (MexAB-OprM-overproducing mutant of PAO1), KG2225 (MexAB-deficient mutant of PAO1) and KG2239 (MexAB-OprM-deficient mutant of PAO1) were used for testing susceptibility.

Antimicrobial Agents

A series of 2-(thiazol-2-ylthio) carbapenem derivatives (Fig. 1) and meropenem were synthesized in the laboratories of the Dainippon Sumitomo Pharma Research Division as previously reported [4]. Tetracycline and chloramphenicol were purchased from Sigma.

Affinity for Pseudomonal PBPs

The affinity for PBPs was estimated by competition assay as follows. The detection of the PBPs of *P. aeruginosa* was carried out using a nonradioactive method with BOCILLIN FL (Molecular Probes, Inc.), a fluorescent penicillin [6, 7]. The membrane fraction of *P. aeruginosa* was prepared as previously reported [8].

First, 17.5 μl of the membrane preparation (175 μg of protein) was incubated with 2.5 μl of different concentrations of test compounds at 35°C for 10 minutes. Next, 10 μl of BOCILLIN FL was added to a final concentration of 12.5 μM, and the reaction mixture was kept at 35°C for 30 minutes. Then, 1.0 μl of a solution containing 15% sodium sarcosine and 45 mg/ml of unlabelled penicillin V (MP Biomedicals, Inc.) was added and the reaction mixture was centrifuged at room temperature for 30 minutes. The supernatant (10 μl) was subjected to SDS-PAGE (10% polyacrylamide gel; Bio-Rad Laboratories). To visualize the labeled PBPs, the gels were directly scanned with a 2D 2920 Master Imager (excitation at 480 nm and emission at 530 nm) (Amersham Pharmacia Biotech). The fluorescence intensity of each band was quantified by Scion Image, which is available on the Internet as a free download from Scion Corporation (http://www.scioncorp.com). Binding affinity was expressed as the IC_{50}: the concentration (in micrograms per milliliter) that inhibited BOCILLIN FL from binding by 50% compared with the control.

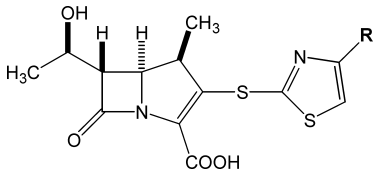
Susceptibility Testing

MICs were determined by the serial agar dilution method [9], with Mueller-Hinton agar (Difco). The final inocula comprised approximately 10^4 cfu. Agar plates were incubated at 37°C for 17 to 24 hours. The MIC was defined as the lowest drug concentration that completely prevented visible growth. Disodium EDTA or sodium hexametaphosphate (NaHMP) was used as an outer membrane permeabilizer for testing MICs under conditions where the outer membrane permeant barrier was reduced.

Results and Discussion

Correlation between Antipseudomonal Activity and Physicochemical Property

The MIC of each derivative against strain IFO3451 is shown in Table 1. MIC values ranged from 4 to 32 μg/ml. It has been previously reported that antipseudomonal activity of carbapenem is closely related to its basicity and/or lipophilicity of C-2 side chain substituents [4, 5]. However, there was no correlation between the antipseudomonal activity and cp*K*a value or clogP value of these eleven carbapenems. On the other hand, there was a good correlation between the antipseudomonal activities and molecular weights of these carbapenems (Fig. 2). Six derivatives with high molecular weights (MW=



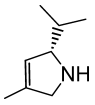
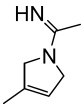
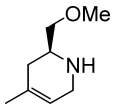
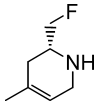
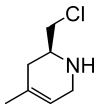
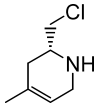
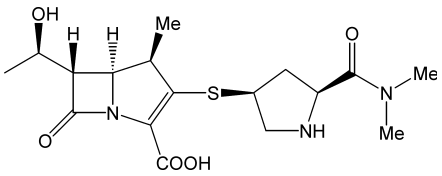
R	R	
1		cp*K*a=8.51 clogP=−0.15 M.W.=393.48
2 (SMP-601)		cp*K*a=8.58 clogP=0.34 M.W.=407.51
3		cp*K*a=8.58 clogP=0.34 M.W.=407.51
4		cp*K*a=8.45 clogP=1.22 M.W.=435.57
5		cp*K*a=11.15 clogP=−1.71 M.W.=434.54
6		cp*K*a=9.12 clogP=0.18 M.W.=407.51
7		cp*K*a=9.19 clogP=0.67 M.W.=421.54
8		cp*K*a=8.43 clogP=−0.06 M.W.=451.56
9		cp*K*a=7.97 clogP=0.26 M.W.=439.53
10		cp*K*a=8.06 clogP=0.64 M.W.=455.98
11		cp*K*a=8.06 clogP=0.64 M.W.=455.98
Meropenem		cp*K*a=8.34 clogP=−3.13 M.W.=383.51

Fig. 1 Structures of 2-(thiazol-2-ylthio)-1β-methylcarbapenem derivatives and meropenem.

434.54～455.98) showed high MICs (16～32 μg/ml). In contrast to these compounds, five derivatives with relatively low molecular weights (MW=393.48～421.54) showed relatively high antipseudomonal activity (4～8 μg/ml). In fact, meropenem (Table 1, MW=383.51) and other excellent antipseudomonal carbapenems (imipenem (MW=299.34), panipenem (MW=339.42), biapenem (MW=350.40)) have relatively low molecular weights [10～12].

From these results, it was suggested that the molecular weight of 2-(thiazol-2-ylthio)-1β-methylcarbapenems with a certain range of cp*K*a value or clogP value is an important determinant of antipseudomonal activity.

In order to clarify the cause of the difference of antipseudomonal activity among these derivatives and the mode of action of this series of carbapenems against *P. aeruginosa*, we examined the contribution of the affinity for PBPs, outer membrane permeability, and the effect of the MexAB-OprM efflux system on the antipseudomonal activities of these carbapenem derivatives.

Affinity for PBPs

The affinity (IC_{50}) of each derivative for pseudomonal PBPs is shown in Table 1.

Among eleven compounds (dihydropyrrole derivatives: **1**～**5**; tetrahydropyridine derivatives: **6**～**11**), only a slight difference of affinity for each pseudomonal PBP was observed. All derivatives also showed relatively high

affinity, slightly lower or equivalent to those of meropenem for essential PBPs (PBP1A, 1B, 2, 3) and PBP4. Therefore, no significant correlation between the affinities for each pseudomonal PBP and antipseudomonal activities was observed in this series of carbapenems. Almost the same results were observed in preliminary experiments using strain NCTC10490 (data not shown).

From these results, it was shown that the C-2 side chain substitutions with the change in physicochemical properties did not significantly affect the affinity for pseudomonal PBPs of these derivatives, and it was confirmed that the affinity for PBPs was not an important determinant of the antipseudomonal activity of this series of carbapenems.

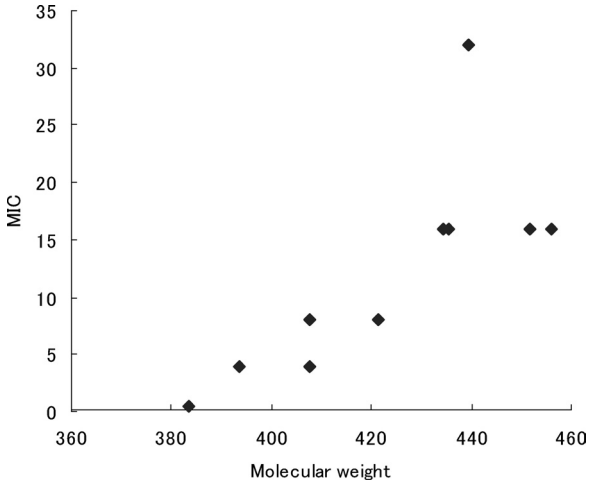


Fig. 2 Correlation between molecular weight and MIC against *Pseudomonas aeruginosa* IFO3451 in 2-(thiazol-2-ylthio)-1β-methylcarbapenems and meropenem.

Effect of Outer Membrane Permeability and MexAB-OprM Efflux System

It was reported that pre-existing carbapenems (imipenem, meropenem, panipenem, biapenem) mainly pass through the outer membrane of *P. aeruginosa via* a specific porin channel, outer membrane protein D_2 (OprD), and some are excreted by multidrug efflux systems such as the MexAB-OprM efflux system of *P. aeruginosa* [13～16]. The MICs of derivatives **1**～**11** against various OprD or MexAB-OprM mutants of *P. aeruginosa* PAO1 are shown in Table 2.

(1) Effect of OprD Expression

The MICs against PAO1 ranged from 8 to 32 μg/ml. For all derivatives, MICs against OprD-deficient mutants (IMP3 and KG2603) were not significantly increased in comparison with those against the parent strain PAO1. This indicated that these carbapenem derivatives, the molecular weight of which was larger than those of pre-existing carbapenems, did not utilize the OprD channel significantly to pass through the outer membrane of *P. aeruginosa*, unlike other carbapenems.

(2) Effect of MexAB-OprM Expression

The effect of the expression of the MexAB-OprM efflux system on the antipseudomonal activity of the carbapenems was investigated. The MICs of the six derivatives (**1**, **2**, **3**, **5**, **6** and **7**) against MexAB-OprM overproducing strains

Table 1 Antimicrobial activity and affinity for PBPs of *P. aeruginosa* IFO3451

Compound No.	IC_{50} (μg/ml)						MIC (μg/ml)
	1A	1B	2	3	4	5/6	
1	0.13	0.19	0.11	0.01	<0.01	0.39	4
2 (SMP-601)	0.32	0.27	0.17	0.03	0.02	0.43	8
3	0.42	0.26	0.22	0.02	0.01	0.38	4
4	0.27	0.26	0.22	0.03	0.02	0.33	16
5	0.28	0.24	0.27	0.08	0.02	0.56	16
6	0.45	0.32	0.18	0.02	<0.01	0.36	4
7	0.21	0.20	0.24	0.03	0.01	0.42	8
8	0.33	0.25	0.32	0.06	0.04	0.58	16
9	0.53	0.55	0.21	0.15	0.03	2.77	32
10	0.43	0.32	0.30	0.12	0.03	0.43	16
11	0.94	0.32	0.32	0.07	0.02	0.39	16
Meropenem	0.25	0.30	0.03	0.03	0.02	8.24	0.5

Table 2 Contribution of OprD and the MexAB-OprM efflux system to antipseudomonal activity

Compound No.	MIC (μg/ml)						
	PAO1 (parent)	IMP3 (OprD-)	KG2603 (OprD-)	OCR1 (MexAB-OprM+)	KG2212 (MexAB-OprM+)	KG2225 (MexAB-)	KG2239 (MexAB-OprM-)
1	8	8	8	8	8	8	8
2 (SMP-601)	16	16	8	16	16	8	8
3	8	8	8	16	16	8	8
4	16	16	16	32	32	8	8
5	32	32	32	32	32	16	16
6	8	8	8	8	8	8	8
7	16	16	16	32	16	16	16
8	32	>32	32	>32	>32	16	16
9	32	>32	32	>32	>32	16	16
10	32	64	32	64	64	16	16
11	32	32	32	128	64	16	16

were equal or slightly higher (by one dilution) than those against MexAB-deficient or MexAB-OprM deficient strains. On the other hand, the MICs of all other five derivatives (**4**, **8**, **9**, **10** and **11**) against MexAB-OprM overproducing strains were significantly higher (more than two dilutions) than those against MexAB-deficient or MexAB-OprM-deficient strains. The former derivatives have a relatively low molecular weight (393.48～434.54) and high p*K*a (8.51～11.15), and the latter derivatives have a relatively high molecular weight (435.57～455.98) and low p*K*a (7.97～8.45). The relationships between these physicochemical properties and the effect of the MexAB-OprM efflux system on the antipseudomonal activity of the carbapenems, and the contribution of the MexAB-OprM efflux system on the antipseudomonal activity of these carbapenems have not been sufficiently clarified in this study; however, these findings are very interesting and valuable for further studies.

(3) Effect of Outer Membrane Permeabilizer

The MICs of these carbapanems against MexAB-deficient or MexAB-OprM-deficient mutants were higher than the IC_{50}s for pseudomonal PBPs, and it was possible that outer membrane permeability greatly contributed to anti-pseudomonal activity. Therefore, we investigated the effect of outer membrane permeabilizers on the antipseudomonal activity of these carbapenems. It was reported that the function of the outer membrane as a barrier to a variety of antibiotics, such as tetracycline and chloramphenicol, was reduced by EDTA (2.5 mM) or NaHMP (25 mM) as a divalent cation chelator [17, 18].

The MICs of the seven carbapenem derivatives against each strain decreased 8- to 256-fold in the presence of 2.5 mM EDTA or 25 mM NaHMP (Table 3). Therefore, it was found that disruption of the outer membrane barrier by the addition of a cation chelator caused a significant increase of antipseudomonal activities in this series of carbapanems. This result strongly suggests that the cause of the relatively low antipseudomonal activity in this series of carbapenems is their low permeability through the outer membrane of *P. aeruginosa*.

(4) Effect of MexAB-OprM Expression in the Presense of Outer Membrane Permeabilizer

The effect of the expression of the MexAB-OprM efflux system on the antipseudomonal activity of the carbapenems in the presence of an outer membrane permeabilizer was investigated (Table 3). In the presence of chelators, the MICs of all derivatives against MexAB-OprM-overproducing strains were significantly higher (more than two dilutions) than those against MexAB-deficient or MexAB-OprM-deficient strains. Namely, in the presence of an outer membrane permeabilizer, the effect of MexAB-OprM on antipseudomonl activity was observed in a wider range of derivatives in this series of carbapanems. Also, the relationships between these physicochemical properties (molecular weight, cp*K*a) and the effect of the MexAB-OprM efflux system on the MIC was not clear in this condition in comparison with the result of the MIC without outer membrane permeabilizer. Therefore, it was suggested that the relationships between these physicochemical properties (molecular weight, cp*K*a) and the effect of the

Table 3 Effect of outer membrane permeabilizers on antipseudomonal activity

Compound No.	Permeabilizer	MIC (μg/ml)						
		PAO1 (parent)	IMP3 (OprD-)	KG2603 (OprD-)	OCR1 (MexAB-OprM+)	KG2212 (MexAB-OprM+)	KG2225 (MexAB-)	KG2239 (MexAB-OprM-)
1	none	8	16	8	16	16	8	8
	EDTA	0.5	0.5	0.5	1	1	0.25	0.125
	NaHMP	0.25	0.5	0.25	2	0.5	≦0.063	≦0.063
2	none	16	16	16	16	16	8	8
(SMP-601)	EDTA	1	0.5	1	2	2	0.25	0.125
	NaHMP	0.5	0.5	0.5	4	1	≦0.063	≦0.063
3	none	16	16	16	16	16	8	8
	EDTA	1	1	1	2	2	0.125	≦0.063
	NaHMP	0.5	0.5	0.5	8	1	≦0.063	≦0.063
4	none	32	32	32	64	32	16	8
	EDTA	4	2	4	8	4	0.25	0.125
	NaHMP	2	2	2	16	4	0.125	≦0.063
6	none	8	16	8	16	16	8	8
	EDTA	0.5	0.5	0.5	1	1	0.25	0.125
	NaHMP	0.5	0.5	0.25	4	1	≦0.063	≦0.063
7	none	16	32	16	32	32	16	16
	EDTA	2	2	2	2	2	0.5	0.125
	NaHMP	1	1	1	8	2	0.125	≦0.063
10	none	32	64	32	128	64	16	16
	EDTA	8	4	8	16	8	0.25	0.125
	NaHMP	2	4	2	32	8	0.125	≦0.063
Meropenem	none	0.5	4	2	2	1	0.25	0.25
	EDTA	0.5	0.5	1	1	1	≦0.063	≦0.063
	NaHMP	0.25	1	0.5	2	0.5	≦0.063	≦0.063
Tetracycline	none	16	16	16	64	32	2	0.5
	EDTA	0.5	0.25	0.5	2	1	≦0.063	≦0.063
	NaHMP	0.25	0.25	0.25	2	0.5	≦0.063	≦0.063
Chloramphenicol	none	64	64	64	512	128	4	2
	EDTA	2	1	2	4	2	0.5	≦0.25
	NaHMP	2	2	2	16	4	0.5	0.5

MexAB-OprM efflux system on antipseudomonl activity without an outer membrane permeabilizer was caused by the difference in outer membrane permeability among these carbapenam derivatives.

On the other hand, in the presence of these chelators, the MICs against MexAB-deficient or MexAB-OprM-deficient mutants remarkably decreased. These MIC values were very close to the values of IC_{50}s for pseudomonal PBPs, and there was not much difference in comparison with meropenem under the same conditions. This result indicated that the antipseudomonal activity of these carbapenem derivatives reflected the affinity for pseudomonal PBPs when there was no effect of the outer membrane barrier and the MexAB-OprM efflux system. From these results, it was clear that the effect of the MexAB-OprM efflux system was an important determinant of antipseudomonal activity of these carbapenems.

In conclusion, the major determinants of the antipseudomonal activity of the 2-(thiazol-2-ylthio)-1β-methylcarbapenems are the outer membrane permeability and the effect of the MexAB-OprM efflux system, not the affinity for pseudomonal PBPs.

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