SYNERGISM OF PS-5 WITH PENICILLINS AND CEPHALOSPORINS IN ANTIMICROBIAL ACTIVITY AGAINST β-LACTAM-RESISTANT GRAM-NEGATIVE MICROORGANISMS

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The *in vitro* synergism of PS-5 combined with various penicillins and cephalosporins in antimicrobial activity was examined in detail against β-lactam-resistant Gram-negative bacteria. PS-5 showed a highly significant synergism in antimicrobial action against *Escherichia coli* RGN238 in combination with penicillins; and against *Proteus vulgaris* GN76 and *Serratia marcescens* T55 in combination with cephalosporins. It was moderately synergistic against *Citrobacter freundii* GN346, *Enterobacter cloacae* 45, *Proteus morganii* 111 and *Enterobacter aerogenes* E19, whereas no synergism was observed against *Pseudomonas aeruginosa* E2 and *Klebsiella pneumoniae* 130.

Since the discovery of thienamycin,[1] 38 carbapenem compounds have been isolated from streptomycetes and bacteria.[2] Microbiologically, carbapenem compounds are clearly distinguished from penicillins and cephalosporins in antimicrobial spectra and β-lactamase-inhibitory activities.

Classical penicillins and cephalosporins are generally more active against Gram-positive microorganisms than against Gram-negative ones, whereas carbapenems have an unusually wide spectrum of antimicrobial activity against both Gram-positive and -negative microbes. The increasing frequency of occurrence of β-lactamases in Gram-negative pathogens is a serious problem in chemotherapy. As one of the reasonable approaches to this problem, β-lactamase inhibitors have been screened from natural and synthetic compounds, resulting in the isolation of clavulanates and sulfo-penicillins. The recent development of Augmentin (a combination of amoxicillin with clavulanate) has been proved to be clinically effective against a relatively limited range of bacteria.[3] Since carbapenem antibiotics, which have a potent antimicrobial activity in contrast to clavulanate, are also strong β-lactamase inhibitors,[4~7] it is interesting to examine the possible advantages of carbapenems over clavulanate in combination of carbapenems with traditional penicillins and cephalosporins from the viewpoint of clinical chemotherapy, although the mode of inhibition of β-lactamases by carbapenem compounds appears complicated and hardly analysable by the conventional concept of β-lactamase inhibition.

The object of this communication is to study the synergism of PS-5 combined with various penicillins and cephalosporins with regard to antimicrobial activity against β-lactam-resistant Gram-negative bacteria.

Materials and Methods

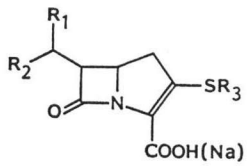
Microorganisms

Microbes used in the present paper are from our culture collection.

Minimum Inhibitory Concentration (MIC)

MIC was measured by the revised method of the Japan Society of Chemotherapy.[8]

Fig. 1. Chemical structures of the carbapenem compounds.



Carbapenem	R_1	R_2	R_3
PS-5	H	CH_3	$CH_2CH_2NHCOCH_3$
PS-6	CH_3	CH_3	$CH_2CH_2NHCOCH_3$
PS-7	H	CH_3	$CH=CHNHCOCH_3$
NS-5	H	CH_3	$CH_2CH_2NH_2$
OA-6129A	H	CH_3	$CH_2CH_2NHCOCH_2CH_2NHCO-CH(OH)-C(CH_3)(CH_3)-CH_2OH$

Synergistic Effect

The synergy test was performed with a 96-well Microtiter plate (Cooke Engineering, U.S.A.) as described in a previous paper.[9] The fractional inhibitory concentration (FIC) index was calculated according to the method of WEINSTEIN *et al.*,[10] and was defined as follows: FIC$\leqq$0.5: synergistic, 0.5$<$FIC$\leqq$1.0: additive, 1.0$<$FIC: antagonistic.

Antibiotics

Naturally-occurring carbapenem compounds were prepared as sodium salts by fermentation as detailed in previous papers.[11~14] The chemical structures of the carbapenem compounds used in the present paper are presented in Fig. 1.

The following penicillins and cephalosporins were obtained from commercial sources: benzylpenicillin (PCG; Meiji Seika Kaisha, Ltd.), ampicillin (ABPC; Toyo Jozo Co., Ltd.), methicillin (DMPPC; Banyu Pharmaceutical Co., Ltd.), oxacillin (MPIPC; Banyu Pharmaceutical Co., Ltd.), cloxacillin (MCIPC; Fujisawa Pharmaceutical Co., Ltd.), piperacillin (PIPC; Toyama Chemical Co., Ltd.), carbenicillin (CBPC; Pfizer International Inc.), sulbenicillin (SBPC; Takeda Chemical Industries, Ltd.), ticarcillin (TIPC; Beecham Yakuhin K. K.), cephalothin (CET; Shionogi & Co., Ltd.), cephaloridine (CER; Shionogi & Co., Ltd.), cefazolin (CEZ; Fujisawa Pharmaceutical Co., Ltd.), cephalexin (CEX; Takara Kohsan Co., Ltd.), cephacetrile (CEC; Takeda Chemical Industries, Ltd.), cefotiam (CTM; Takeda Chemical Industries, Ltd.), cefotaxime (CTX; Hoechst Japan Ltd.), ceftizoxime (CZX; Fujisawa Pharmaceutical Co., Ltd.), cefoperazone (CPZ; Toyama Chemical Co., Ltd.), ceftezole (CTZ; Fujisawa Pharmaceutical Co., Ltd.), cefoxitin (CFX; Daiichi Seiyaku Co., Ltd.) and cefmetazole (CMZ; Sankyo Co., Ltd.).

Results

Table 1 summarizes the antimicrobial spectra of PS-5, PS-6, PS-7, NS-5 (deacetylated PS-5), OA-6129A, ampicillin (reference penicillin) and cefazolin (reference cephalosporin). It is evident from Table 1 that the carbapenem compounds generally exhibit potent antimicrobial activities against a wide variety of Gram-positive and -negative microorganisms including β-lactamase-producing strains.

With the possibility that the carbapenem compounds might be utilized as β-lactamase inhibitors in combination with known penicillin and cephalosporin compounds, the following β-lactamase-producing microbes were chosen for a detailed examination of the synergistic effect of PS-5 combined

Table 1. MIC's of PS-5, PS-6, PS-7, NS-5, OA-6129A, ampicillin and cefazolin.

Microorganism	PS-5	PS-6**	PS-7**	NS-5	OA-6129A	ABPC	CEZ
Bacillus subtilis ATCC 6633	0.10	0.78	0.39	0.05	0.39	0.05	0.10
Micrococcus luteus S19	0.10	0.39	0.20	0.024	0.78	0.013	0.39
Staphylococcus aureus FDA209P	0.024	0.10	0.39	0.001	0.78	0.013	0.10
S. aureus Smith	0.20	0.20	0.39	0.003	1.56	0.05	0.20
S. aureus Russell*	0.20	0.20	0.39	0.024	0.78	25	0.20
S. epidermidis	0.20	0.39	0.20	0.05	1.56	0.024	0.20
Alcaligenes faecalis A1	0.78	1.56	0.78	1.56	1.56	3.13	3.13
Citrobacter freundii GN346*	3.13	12.5	3.13	6.25	50	>400	>400
Comamonas terrigena B-996	0.012	0.10	0.05	0.05	0.05	0.05	0.05
Enterobacter aerogenes E19*	3.13	25	3.13	6.25	25	>400	>400
E. cloacae 45*	3.13	25	3.13	12.5	50	>400	>400
Enterobacter sp. E8*	3.13	6.25	1.56	6.25	12.5	6.25	3.13
Escherichia coli K-12	1.56	6.25	0.78	6.25	12.5	3.13	0.78
E. coli RGN238*	3.13					1,250	1.3
E. coli RGN823*	3.13	6.25	1.56	3.13	12.5	400	1.56
Klebsiella pneumoniae 130*	6.25					10,000	39
K. pneumoniae K13*	3.13	25	3.13	6.25	50	400	25
Proteus mirabilis P6	6.25	12.5	6.25	12.5	50	3.13	3.13
P. rettgeri P7	3.13	6.25	1.56	12.5	25	>400	3.13
P. vulgaris GN76*	6.25	12.5	12.5	12.5	100	>400	>400
P. morganii 111*	6.25					625	78
Proteus sp. P22*	6.25	25	12.5	12.5	100	>400	>400
Pseudomonas aeruginosa IFO3445	12.5	50	12.5	3.13	>100	>400	>400
P. aeruginosa NCTC10490	12.5	50	6.25	0.78	>100	>400	>400
P. aeruginosa E2*	400					1,250	10,000
Serratia marcescens S18*	3.13	25	1.56	12.5	100	200	>400
S. marcescens T55*	6.25	50	3.13	12.5	100	50	>400

Inoculum size: 10^8 cells/ml (** 10^5 cells/ml).
*: β-Lactamase producer.

Table 2. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *E. coli* RGN238 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	625	0.10	+	20	0.06
ABPC	1,250	0.05	+	5	0.02
MCIPC	5,000	0.05	+	625	0.14
MPIPC	2,500	0.10	+	313	0.16
SBPC	625	0.10	+	5	0.03
CBPC	5,000	0.10	+	78	0.05
TIPC	2,500	0.10	+	20	0.03
PIPC	39	0.05	+	1.3	0.02
CET	20	0.78	+	2.5	0.38
CER	5	0.78	+	1.3	0.50
CEX	20	0.78	+	2.5	0.38
CEC	5	0.78	+	2.5	0.75
PS-5	3.13				

with various penicillins and cephalosporins: *Escherichia coli* RGN238, *Proteus vulgaris* GN76, *Serratia marcescens* T55, *Citrobacter freundii* GN346, *Enterobacter cloacae* 45, *Proteus morganii* 111, *Enterobacter aerogenes* E19, *Klebsiella pneumoniae* 130, *Pseudomonas aeruginosa* E2.

Table 3. Summary of the synergism of PS-5, NS-5 and OA-6129A with β-lactam antibiotics in antimicrobial activities against *P. vulgaris* GN76 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	5,000	1.56	+	313	0.19
ABPC	1,250	0.39	+	78	0.12
MCIPC	625	0.39	+	313	0.56
MPIPC	1,250	1.56	+	313	0.50
DMPPC	313	0.20	+	156	0.53
CBPC	5	1.56*	+	1.3	0.31
TIPC	2.5	3.13*	+	0.63	0.50
CEZ	625	0.39	+	39	0.12
CER	1,250	0.39	+	39	0.06
CEX	1,250	0.39	+	78	0.12
CET	1,250	0.78	+	39	0.16
CEC	625	0.39	+	39	0.12
CTZ	156	0.78	+	10	0.19
CTM	78	0.39	+	2.5	0.09
PS-5	6.25				
ABPC	1,250	1.56	+	78	0.12
CER	1,200	1.56	+	39	0.08
NS-5	25				
ABPC	1,250	6.25	+	39	0.16
CER	1,250	6.25	+	39	0.16
OA-6129A	50				

* MIC of PS-5: 12.5 μg/ml.

Table 4. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *S. marcescens* T55 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	1,250	0.39	+	156	0.19
MCIPC	1,250	0.20	+	625	0.52
MPIPC	1,250	0.78	+	625	0.63
DMPPC	5,000	3.13	+	625	0.63
ABPC	39	0.78	+	10	0.38
CER	10,000	0.20	+	156	0.05
CET	10,000	0.39	+	313	0.09
CEZ	10,000	0.78	+	78	0.13
CEX	625	0.78	+	156	0.38
CEC	5,000	0.20	+	313	0.09
CTZ	2,500	0.78	+	78	0.16
PS-5	6.25				

The synergistic effect of PS-5 against *E. coli* RGN238 is presented in Table 2. *E. coli* RGN238 produces plasmid-mediated β-lactamase (penicillinase).[15] The synergism of PS-5 is clearly more significant with the penicillin compounds than with the cephalosporin compounds.

The MIC values against *P. vulgaris* GN76 (Table 3) were obtained with the combinations of PS-5 with the penicillins and the cephalosporins. This strain was already reported to be sensitive to the synergism of several carbapenem compounds with penicillins and cephalosporins.[4,7,16] As it produces

Table 5. Summary of the synergism of PS-5 and OA-6129A with β-lactam antibiotics in antimicrobial activities against *C. freundii* GN346 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
ABPC	1,250	0.78	+	156	0.38
MCIPC	625	0.39	+	156	0.38
MPIPC	313	1.56	+	10	0.53
DMPPC	2,500	1.56	+	78	0.53
CBPC	156	0.39	+	78	0.63
TIPC	156	1.56	+	20	0.63
PIPC	78	1.56	+	5	0.56
SBPC	625	0.39	+	156	0.38
PCG	5,000	1.56	+	625	0.50
CER	1,250	0.78	+	20	0.27
CEX	2,500	0.78	+	313	0.38
CET	5,000	0.78	+	625	0.38
CEZ	2,500	0.39	+	625	0.38
CFX	313	0.20	+	156	0.56
CMZ	156	0.78	+	39	0.50
PS-5	3.13				
ABPC	1,250	6.25	+	313	0.38
CER	1,250	6.25	+	313	0.38
OA-6129A	50				

Table 6. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *E. cloacae* 45 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	10,000	1.56	+	1,250	0.38
ABPC	2,500	1.56	+	156	0.31
DMPPC	5,000	3.13	+	625	0.63
CET	10,000	1.56	+	1,250	0.38
CEX	5,000	3.13	+	20	0.50
CER	1,250	0.78	+	156	0.25
CEZ	2,500	1.56	+	156	0.31
CEC	2,500	0.78	+	1,250	0.63
CTZ	1,250	1.56	+	78	0.31
CFX	156	3.13	+	39	0.75
CMZ	78	0.78	+	39	0.63
PS-5	6.25				

type Ic cephalosporinase,[17] and as OKAMURA *et al.* described that PS-5 inhibited the β-lactamase of *P. vulgaris*,[18] it will be interesting to study the possible correlation of the synergistic effect with the β-lactamase-inhibitory effect. Table 3 demonstrates that PS-5 is more synergistic with the cephalosporins than with the penicillins. Although the available data are scarce, NS-5 and OA-6129A seem similarly synergistic in antimicrobial activity against this microorganism, when they are combined with penicillins and cephalosporins.

Table 4 reveals that *S. marcescens* T55 is roughly as sensitive as *P. vulgaris* GN76 to the synergism of PS-5 with the known β-lactam compounds.

Table 7. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *P. morganii* 111 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	625	1.56	+	78	0.38
ABPC	625	1.56	+	78	0.38
SBPC	1,250	1.56	+	625	0.75
MPIPC	2,500	0.39	+	625	0.31
TIPC	625	1.56	+	78	0.38
CBPC	1,250	0.78	+	313	0.38
MCIPC	2,500	1.56	+	625	0.50
CEX	5,000	1.56	+	1,250	0.50
CEC	625	1.56	+	156	0.50
CER	78	0.78	+	20	0.38
CEZ	78	1.56	+	20	0.50
CET	78	1.56	+	39	0.75
CTZ	20	1.56	+	10	0.75
PS-5	6.25				

Table 8. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *E. aerogenes* E19 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
PCG	2,500	0.78	+	625	0.50
MPIPC	313	1.56	+	156	0.63
CEZ	1,250	3.13	+	313	0.50
CER	625	1.56	+	78	0.38
CET	5,000	3.13	+	1,250	0.50
CEX	625	0.78	+	625	1.06
CEC	1,250	1.56	+	78	0.56
CTZ	625	0.78	+	78	0.38
PS-5	3.13				

Table 9. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *K. pneumoniae* 130 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
TIPC	10,000	6.25	+	10,000	
PCG	10,000	3.13	+	5,000	1.0
ABPC	10,000	3.13	+	5,000	1.0
CBPC	10,000	3.13	+	10,000	
CER	156	1.56	+	39	0.50
CET	78	1.56	+	39	0.75
CTZ	20	1.56	+	10	0.75
CEC	78	1.56	+	39	0.75
PS-5	6.25				

C. freundii GN346 is a producer of type Ia cephalosporinase.[10] The FIC indices in Table 5 indicate that the combination effects of PS-5 with the penicillins and cephalosporins are moderately synergistic. OA-6129A also seems moderately synergistic.

Table 10. Summary of the synergism of PS-5 with β-lactam antibiotics in antimicrobial activities against *P. aeruginosa* E2 (MIC in μg/ml).

Antibiotic	Alone	In combination			FIC index
		PS-5	+	β-Lactam	
ABPC	625	>400	+	156	
DMPPC	1,250	>400	+	625	
MPIPC	1,250	>400	+	625	
PCG	5,000	>200	+	2,500	
PS-5	>400				

Table 11. Conclusion on the synergistic activity of PS-5 with penicillins and cephalosporins.

Microorganism	Penicillins		Cephalosporins	
	FIC ≦0.50	<0.125	≦0.50	<0.125
E. coli RGN238	8/8	6/8	2/4	0/4
P. vulgaris GN76	3/7	2/7	7/7	5/7
S. marcescens T55	2/5	1/5	6/6	3/6
C. freundii GN346	3/9	0/9	4/6	0/6
E. cloacae 45	2/3	0/3	4/8	0/8
P. morganii 111	5/7	0/7	1/6	0/6
E. aerogenes E19	0/2	0/2	2/6	0/6
K. pneumoniae 130	0/4	0/4	0/4	0/4
P. aeruginosa E2	0/4	0/4		

The synergy tests against *E. cloacae* 45, *P. morganii* 111 and *E. aerogenes* E19 are summarized in Tables 6, 7 and 8 respectively. On these organisms the combinations of PS-5 with the penicillins and cephalosporins produce moderately synergistic effects except for several cases of additive effects.

All the synergy tests against *K. pneumoniae* 130 show additive effects (Table 9).

Against *P. aeruginosa* E2, as the MIC value of PS-5 is above 400 μg/ml, the combination effect of PS-5 with the known β-lactam compounds is hardly conclusive from Table 10, although it is likely to be additive or slightly synergistic.

Table 11 summarizes the overall results of the synergy tests of PS-5 against the nine β-lactamase-producing microbes. It is apparent from Table 11 that PS-5 is highly synergistic with the penicillins against *E. coli* RGN238; and with the cephalosporins against *P. vulgaris* GN76 and *S. marcescens* T55.

Discussion

Synergism of antimicrobial activity is well documented in combinations of depsipeptide antibiotics such as viridogrisein with non-peptidyl macrocyclic lactone antibiotics such as griseoviridin.[20] The mechanism of action responsible for the antimicrobial synergy is due to the inhibition of protein synthesis at two different sites of peptide elongation. Since carbapenem compounds are β-lactamase inhibitors,[18,21~23] and as the synergism is observed only against β-lactamase-producing microbes, it is considered likely that the inhibition of β-lactamase is the primary cause for the antimicrobially synergistic effect of carbapenems with penicillins and cephalosporins. For example, OKAMURA *et al.* reported a close correlation of β-lactamase inhibition by PS-5 with antimicrobial synergy.[4]

Clavulanate is least antimicrobial, but acts on some types of β-lactamases as a strong inhibitor.[24] Augmentin, a combined preparation of amoxicillin with clavulanate, effectively reduces the MIC values of amoxicillin against several pathogenic microbes.[25] In contrast to clavulanate, carbapenem compounds have strong antimicrobial activities together with potent β-lactamase-inhibitory properties.

Therefore it is essential to differentiate their antimicrobial activity from their β-lactamase-inhibitory activity in the examination of the synergistic effect.

Taken together with the results reported in previous papers,[4,9,26,27] the experimental findings in the present paper clearly indicate that the inhibition of β-lactamase is primarily responsible for the antimicrobial synergism. It is important to note, however, that the antimicrobial synergism of PS-5 varies depending on the types of β-lactamases and combined penicillin and cephalosporin compounds. In general, *E. coli* RGN238 producing penicillinase is synergistically more sensitive to the combination of PS-5 with the penicillins than with the cephalosporins, while the reverse is observed with *P. vulgaris* GN76 and *S. marcescens* T55 which are seemingly cephalosporinase producers, suggesting that the significance of inhibition of the responsible enzyme by PS-5 is more profound for good substrates than for poor substrates. Furthermore, the extent of antimicrobial synergism seems to depend on the substrate profile of the enzyme. As β-lactam-resistant pathogens most often produce β-lactamases with unknown substrate profiles, it is ideal to choose an appropriate penicillin or cephalosporin compound which is the most synergistic in combination with a given carbapenem compound so that the best therapeutic results can be obtained clinically. For detailed analysis of the probable correlation of the antimicrobial synergism with the β-lactamase inhibition, the modes of inhibition of the involved β-lactamases by PS-5 are under study using highly or partially purified preparations of these enzymes.

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