STUDIES ON THE OA-6129 GROUP OF ANTIBIOTICS, NEW CARBAPENEM COMPOUNDS

II. *IN VITRO* EVALUATION

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The OA-6129 group of antibiotics, new carbapenem compounds, had relatively potent antimicrobial activities against Gram-positive and Gram-negative bacteria. Synergism of compound OA-6129A in combination with conventional β-lactam antibiotics was observed in antimicrobial activity against β-lactamase producers such as *Proteus vulgaris* GN 76 and *Citrobacter freundii* GN 346. The new carbapenem compounds were slightly superior to PS-5 in stability to kidney homogenates of various animal species.

In the last several years, a number of new β-lactam antibiotics that are characterized by a 7-oxo-1 azabicyclo[3.2.0]hept-2-ene ring system have been isolated from streptomycetes[1~11]. This family of antibiotics has been named carbapenem. We have recently isolated new members of this family, compounds OA-6129A, B_1, B_2 and C, from the culture broth of *Streptomyces* sp. OA-6129. Fermentation, isolation and physico-chemical properties of these compounds have been described in the preceding paper[12]. This paper deals with the *in vitro* evaluation of the OA-6129 group of carbapenem compounds in comparison with PS-5.

Materials and Methods

Antibiotics

Carbapenem compounds OA-6129A, B_1, B_2, C[12] and PS-5[4] (sodium salts) were fermentatively prepared in our laboratories. Compound (−)OA-6129A, a synthetic carbapenem derivative, was prepared as described elsewhere[13]. Cefazolin sodium (CEZ) was obtained from Fujisawa Pharmaceuticals Co., Ltd., ampicillin sodium (ABPC) from Toyo Jozo Co., Ltd. and cephaloridine (CER) from Shionogi & Co., Ltd.

Bacterial Strains

The test organisms employed in this paper were from the stock culture collection in our laboratories.

Determination of Minimum Inhibitory Concentrations (MIC's)

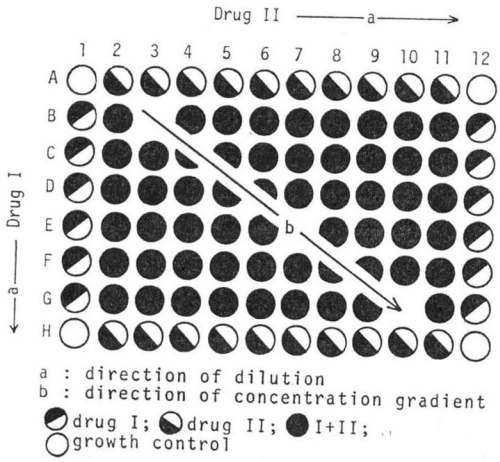
Heart infusion agar (Difco Laboratories, U.S.A.) was used, unless otherwise specified[14]. An appropriate dilution (10^6 cells/ml) of a fresh overnight culture of the test microbe was prepared and 1 μl of the dilution was inoculated on the agar plate containing a test antibiotic. The agar plates were incubated at 35°C for 18 hours. The MIC value was defined as the lowest concentration of antibiotic that inhibited the development of visible growth of the test microorganism.

Synergistic Effect

The synergy test was performed with a 96-well Microtiter® plate (Cooke Engineering, U.S.A.). Plate layout of the assay is given in Fig. 1.

A two-fold dilution series of drug I was prepared in Heart infusion broth (HIB, Difco) and 50 μl each of the drug I dilutions was pipetted into all wells of row B through row G. Then, two-fold serial

Fig. 1. Plate layout of Microtiter plate synergy assay.



Medium: Heart infusion broth (Difco).
Inoculum size: 10^5 cells/ml.
Final volume of dilution: 200 μl.

dilutions of drug II were made and 50 μl each of the dilutions was pipetted into all wells of column 2 through column 11. Each well in the Microtiter® plate was inoculated with 100 μl of growth medium (HIB) containing 2×10^5 cells/ml of the test organism. The final volume of the mixture in a well was 200 μl. The four corner wells of the plate contained no drug and used as positive growth controls. The MIC of drug I alone was determined in columns 1 and 12. The MIC of drug II alone was determined in rows A and H.

The fractional inhibitory concentration (FIC) index was calculated by the method of WEINSTEIN *et al.*[15] as follows:

$$\text{FIC index} = \frac{\text{MIC of drug I in combination}}{\text{MIC of drug I alone}} + \frac{\text{MIC of drug II in combination}}{\text{MIC of drug II alone}}$$

The effect was defined as synergistic when the FIC index was equal to or less than 0.5; as additive when 0.5~1.0; and as antagonistic when more than 1.0.

Stability Test in Kidney Homogenates

Mouse kidneys were excised from male *dd*Y mice (aged 5 weeks; weighing 19~21 g) and dog kidneys from a female mongrel dog (weighing 12 kg). Seemingly healthy portions of renal cortex were collected from urologically excised human kidneys.

Fresh kidneys were homogenized in 5 volumes of 0.02 M phosphate buffer, pH 7.5, with a Teflon® Potter homogenizer, and centrifuged at 0°C and $8,000\times g$ for 20 minutes to give a kidney homogenate. For removal of possibly remaining antimicrobial agents, the human kidney homogenate was dialyzed at 4°C for 18 hours in 0.02 M phosphate buffer, pH 7.5.

Reaction mixtures containing one volume each of a kidney homogenate and an antibiotic solution were incubated at 37°C for 60 minutes. Enzyme reactions were stopped by heating the reaction mixtures at 100°C for 15 seconds at the end of incubation. The concentration of the antibiotic remaining in the reaction mixture was determined by bioassay using *Comamonas terrigena* B-996[4].

Plasma Levels in Mice

Male *dd*Y mice (aged 5 weeks; weighing 19~21 g) were used in groups of 5 mice each. A single dose of an antibiotic was given intravenously. Blood samples were taken from the retroorbital sinus at indicated times with heparinized capillary tubes. Each blood sample was centrifuged at $1,500\times g$ for 5 minutes and the supernatant was assayed by the disk-agar diffusion method with *C. terrigena* B-996 as the assay organism.

Results

Antimicrobial Activity

Table 1 summarizes the MIC's of the OA-6129 group of carbapenem compounds and related antibiotics against Gram-positive bacteria.

Compound OA-6129A which differs from PS-5 in the C-3 pantetheinyl side chain (accordingly it is 1.5-fold larger in molecular weight than PS-5) is 4~32-fold less active than PS-5[16]. The most active in the OA-6129 group of carbapenems is compound OA-6129B_1 which has the 8-(*S*)-hydroxyl group and the 5,6-*cis* configuration. Compound OA-6129B_2, a 6-(*R*)-epimer of compound OA-6129B_1, is

Table 1. Comparative antimicrobial activities of the OA-6129 group of carbapenem compounds, PS-5 and cefazolin (CEZ) against Gram-positive bacteria (MIC μg/ml).

Microorganism	OA-6129A	OA-6129B$_1$	OA-6129B$_2$	OA-6129C	(−)OA-6129A	PS-5	CEZ
Bacillus subtilis ATCC 6633	0.39	0.05	1.56	0.10	3.13	0.10	0.10
Micrococcus luteus S19	0.78	0.10	6.25	0.78	3.13	0.10	0.39
Staphylococcus aureus FDA 209P	0.78	0.10	6.25	0.10	0.78	0.024	0.10
Staphylococcus aureus Smith	1.56	1.56	12.5	0.78	3.13	0.20	0.20
Staphylococcus aureus Russell*	0.78	0.78	12.5	3.13	3.13	0.20	0.20
Staphylococcus epidermidis	1.56	0.78	12.5	3.13	3.13	0.20	0.20

* β-Lactamase producer.
Medium: Heart infusion agar (Difco); Inoculum size: 10^6 cells/ml.

8~64-fold less active than compound OA-6129B$_1$. Compound OA-6129C which is sulfated compound OA-6129B$_1$ seems slightly less active than compound OA-6129B$_1$.

Even if the relative increase of the OA-6129 group in molecular weight is taken into consideration, the C-3 pantetheinyl side chain clearly reduces the antimicrobial activity of carbapenems against Gram-positive bacteria. It is particularly apparent from the comparison of compound OA-6129A with PS-5 which has the C-3 acetylcysteaminyl side chain.

Table 2 presents the antimicrobial activities of the OA-6129 group of carbapenems against Gram-negative bacteria. As is also seen in Table 1, the exchange of the acetylcysteaminyl side chain with

Table 2. Comparative antimicrobial activities of the OA-6129 group of carbapenem compounds, PS-5 and cefazolin (CEZ) against Gram-negative bacteria (MIC μg/ml).

Microorganism	OA-6129A	OA-6129B$_1$	OA-6129B$_2$	OA-6129C	(−)OA-6129A	PS-5	CEZ
Alcaligenes faecalis Al	1.56	0.20	6.25	0.39	3.13	0.78	3.13
Citrobacter freundii GN 346*	50	6.25	25	50	25	3.13	>400
Comamonas terrigena B-996	0.05	0.012	0.39	0.10	0.10	0.012	0.05
Enterobacter aerogenes E19*	25	3.13	25	6.25	12.5	3.13	>400
Enterobacter cloacae 45*	50	6.25	50	50	25	3.13	>400
Enterobacter sp. E8*	12.5	0.39	25	1.56	12.5	3.13	3.13
Escherichia coli K-12	12.5	0.39	12.5	0.78	12.5	1.56	0.78
Escherichia coli RGN 823*	12.5	0.39	12.5	0.39	12.5	3.13	1.56
Klebsiella pneumoniae K13*	50	>50	12.5	12.5	50	3.13	25
Proteus mirabilis P6	50	0.78	50	0.78	25	6.25	3.13
Proteus rettgeri P7	25	0.78	25	0.20	25	3.13	3.13
Proteus vulgaris GN 76*	100	50	50	1.56	100	6.25	>400
Proteus sp. P22*	100	>50	50	0.78	100	6.25	>400
Providencia sp. P8	12.5	0.20	6.25	0.20	12.5	3.13	1.56
Pseudomonas aeruginosa IFO 3445	>100	>50	>100	50	200	12.5	>400
Pseudomonas aeruginosa NCTC 10490	>100	>50	>100	>50	200	12.5	>400
Serratia marcescens S18*	100	6.25	25	12.5	50	3.13	>400
Serratia marcescens T55*	100	12.5	50	12.5	100	6.25	>400

* β-Lactamase producer.
Medium: Heart infusion agar (Difco); Inoculum size: 10^6 cells/ml.

the pantetheinyl one results in 4~16-fold reduction of antimicrobial activity against Gram-negative pathogens. Quantitative comparison of antibiotic activity is possible between compound OA-6129A and PS-5 in Table 2. The presence of the C-3 pantetheinyl side chain also seems to be related to reduced antibiotic potency against some of the β-lactamase-producing bacteria. In general, the OA-6129 group of carbapenems have no anti-pseudomonal activity.

Influence of the Stereochemistry of the C-3 Pantetheinyl Side Chain on Antimicrobial Activity

The OA-6129 group of carbapenems contain the natural type (or *R*-type) of pantoate. For comparison purpose, compound (−)OA-6129A, a synthetic carbapenem containing the *S*-type of pantoate, was synthesized from *p*-nitrobenzyl PS-5 S-oxide by replacing with *S*-pantetheine[13]. The antimicrobial spectrum of compound (−)OA-6129A is included in Tables 1 and 2. Although there is no difference noted between the two isomers in antimicrobial activity against Gram-negative bacteria, the natural isomer of compound OA-6129A is more active against Gram-positive bacteria than the synthetic counterpart.

Synergism

Synergism in antimicrobial activity of carbapenem compounds combined with conventional penicillins and cephalosporins is at least partly ascribed to the β-lactamase-inhibitory activity of carbapenems[17]. Table 3 shows the synergistic effects of compound OA-6129A combined with ampicillin and cephaloridine on antimicrobial activity against β-lactamase-producing bacteria.

Similar to PS-5, compound OA-6129A has a marked synergism with ampicillin and cephaloridine in antibiotic activity against type Ic β-lactamase-producing *Proteus vulgaris* GN 76.

Stability in Kidney Homogenates

Because of the unexpected susceptibility to renal dipeptidase[18~21] the hitherto-reported carbapenem compounds are least stable in kidney homogenates of various animal species. Table 4 presents the stability data of the OA-6129 group of carbapenem compounds in kidney homogenates of mouse, dog and man.

Table 3. Synergism of OA-6129A and PS-5 with cephaloridine (CER) and ampicillin (ABPC) in antimicrobial activities against *Proteus vulgaris* GN 76 and *Citrobacter freundii* GN 346 (β-lactamase producers) (MIC μg/ml).

	β-Lactam	Alone	In combination	
			Carbapenem+CER (FIC index)	Carbapenem+ABPC (FIC index)
(1) *Proteus vulgaris* GN 76	CER	1,250		
	ABPC	1,250		
	OA-6129A	50	6.25+39 (0.16)	6.25+39 (0.16)
	(−)OA-6129A	100	6.25+39 (0.09)	6.25+20 (0.08)
	PS-5	12.5	0.39+39 (0.06)	0.39+78 (0.12)
(2) *Citrobacter freundii* GN 346	CER	1,250		
	ABPC	1,250		
	OA-6129A	50	6.25+313 (0.38)	6.25+313 (0.38)
	(−)OA-6129A	25	0.78+313 (0.28)	6.25+313 (0.50)
	PS-5	3.13	0.78+ 20 (0.27)	0.20+313 (0.31)

Table 4. Comparative stabilities of the OA-6129 group of carbapenem compounds, PS-5 and cefazolin (CEZ) in kidney homogenates of various animal species.

Animal species	Percent amount of unchanged β-lactam						
	OA-6129A	OA-6129B_1	OA-6129B_2	OA-6129C	(−)OA-6129A	PS-5	CEZ
PBS (control)	100	100	100	100	100	100	100
Mouse	7.0	13.2	7.8	0	8.4	3.4	100
Dog	25.0	64.0	27.0	1.8	10.8	5.6	100
Man	57	86	70	32	18.0	37	100

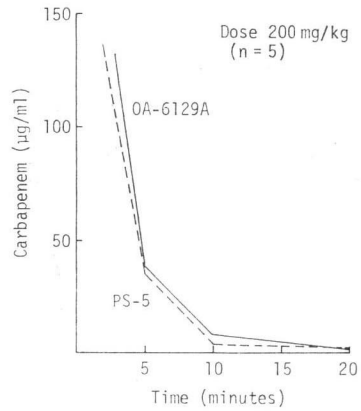
Reaction mixture: β-Lactam (100 μg/ml) 0.1 ml.
Kidney homogenate 0.1 ml.
Incubated at 37°C for 60 minutes and then bioassayed by *Comamonas terrigena* B-996.

It is interesting to note that compounds OA-6129A, B_1 and B_2 are more stable than PS-5, whereas compound OA-6129C seems more labile than PS-5. In common to the four compounds of the OA-6129 group as well as other carbapenems, the mouse kidney homogenate showed the strongest inactivating activity.

Plasma Level in Mice

Fig. 2 illustrates the comparative mean plasma levels of carbapenems OA-6129A and PS-5 after single intravenous doses of 200 mg/kg to groups of 5 mice. As is expected from the stability data in Table 4, compounds OA-6129A and PS-5 show the practically same pharmacokinetic properties. The short half-life and poor urinary recovery of OA-6129A is considered to result from the enzymatic breakdown by renal dipeptidase[20].

Fig. 2. Mean plasma concentrations of OA-6129A and PS-5 after a single intravenous administration to mice.



	OA-6129A	PS-5
C_{max} (μg/ml)	131 (3 minutes)	137 (2 minutes)
$t_{1/2}$ (minutes)	1.2	1.5
Urinary recovery (%) (0~6 hours)	0.35	0.1

Discussion

Except for difference in the C-3 side chain, compounds OA-6129A, B_1, B_2, and C have the same absolute structures as PS-5, epithienamycins A and C and MM 17880, respectively. As far as compound OA-6129A is compared with PS-5, the introduction of the pantetheinyl side chain at C-3 instead of the acetylcysteaminyl group leads to a significant reduction of antimicrobial activity. Although critical comparison of the other carbapenem compounds has not yet been done because of unavailability of the antibiotic samples and the test strains, it is highly probable that compounds OA-6129B_1, B_2 and C are about 10-fold-less active than epithienamycins A and C and MM 17880, respectively. In addition, the increasing order of specific antimicrobial activity observed among the OA-6129 group of carbapenems (OA-6129B_2<OA-6129A<OA-6129C<OA-6129B_1) is the same as what is established among PS-5, epithienamycins A and C and MM 17880[22]. In other words, when the C-3 side chain is same, the C-6 configuration and the type of the C-8 substituent determine the specific antimicrobial activity of carbapenems[6,16,23].

The OA-6129 group of compounds practically have no antipseudomonal activity, presumably because the pantetheinyl side chain has no basicity (*cf.* thienamycin and NS-5)[24] and poorly penetrates the cell wall and membrane of pseudomonads.

It is well known that pantetheine found in natural products such as CoA contains (*R*)-pantoate, whereas (*S*)-pantetheine has no physiological function in organisms. Contrary to our expectation, the stereochemistry of the C-3 pantetheinyl side chain has no significant influence on antibiotic activity of carbapenems. Compound (−)OA-6129A seems more susceptible to renal dipeptidase than the natural counterpart (Table 4).

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