THE MECHANISM OF ACTION OF PIPERACILLIN-ANALOGUES *IN VITRO*; EFFECT OF THE CARBON NUMBER AT THE N-4 POSITION OF 2,3-DIOXOPIPERAZINE ON THE OUTER MEMBRANE PERMEABILITY, STABILITY TO β-LACTAMASE AND BINDING AFFINITY TO PENICILLIN-BINDING PROTEINS

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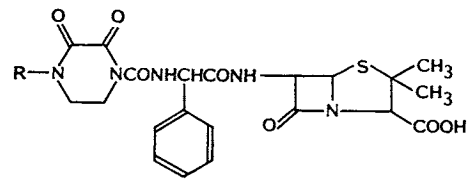
The relationship between the chemical structure and the mode of action of piperacillin-analogues (PIPC-analogues) against *Escherichia coli* and *Klebsiella pneumoniae* were investigated. The antibacterial activity of PIPC-analogues increased with an increase in the number of carbon atoms at the N-4 position of 2,3-dioxopiperazine. Their mode of action is discussed on the basis of the results of studies on outer membrane permeability, stability to β-lactamase and binding affinity to penicillin-binding proteins (PBPs). The outer membrane permeability and stability to β-lactamase were hardly affected by the chain length of the alkyl group at the N-4 position. On the other hand, the affinity to PBPs, especially to PBP 3, became stronger with increase of the number of carbon atoms at N-4 position. These results suggest that increased affinity to PBPs is the main reason for the increased antibacterial activity of the PIPC-analogues reported here.

Piperacillin sodium (PIPC) is a semisynthetic penicillin with broad spectrum activity against Gram-positive and Gram-negative bacteria including anaerobes[1]. The characteristic structure of this molecule is a 2,3-dioxopiperazine moiety attached to the amino group of aminobenzylpenicillin through a ureid bond. With the introduction of this moiety, the antibacterial activity of PIPC was much increased as compared with that of aminobenzylpenicillin. In our laboratory, we synthesized various piperacillin-analogues (PIPC-analogues) to enhance the antibacterial activity. Our efforts toward structural modifications have been focused mainly on N-4 position of 2,3-dioxopiperazine (Fig. 1). In these compound, we found that the antibacterial activity became stronger with the increase of the number of carbon atoms at the N-4 position. Thus, we considered that the introduction of the alkyl groups must play an enhancing effect with regard to the action of β-lactam antibiotics. In this paper, we investigated the effect of the increase

Fig. 1. Chemical structure of 6-[D(−)-α-(4-alkyl-2, 3-dioxo-1-piperazinecarboxamido)phenylactamido]-penicillanic acid.
C-1, C-3, C-4, C-6, C-7: Piperacillin-analogues, C-2: piperacillin.



	R	Code No.
C-1	CH_3	T-1187
C-2	C_2H_5	T-1220
C-3	C_3H_7	T-1224
C-4	C_4H_9	T-1221
C-6	C_6H_{13}	T-1218
C-7	C_7H_{15}	T-1222

in the number of carbon atoms at the N-4 position on the antibacterial activity.

Materials and Methods

Organism

The bacteria employed in this study were *Escherichia coli* W3110 and *Escherichia coli* YA-21, kindly supplied by S. MITSUHASHI, Gunma University, Japan and by S. MIZUSHIMA, Nagoya University, Japan, respectively. Both strains were derivatives of *Escherichia coli* K-12. *Klebsiella pneumoniae* Y-50 was a clinical isolate. For measurement of outermembrane permeability of penicillins using β-lactamase located in the periplasmic space[2], an R-plasmid, RGN823, specifying the TEM-type β-lactamase, was transferred to each of the strains, from an *E. coli* strain carrying the R-plasmid, by conjugation.

Antibiotics

PIPC-analogues (C-1, C-3, C-4, C-6, C-7) were synthesized in our laboratory[3]. Benzylpenicillin (PCG) (Nippon Merck-Banyu Co., Ltd., Osaka, Japan), ampicillin (ABPC) (Toyama Chemical Co., Ltd., Tokyo, Japan) and cephalothin (CET) (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) were commercially available. [^{14}C]Benzylpenicillin (specific activity, 58.9 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England.

Reverse-phase Thin-layer Chromatography (TLC)

The hydrophobic character of various penicillins was expressed as the Rf value which was measured by reverse-phase TLC by a slight modification of the method of SAWAI *et al.*[4]. The polar mobile phase was acetate-veronal buffer (pH 7.0) - MeOH (3 : 2). Merck TLC Silica gel 60F silanized plates were used as the nonpolar stationary phase. The sample for examination was dissolved in the acetate-veronal buffer to give a concentration about 3 mg/ml, and 1 to 2 μl of the solution was then located on the TLC plate. After development at room temp, the antibiotics were detected on the plate by using UV light.

Measurement of Bacterial Susceptibility

The susceptibility of bacteria was measured by agar dilution method, and the susceptibility was expressed as the minimum inhibitory concentration (MIC). An overnight culture of the bacterial strain in peptone broth (Polypeptone 10 g, NaCl 5 g/liter) was diluted to give a final concentration of 10^6 cells/ml, and one loopful (about 0.005 ml) of each culture was inoculated on heart infusion agar (Eiken, Tokyo) plates using a Microplanter (Sakuma, Tokyo). The MICs were determined after overnight incubation at 37°C.

Assay of Outer Membrane Permeability

The outer membrane permeability was carried out using the method of SAWAI *et al.*[4], as expressed by the parameter "C" (cm^3/minute/μg of dry cell)[5].

Assay of Penicillin-binding Proteins (PBPs)

Penicillin-binding proteins of *E. coli* YA-21 were detected according to the SPRATT method[6] with slight modifications. The molar concentration of antibiotic required to inhibit the [^{14}C]benzylpenicillin binding by 50% (ID_{50}) was determined from the densitometric tracing of radioactive PBP bands on an X-ray film.

Preparation of Crude β-Lactamase and Measurement of β-Lactamase Activity

An overnight culture of each strain was diluted 10-fold into fresh heart infusion broth (Eiken, Tokyo) and incubated with shaking at 37°C for 4 hours. Cells were washed once with 5 ml of 0.1 M phosphate buffer (pH 7.0), resuspended in 5 ml of the same buffer and disrupted sonically for 2 minutes at 4°C. The disrupted cells were centrifuged at 100,000×g for 20 minutes at 4°C, and the resulting supernatant was used for the enzyme assay and protein determination. β-Lactamase activity was assayed by the micro iodometric method of NOVICK[7] with slight modifications. Protein assay was performed by the method of LOWRY *et al.*[8].

Results

Antibacterial Activity of PIPC-analogues

Table 1 shows the MIC values of the PIPC-analogues and their hydrophobicity. The hydrophobicity of PIPC-analogues became stronger with increase in the number of carbon atoms at the N-4 position of 2,3-dioxopiperazine. In *E. coli* YA-21, *E. coli* W3110 and *K. pneumoniae* Y-50, the antibacterial activity was greater as the hydrophobicity increased. The MIC value of compound C-7 was about 30 times lower than that of compound C-1.

Outer Membrane Permeability of PIPC-analogues

The penetration rates of PIPC-analogues were measured and were compared with those of ampicillin and benzylpenicillin. The penetration rates of PIPC-analogues showed constant levels regardless of the hydrophobicity of the molecules, though some differences were observed in the penetration rates among the strains (Table 2). In addition, there were no differences in penetration rates between PIPC-analogues and other penicillins. These results indicated that the outer membrane permeability did not contribute to the degree of antibacterial activity, and the structural differences of the penicillins tested including PIPC-analogues were not related to the outer membrane permeability.

Stability to β-Lactamase

We then examined the influence that the low-level β-lactamase may exert the difference of antibacterial activity. Table 3 shows the kinetic constants for hydrolysis and the affinity of the PIPC-analogues by cephalosporinase and penicillinase, respectively. The PIPC-analogues were more stable

Table 1. MICs of PIPC-analogues and other penicillins.

Compound	Hydrophobicity[a]	MIC (μg/ml)		
		E.c. W3110	*E.c.* YA-21	*K.p.* Y-50
C-1	0.69	3.1	3.1	6.3
C-2	0.63	1.6	0.8	1.6
C-3	0.55	0.8	0.8	1.6
C-4	0.44	0.4	0.4	0.4
C-6	0.21	0.1	0.2	0.2
C-7	0.16	0.1	0.1	0.2
ABPC	0.73	3.1	3.1	25
PCG	0.66	25	25	25

[a] Given as Rf value of reverse-phase TLC.
E.c.: *Escherichia coli*, *K.p.*: *Klebsiella pneumoniae*.

Table 2. Outer membrane permeability of PIPC-analogues.

Strain	Permeability coefficient ($\times 10^{-5}$ cm^3/minute/μg of dry cell)							
	C-1	C-2	C-3	C-4	C-6	C-7	ABPC	PCG
E.c. W3110	219	234	214	207	207	221	192	190
E.c. YA-21	47.6	29.3	20.8	24.5	23.8	22.7	37.9	31.2
K.p. Y-50	34.1	33.4	30.1	26.0	29.9	38.2	56.3	41.1

E.c.: *Escherichia coli*, *K.p.*: *Klebsiella pneumoniae*.

Table 3. Relative rates of hydrolysis and affinities of PIPC-analogues for the crude β-lactamases.

Compound	*E.c.* W3110[a]		*E.c.* YA-21[a]		*K. p.* Y-50[b]	
	Vmax[c]	*Ki* (μM)	Vmax	*Ki* (μM)	Vmax	*Km* (μM)
C-1	3.4	10.8	7.8	4.1	51	52
C-2	3.0	6.3	5.9	3.1	72	33
C-3	3.4	5.0	4.3	2.5	63	22
C-4	3.3	2.9	5.8	2.7	51	25
C-6	3.1	2.5	2.6	2.9	49	23
C-7	3.3	3.6	4.1	2.0	42	18
ABPC	4.9	NT	4.5	13.4	108	20
PCG	31	NT	43	6.3	100 (94)	31
CET	100 (20)[e]	61[d]	100 (11)	34[d]	5	104

[a] Cephalosporinase.
[b] Penicillinase.
[c] Hydrolysis ratio is expressed as relative rate of hydrolysis, taking the absolute rate of PCG as 100 for penicillinase, CET as 100 for cephalosporinase.
[d] *Km* value (μM).
[e] Specific activity ($\times 10^{-3}$ unit/mg protein).
NT: Not tested. *E.c.*: *Escherichia coli*, *K.p.*: *Klebsiella pneumoniae*.

Table 4. Competition of PIPC-analogues with ^{14}C-labeled benzylpenicillin for binding to PBPs of *Escherichia coli* YA-21.

Compound	ID_{50}*					
	1a	1bs	2	3	4	5/6
C-2	3.6	0.46	0.25	0.23	>8	>8
C-4	1.7	0.44	0.17	0.03	4.6	3.5
C-7	0.57	0.12	0.07	0.005	3.9	1.4

* Concentration as a molar ratio to ^{14}C-labeled benzylpenicillin required for 50% inhibition of labeled benzylpenicillin binding.

than benzylpenicillin and ampicillin, but no differences in their stability can be observed in the PIPC-analogues. These results suggested that increased in antibacterial activity was not related to the stability to β-lactamase.

Affinity to PBPs

To confirm the enhanced antibacterial effects of these compounds, the affinity for the target enzymes, namely PBPs, was determined by competition with [^{14}C]benzylpenicillin in *E. coli* YA-21. PIPC-analogues showed very high affinity for PBPs 2 and 3, moderate affinity for 1bs, and low affinity for 1a, 4 and 5/6, and the affinity became stronger with the increase in the number of carbon atoms at N-4 position of 2,3-dioxopiperazine (Table 4). The ID_{50} values of compound C-7 for PBPs 1a, 2, 4 and 5/6 were about 2 to 6 times lower, and for PBP 3 about 40 times lower, than that of compound C-2 (PIPC). The increased affinities of the PIPC-analogues were well reflected in their increase in antibacterial activity.

Discussion

It is obvious from our earlier study on the structure-activity relationships that the number of carbon atoms at the N-4 position of 2,3-dioxopiperazine exerts a considerable effect on antibacterial activity against *E. coli* and *K. pneumoniae*[9]. In this paper, we investigated the factors responsible

for the antibacterial activity of PIPC-analogues. The antibacterial activity of PIPC-analogues against the test organisms became stronger with the increase in the hydrophobicity of the compounds. Thus, we analyzed the correlation between the number of carbon atoms at the N-4 position and the following parameters, i) outer membrane permeability, ii) stability to β-lactamase and iii) affinity to PBPs. First, we showed that the ability of PIPC-analogues to penetrate the outer membrane was almost constant, independently of their hydrophobicity. SAWAI *et al.*[5] suggested that penicillins could pass through the outer membrane *via* a non-porin pathway, and that their penetration rates were hardly affected by their hydrophobicity. A similar assumption is also applicable to PIPC-analogues. Second, we showed that the stability of PIPC-analogues was also constant against the β-lactamase produced by strains used in this study. These observations may indicate that the increased antibacterial activity of PIPC-analogues was hardly affected by either of these factors.

Finally, we showed that the increase of the carbon number at N-4 position resulted the increased affinities to PBPs, and there was a good correlation between the MIC and ID_{50} value in PBPs 3. The increased affinity for the PBPs in *E. coli* W3110 and *K. pneumoniae* Y-50 was also shown in a similar manner (unpublished data).

In summary, we conclude that the increase of the carbon number in the range of $CH_3 \sim C_7H_{15}$ at N-4 position of 2,3-dioxopiperazine plays an important role in enhancing the affinity to PBPs, especially to PBP 3.

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