MUREIDOMYCINS A~D, NOVEL PEPTIDYLNUCLEOSIDE ANTIBIOTICS WITH SPHEROPLAST FORMING ACTIVITY

III. BIOLOGICAL PROPERTIES

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(Received for publication December 28, 1988)

Mureidomycins (MRD's) A~D were specifically active against *Pseudomonas aeruginosa*. Among them, MRD C was most active, with MICs of 0.1 to 3.13 μg/ml against many strains of the target organism. Its activity was comparable to that of cefoperazone, ceftazidime and cefsulodin. MRD C-resistant mutants of *P. aeruginosa* appeared spontaneously at a high frequency when cultured in the presence of the antibiotic. No cross-resistance was observed with β-lactam antibiotics. A rapid decrease of turbidity along with spheroplast formation and cell lysis was observed when cells of *P. aeruginosa* were grown in the presence of MRD C. The compounds exhibited low toxicity and protected mice from experimental infection with *P. aeruginosa*. The urinary and fecal recoveries of MRD C given subcutaneously were 5 and 18%, respectively.

Although many new β-lactam antibiotics with activity against *Pseudomonas aeruginosa* have been synthesized, the infectious diseases caused by this species of bacteria still pose serious problems throughout the world.

As previously reported, new peptidylnucleoside antibiotics named MRD's A~D were produced by *Streptomyces flavidovirens* SANK 60486. Their isolation, characterization and structural elucidation revealed their unique physico-chemical properties[1,2]. In this paper, the biological properties of MRD's are reported.

Materials and Methods

Antibiotics

MRD's were prepared by Fermentation Research Laboratories, Sankyo Co., Ltd. Fosfomycin (FOM), cefsulodin (CFS), ceftazidime (CAZ), cefoperazon (CPZ), carbenicillin (CBPC), sulbenicillin (SBPC), piperacillin (PIPC) and cefotaxime (CTX) are commercially available antibiotics.

Antimicrobial Activities

For *in vitro* studies, the strain of *P. aeruginosa* SANK 75775 was grown in Nutrient broth (Eiken) at 37°C with vigorous shaking. In the case of morphological studies, nutrient broth was supplemented with 12% sucrose and 0.1 M $MgSO_4 \cdot 7H_2O$. MICs were determined by the agar dilution method using Mueller-Hinton agar. Turbidity of the culture was monitored on a Shimadzu Spectronic 20 at absorbance of 550 nm. The bactericidal activity of the antibiotics was measured as follows: 0.1 ml of a logarithmic phase culture of *P. aeruginosa* SANK 75775 was transferred into the test tubes with 9.9 ml of pre-warmed medium containing finally 0, 1, 2, 4 and 8 times MICs of the antibiotics. The tubes were shaken vigorously and aliquots of the culture were withdrew at 1, 2, 4 and 6 hours after inoculation to determine the viable cell numbers after dilution with 0.85% NaCl. Antibiotic resistant

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mutants of *P. aeruginosa* SANK 75775 were selected from the colonies grown on the nutrient agar medium containing antibiotics at 4 times MICs.

In Vivo Experiments

The *dd*Y female mice were infected intravenously with *P. aeruginosa* SANK 75775 grown overnight at 37°C and treated with antibiotics subcutaneously twice at immediately and 4 hours after infection. ED_{50}'s were determined on day 7th after infection. For determination of concentrations of MRD's A and C in urine, feces, serum or tissue extract, a paper-disc agar diffusion assay was employed using *P. aeruginosa* SANK 70579 as the test organism.

Results

Antimicrobial Activity of MRD's

MRD's were active against only few Gram-positive and Gram-negative bacteria tested, except

Table 1. Antimicrobial activities of MRD's A~D.

Test organism	MIC (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA 209P JC-1	200	>200	>200	>200
Escherichia coli NIHJ JC-2	>200	>200	>200	>200
Proteus mirabilis B-30-1	>200	>200	>200	>200
Serratia marcescens	>200	>200	>200	>200
Klebsiella pneumoniae PCI 602	25	25	12.5	25
Pseudomonas aeruginosa 1046	6.25	25	1.56	6.25
P. aeruginosa SANK 75775	6.25	12.5	1.56	6.25
P. aeruginosa 1080	25	50	3.13	12.5
P. aeruginosa SC 8753	12.5	25	3.13	12.5
P. aeruginosa SANK 73279	12.5	25	1.56	6.25
P. aeruginosa NRRL B1000	25	50	3.13	6.25
P. aeruginosa ATCC 13388	25	50	3.13	6.25
P. aeruginosa SANK 70479	6.25	12.5	1.56	6.25
P. aeruginosa SANK 70579	<0.1	0.2	<0.1	6.25
P. aeruginosa NCTC 10490	12.5	25	0.4	6.25

Mueller-Hinton agar. Inoculum size 10^6 cells/ml.

Table 2. Antipseudomonal activities of MRD C, CBPC, SBPC, CTX, PIPC and CPZ.

Test organism	MIC (μg/ml)					
	MRD C	CBPC	SBPC	CTX	PIPC	CPZ
Pseudomonas aeruginosa 433	3.13	12.5	NT	NT	0.78	1.56
P. aeruginosa 638	6.25	50	NT	NT	3.13	6.25
P. aeruginosa 1008	3.13	50	NT	NT	3.13	3.13
P. aeruginosa 1872	6.25	100	NT	NT	3.13	3.13
P. aeruginosa SANK 75775	3.13	50	NT	NT	1.56	3.13
P. aeruginosa SC 8753	1.56	>200	>200	>200	NT	NT
P. aeruginosa B2-5	3.13	50	50	6.25	NT	NT
P. aeruginosa 1080	3.13	50	50	12.5	NT	NT
P. aeruginosa ATCC 13388	3.13	100	50	6.25	NT	NT
P. aeruginosa NRRL B1000	3.13	100	50	12.5	NT	NT
P. aeruginosa SANK 70479	1.56	25	12.5	3.13	NT	NT
P. aeruginosa SANK 70579	<0.1	<0.2	<0.2	<0.2	NT	NT
P. aeruginosa NCTC 10490	0.4	<0.2	0.4	<0.2	NT	NT

Inoculum size 10^6 cells/ml. Mueller-Hinton agar.
NT: Not tested.

Table 3. MICs of MRD C, CPZ, CFS and CAZ against antibiotic-resistant mutants of *Pseudomonas aeruginosa* SANK 75775.

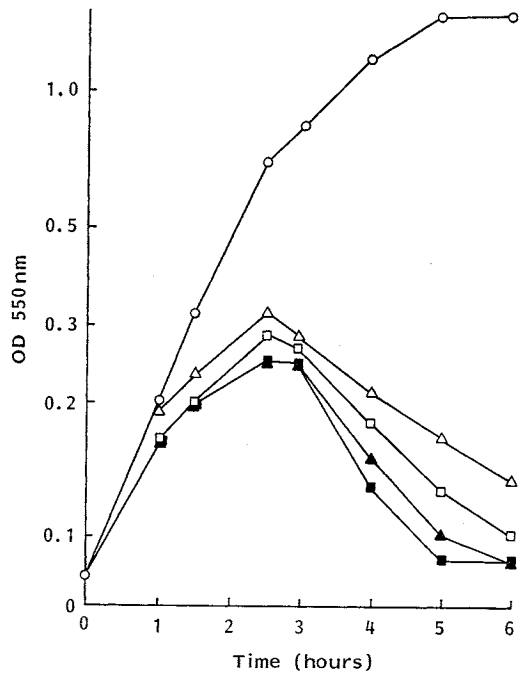
	MIC (μg/ml)			
	MRD C	CPZ	CFS	CAZ
P. aeruginosa SANK 75775	1.56	3.13	0.78	0.78
CPZ-1[a]	1.56	25	3.13	6.25
CPZ-2	1.56	25	3.13	6.25
CPZ-3	1.56	25	6.25	6.25
CFS-1	3.13	25	6.25	1.56
CFS-2	3.13	25	6.25	1.56
CFS-3	1.56	25	12.5	0.39
CAZ-1	1.56	50	6.25	12.5
CAZ-2	1.56	50	6.25	12.5
CAZ-3	3.13	>100	25	25

[a] Antibiotic-resistant mutant.

Klebsiella pneumoniae PCI 602 and *P. aeruginosa* (Table 1). MRD's inhibited the growth of *P. aeruginosa* at the range from <0.1 to 100 μg/ml. Among them, MRD C was most active and almost all strains of *P. aeruginosa* were inhibited at concentrations of 3.13 μg/ml or less. MRD C's antipseudomonal activity was compared with several β-lactam antibiotics active against *P. aeruginosa* (Table 2). MRD C was more active than CBPC or SBPC. CTX, PIPC and CPZ showed almost the same activity as MRD C. It is worth to note that MRD C was not only active against *P. aeruginosa* SC 8753 which was isolated clinically as β-lactam-resistant one but also against *P. aeruginosa* 433, 638, 1008 and 1872 which were fresh clinical isolates.

Fig. 1. The effect of MRD C on the growth of *Pseudomonas aeruginosa* SANK 75775.

○ Control, △ 1×MIC, □ 2×MIC, ▲ 4×MIC, ■ 8×MIC.



Emergence of MRD-resistant *P. aeruginosa*

The spontaneous frequency of acquisition of resistance to MRD C of *P. aeruginosa* SANK 75775 was 1.2×10^{-6} at MIC and 1.0×10^{-6} at 2 and 4 times MICs, respectively. This frequency was similar to that of FOM (5×10^{-6} at 2 times MICs) but higher than those of CBPC (4.5×10^{-8} at 4 times MICs) and CTX (5.9×10^{-8} at 4 times MICs).

To test the cross resistance of MRD's to β-lactam antibiotics, each antibiotic-resistant mutants were selected at 4 times MICs. As shown in Table 3, CPZ-resistant (CPZ-1 ~ CPZ-3), CFS-resistant (CFS-1 ~ CFS-3) and CAZ-resistant isolates (CAZ-1 ~ CAZ-3) were also resistant to each other β-lactam antibiotics, but MRD C was as active as the parent strain against these resistant bacteria, indicating no cross resistance between MRD's and β-lactam antibiotics.

Fig. 2. Bactericidal activities of MRD C and CBPC against *Pseudomonas aeruginosa* SANK 75775.
(A) CBPC, (B) MRD C.
○ Control, △ 1×MIC, □ 2×MIC, ▲ 4×MIC, ■ 8×MIC.

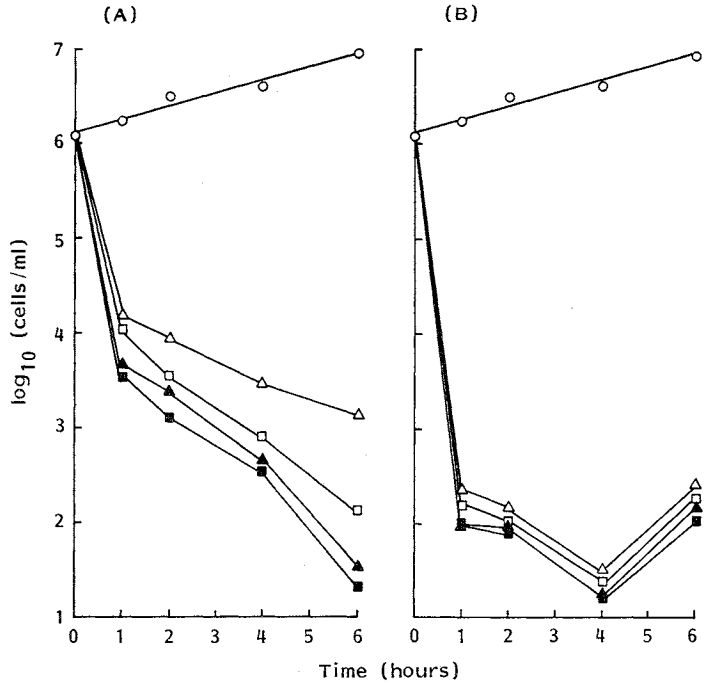
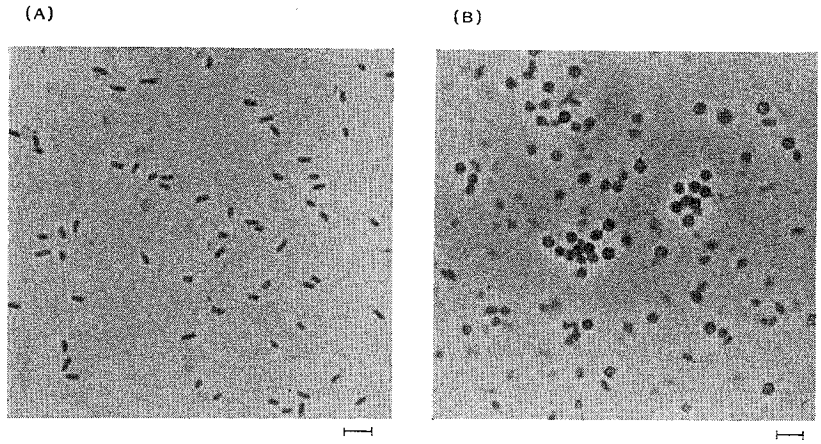


Fig. 3. The morphological change of *Pseudomonas aeruginosa* SANK 75775 caused by MRD C.
(A) Control, (B) treated. A bar indicates 1 μm.



Effects of MRD C on the Growth and the Viability of *P. aeruginosa*

To determine the effects of MRD C on the growth of *P. aeruginosa* SANK 75775, cells were grown at 37°C with vigorous shaking in the presence or absence of the antibiotic. As shown in Fig. 1, at the beginning of incubation, turbidity increased for 2 hours in the presence of MRD C at 1, 2, 4 and 8 times MICs, then it began to decrease rapidly with the cell lysis. The bactericidal activity of MRD C

was indicated by a time-dependent decrease in the viable cell number, which, however, slightly increased at 6 hours after inoculation, indicating regrowth of the resistant bacteria even at 8 times MICs (Fig. 2). MRD C decreased the viable cell number about to 10^{-4} of the initial, but CBPC did about only to 10^{-2}, showing that MRD C could kill bacteria in a shorter time than CBPC. Spheroplast formation was detected under the microscope when cells were grown in hypertonic medium containing MRD C (Fig. 3). These facts point to the inhibition of the cell wall peptidoglycan synthesis[3] as the mechanism of action of MRD's.

Toxicity

Mice tolerated a single intravenous injection of MRD A or C at 400 mg/kg without any toxic symptoms for 14 days.

In Vivo Protection Test

Mice infected with *P. aeruginosa* SANK 75775 intravenously were treated subcutaneously with MRD's immediately and 4 hours after infection. ED_{50}'s of MRD's A~D were determined to be 69, 75, 50 and >100 mg/kg, respectively. Under the same condition, those of CFS and CAZ were 27 and 36 mg/kg, respectively, but CPZ, CBPC, SBPC and PIPC were ineffective even at 400 mg/kg.

Pharmacokinetics in Mice

To investigate the pharmacokinetics of MRD's A and C in mice, they were injected subcutaneously at the dose of 100 mg/kg. As shown in Fig. 4, both antibiotics had concentrations above the MIC levels in serum for about 1 hour. Their half-lives were about 0.7 hour. After subcutaneous administration of MRD's A and C, the urinary recoveries during 24 hours were 5% for both and the fecal recoveries were 30 and 18%, respectively. When tissue distribu-

Fig. 4. Mean serum concentrations of MRD's A and C in mice after single subcutaneous injection of 100 mg/kg.

○ MRD A, ● MRD C.

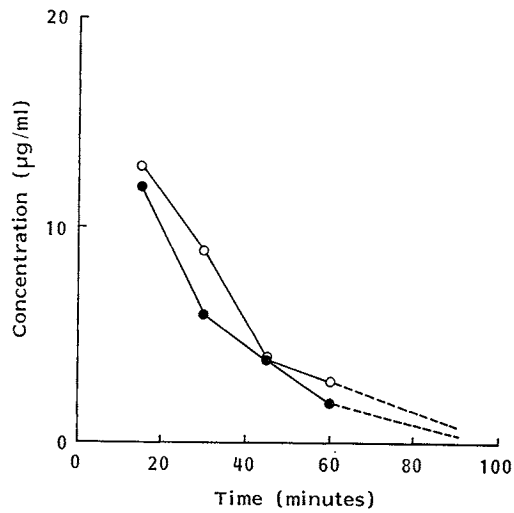


Table 4. Tissue distribution of MRD's A and C in mice.

	A (μg/ml)			C (μg/ml)		
	0.5 hour	1 hour	24 hours	0.5 hour	1 hour	24 hours
Heart	ND	ND	ND	ND	ND	ND
Liver	36	10	ND	18	5	ND
Kidney	17	ND	ND	10	ND	ND
Lung	ND	ND	ND	ND	ND	ND
Spleen	ND	ND	ND	ND	ND	ND

Mice were treated with 100 mg/kg of antibiotics subcutaneously and each tissue was removed from sacrificed mice at 0.5, 1 and 24 hours after treatment. The antibiotics were extracted with 50% acetone after homogenization.

ND: Not detected.

tions of MRD's A and C, given subcutaneously at 100 mg/kg in mice, were measured, levels could be detected only in liver and kidney up to 1 hour, but no activity could be detected in any tissues after 24 hours (Table 4).

Discussion

It is very interesting to note that MRD's are selectively active against *P. aeruginosa* which is known to be generally resistant to many antibiotics, as a consequence of their intrinsic permeability barrier[4]. Several antibiotics have been found to be active against *P. aeruginosa* and against many other bacteria, but antibiotics with specific activity against *P. aeruginosa* have not commonly been found.

The biological activities of MRD's can be summarized as follows: They are bactericidal, they induce spheroplast formation of the sensitive bacteria and they cause cell lysis. From these results, the mechanism of action of MRD's is supposed to be inhibition of peptidoglycan synthesis of the cell wall. In the cell free system for peptidoglycan synthesis, MRD C strongly inhibited the incorporation of ^{14}C-UDP-MurNAc-pentapeptide into cold TCA insoluble fractions at a concentration below MIC (data not shown).

It is very important to study the reason why MRD's are specifically active against *P. aeruginosa*. The peptidoglycan synthetic machinary of *P. aeruginosa* may be different from that of other bacteria or their specific permeability system may allow the influx of MRD's but not of other well-known antibiotics[5,6].

The ability to decrease the viable cell number in a short period may overcome the potential drawback of rapid emergence of resistant cells, as the immune system of the host may eliminate the small resistant population.

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