MODE OF ACTION OF THE PROTEIN, SP127, WHICH ENHANCES THE ACTIVITY OF MACROLIDE ANTIBIOTICS AGAINST *PSEUDOMONAS AERUGINOSA*

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Antibiotics, the activity of which was enhanced against *Pseudomonas aeruginosa* by SP127, were restricted to the basic macrolide antibiotics such as erythromycin, maridomycin and oleandomycin, the neutral macrolide antibiotics such as lankamycin and lankacidin C, vancomycin and enramycin. Synergistic activity of SP127 with the above antibiotics was found against *Pseudomonas aeruginosa* and several strains of *Escherichia coli*, but not against *Proteus vulgaris* and macrolide-resistant *Staphylococcus aureus*. SP127 had extremely weak proteolytic but no lytic activity. From the isotopic experiments, the action of SP127 was partially attributed to the promotion of antibiotic penetration to cells of *Pseudomonas aeruginosa*.

In the previous study[1], the protein SP127, which enhances the activity of erythromycin (EM) against *Pseudomonas aeruginosa*, was isolated from culture broth of *Bacillus brevis* ATCC 8185 and its physicochemical properties were presented.

The purpose of the present study is to elucidate the mode of action of SP127.

Materials and Methods

Test organisms

P. aeruginosa IFO 3080 was mainly used as described previously[1]. As the target organisms, IFO strains of *P. aeruginosa, Escherichia coli* and *Proteus vulgaris*, and macrolide-resistant (MAC^r) strains of *Staphylococcus aureus* were employed. MAC^r strains were kindly provided by Mr. M. KIDA and Dr. H. ONO of our Central Research Division.

Determination of MIC

The minimum inhibitory concentration (MIC) was measured as follows: Bouillon agar (100 ml) was mixed with 2 ml of test organism suspension (optical density at 600 nm of 0.05) and then mixed with each antibiotic tested and SP127 to give various concentrations, respectively. The agar was then poured into a Petri-dish. After solidification of the agar, it was incubated at 37°C for 18~20 hours. The minimum inhibitory concentration for each antibiotic was defined as the lowest concentration of each antibiotic to inhibit the growth of the test organism in the presence of SP127.

Assay of Lytic Activity

Lytic activity was assayed by measuring the decrease of turbidity associated with lysis of cells. The reaction mixture contained cells only, cells and SP127 or cells, SP127 and maridomycin (or EM) in 0.05 M phosphate buffer (pH 7.0). After the mixtures were kept for 3 hours at 37°C, turbidity at 600 nm was measured. One unit was defined as the amount which caused a decrease in 0.001 of turbidity at 600 nm per minute. Various types of cells prepared by the conventional method were used as the enzyme substrates.

Assay of Proteolytic Activity

Proteolytic activity was assayed according to the modified method of KUNITZ[2]. The reaction mixture containing 1 ml of 2% casein, 0.5 ml of 0.2 M buffer and 0.5 ml of the sample was incubated at 36.5°C for 20 minutes, followed by addition of 3 ml of TCA. The reaction mixture was passed through

Whatman filter paper No. 5C and the absorbance of the resulting filtrate was measured at 275 nm. One unit was defined as the amount which caused an increase in 1.0 of absorbance at 275 nm per minute.

Uptake of Maridomycin

P. aeruginosa IFO 3080 was grown in 20 ml of bouillon in a 200-ml flask at 37°C. When OD_{590} reached 0.22, ^{14}C-maridomycin or ^{14}C-maridomycin and SP127 were added. After certain intervals, the uptake of ^{14}C-maridomycin from the extracellular medium by *P. aeruginosa* was measured as follows: One ml of broth was filtered through a Millipore membrane filter (0.45 μ pore size). After the filter membrane was washed twice with 5 ml of ice-cold 0.1 M phosphate buffer (pH 7.0), it was transferred to a vial containing 6 ml of a scintillator (POPOP, 0.2 g; PPO, 4 g; ethyleneglycol, 20 ml; naphthalene, 6.0 g; methanol, 20 ml; dioxane, 1,000 ml). Radioactivity was determined by an Aloka liquid scintillation counter. The uptake of maridomycin was compared by cpm of the cells per ml broth. ^{14}C-Maridomycin (1.1 μC/mg) was a gift from Dr. M. YONEDA of our Central Research Division.

Antibiotics

Almost all antibiotics used in the experiments were obtained from our Central Research Division. Vancomycin was the product of Eli Lilly & Co.

Results

Enhancement of Activity of Various Antibiotics against *P. aeruginosa* by SP127

Table 1 indicates the effect of SP127 on the activity of various antibiotics against *P. aeruginosa*. Interestingly, the antibiotics, the activity of which was enhanced against *P. aeruginosa* by SP127, were restricted to basic macrolide antibiotics (MAC) such as erythromycin, maridomycin (MDM) and oleandomycin, neutral macrolide antibiotics such as lankamycin and lankacidin C, vancomycin and enramycin. Activities of vancomycin and enramycin were enhanced more than 100-fold. Synergy by SP127 and antibiotic such as β-lactam antibiotics, peptide antibiotics, aminoglycosidic antibiotics, polyene antibiotics, tetracycline antibiotics and chloramphenicol was not observed.

From the results, it is clear that the synergism is restricted by the kind of antibiotic.

Table 1. Change in MIC of antibiotics for *P. aeruginosa* IFO 3080 in the presence of SP127

Antibiotic	MIC (μg/ml)	
	None	SP127 (5 μg)
Erythromycin	60	2
Maridomycin	600	20
Oleandomycin	600	20
Leucomycin	600	20
Josamycin	600	20
Spiramycin	500	20
Lankacidin C	300	15
Lankamycin	>1,000	100
Vancomycin	>3,000	30
Enduracidin (Enramycin)	700	6

Synergy by SP127 and each of the following antibiotics was not observed: β-lactam antibiotics such as penicillin G, cephalosporin C, aminobenzylpenicillin and cephalexin, peptide antibiotics such as polymyxin B and gramicidin J, aminoglycosidic antibiotics such as kanamycin, streptomycin, neomycin and validamycin, polyene antibiotics such as fungicidin and trychomycin, tetracycline antibiotics and chloramphenicol.

Enhancement of Antibiotic Activity of Maridomycin and Vancomycin against Gram-negative Bacteria by SP127

Among the antibiotics showing synergism with SP127, the combined effect of maridomycin or vancomycin with SP127 was examined against gram-negative bacteria (Tables 2 and 3). Antibiotic activity of these agents was enhanced 30- to 300-fold against *P. aeruginosa* and *E. coli* in the presence of SP127. Synergy by SP127 and these antibiotics was, however, not observed

Table 2. Change in MIC of maridomycin for gram-negative bacteria in the presence of SP127

Strain	MIC of maridomycin (μg/ml)		
	None	SP127 (5 μg)	SP127 (30 μg)
Pseudomonas aeruginosa IFO 3080	600	20	8
Pseudomonas aeruginosa IFO 3448	>1,000	40	20
Pseudomonas aeruginosa NCTC 10490	600	8	5
Escherichia coli IFO 3044	>1,000	40	20
Escherichia coli IFO 3544	400	400	400
Proteus vulgaris IFO 3167	1,000	1,000	1,000
Proteus vulgaris IFO 3988	600	600	600

Table 3. Change in MIC of vancomycin for gram-negative bacteria in the presence of SP127

Strain	MIC of vancomycin (μg/ml)		
	None	SP127 (5 μg)	SP127 (30 μg)
Pseudomonas aeruginosa IFO 3080	>3,000	30	10
Pseudomonas aeruginosa IFO 3448	3,000	300	60
Pseudomonas aeruginosa NCTC 10490	2,000	20	8
Escherichia coli IFO 3044	>3,000	400	100
Escherichia coli IFO 3544	300	300	300
Proteus vulgaris IFO 3167	600	600	600
Proteus vulgaris IFO 3512	1,000	1,000	1,000

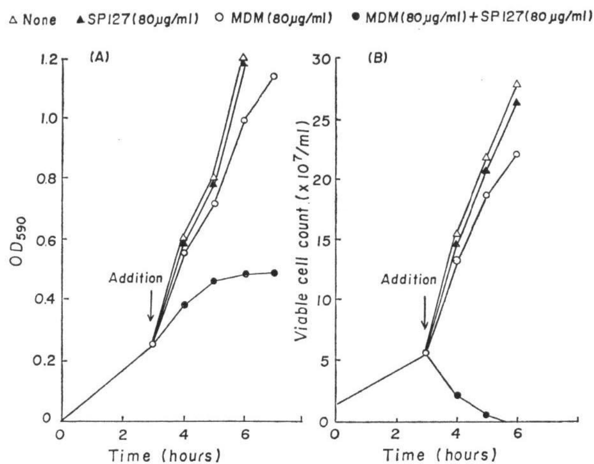
against *Proteus vulgaris*. This was also the case with gram-positive bacteria. These results indicate that the synergism is restricted by target organisms.

Effect of SP127 on MAC^r Strains of *S. aureus*

In the experiments, the following three kinds of MAC^r strains of *S. aureus* were used as a target organisms. Strains FS 4009 and FS 4014 are constitutive MAC^r, strains FS 4044 and FS 4083 are EM-

Fig. 1. Synergistic effect of maridomycin and SP127 on the growth (A) and the viable cell count (B) of *P. aeruginosa*

P. aeruginosa IFO 3080 was grown in 20 ml of bouillon medium in a 200-ml flask at 37°C. When OD_{590} reached 0.22, maridomycin or maridomycin and SP127 were added. Details are in the text.



inducible MAC^r and CS 462 is MAC^r which was derived *in vitro* from *S. aureus* 209P. Strains FS 4009, FS 4014, FS 4044 and FS 4083 are clinical isolates. A change in target site is responsible for MAC^r of these four strains and the change of the permeability is for MAC^r of strain CS 462 (ONO, H.; personal communication).

There was found no synergistic action of SP127 with EM or maridomycin against these five strains. The reason why the synergism was not observed against permeability-changed strain CS 462 may be attributable to a difference in membrane structures between *S. aureus* and *P. aeruginosa*.

Combined Effect of SP127 and Maridomycin on the Growth of *P. aeruginosa*

Fig. 1 indicates the change in the turbidity and viable cell counts of the medium supplemented by MDM, SP127, or MDM and SP127 after 3 hours cultivation. Addition of MDM or SP127 to the concentration of 80 μg/ml to the medium permitted a normal increase in turbidity and viable cell counts. Simultaneous addition of MDM and SP127 to the medium, however, inhibited the increase in turbidity and decreased the viable cell counts. This fact shows that synergism between SP127 and MDM was bacteriocidal.

Lytic Activity of SP127

To elucidate the mode of action of SP127, the lytic activity of SP127 was examined. Lytic activity was 1.2~3.5 u/mg of protein to *P. aeruginosa* IFO 3080 and *E. coli* IFO 3044. This value shows that SP127 has no lytic activity. The same results were obtained with *Proteus vulgaris* IFO 3045, *Bacillus subtilis* PCI 219, *S. aureus* 209P as the substrate.

TAKAHARA *et al.*[3] pointed out that B enzyme produced by *B. subtilis* lyzed *P. aeruginosa* in the presence of Negative-cell-Lytic Factor, however lytic activity of SP127 in the presence of MAC was not observed. Since the combined effect of SP127 and an antibiotic is restricted by the antibiotic and the target organism, the mode of action may be due to another mechanism.

Proteolytic Activity of SP127

Proteolytic activity of SP127 was compared with those of authentic proteases (Table 4). Activity

Table 4. Comparison of proteolytic activity

The reaction mixture contained 1 ml of 2% casein, 0.5 ml of 0.5 M buffer and 0.5 ml of enzyme solution in a total volume of 2.0 ml, and was incubated at 36.5°C for 20 minutes.
pH 7.0 phosphate buffer; pH 9.0 Tris buffer.

Enzyme		Proteolytic activity*	
		pH 7.0	pH 9.0
Bromelin	(Daiwa Kasei)	0.42	0.14
Crystalline bacterial alkaline protease	(Katayama Chem. Ind. Co., Ltd.)	2.35	2.70
α-Chymotrypsin	(Sigma Chem. Co.)	1.75	1.30
Trypsin		0.56	0.58
Pancreatinum	(Iwaki Seiyaku)	0.10	0.09
Nagarse	(Nagase and Co.)	2.25	3.25
Pronase	(Kaken Chem. Co.)	1.17	1.41
SP127		0.004	0.01

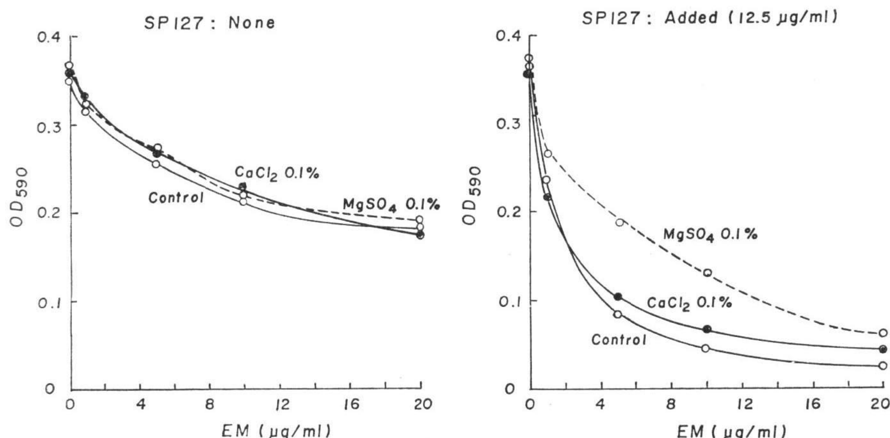
* units/mg of protein

of SP127 was about 1/100 lower than those of Nagarse, Pronase, α-chymotrypsin and about 1/10 lower than those of bromelin, trypsin and pancreatinum. The results do not provide evidence for the mode of action of SP127.

Effect of Ca^{++} or Mg^{++} on the Synergism between SP127 and EM

Since the possibility exists that the action of SP127 is a chelation, the effect of representative dicationic ions such as Ca^{++} and Mg^{++} was tested on the synergism. As will be seen from Fig. 2, no effect of these cations was found in the absence of SP127. With the medium containing 12.5 μg/ml of SP127, however, the tendency was observed for the synergism to be inhibited by $MgSO_4$ in the presence of a low concentration of EM. The reason has not yet been ascertained.

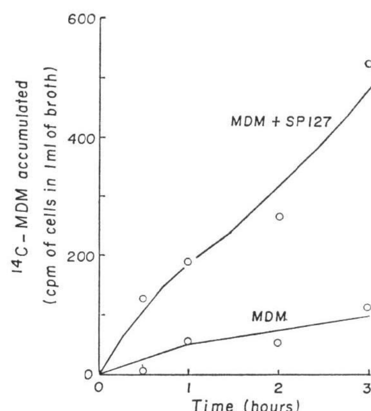
Fig. 2. Effect of Mg^{++} or Ca^{++} on the synergism of erythromycin with SP127.
P. aeruginosa IFO 3080 was grown in bouillon medium containing various concentration of erythromycin at 37°C for 22 hours.



Promotion Effect of SP127 on Maridomycin Penetration

Binding of SP127 to the membrane, binding of SP127 with antibiotic, promotion of antibiotic-penetration and conversion of antibiotic to a more active form are possibly cited as other actions of SP127. Among these, the binding of SP127 with antibiotic and conversion of antibiotic to a more active form were found not to be operative (data not presented). We then tested the effect of SP127 on the penetration of ^{14}C-maridomycin, according to the method in Fig. 1. As shown in Fig. 3, ^{14}C-maridomycin or ^{14}C-maridomycin and SP127 was added to the

Fig. 3. Effect of SP127 on maridomycin uptake by *P. aeruginosa*.
P. aeruginosa IFO 3080 was grown in bouillon medium at 37°C. The 5.3×10^4 cpm of ^{14}C-maridomycin (60 μg) or 5.3×10^4 cpm of ^{14}C-maridomycin (60 μg) plus SP127 (80 μg) was added per ml of broth when OD_{590} reached 0.22.



medium when OD_{590} of culture broth reached 0.22 and the incubation was further continued for 3 hours. It is interesting that the accumulation of ^{14}C-maridomycin in the cells increased by the simultaneous addition of SP127. The mechanism, however, is unknown.

Discussion

The discovery of protein SP127, which was first reported in a previous paper[1], has presented many interesting aspects in the fields of chemotherapy, protein chemistry and microbial physiology.

The present report provides evidence for two specificities of the combined action of SP127 and antibiotics. One is that the synergism is restricted by antibiotics, and the other is the limitation of synergism by target organisms. SP127 enhanced the antibiotic-activity against *P. aeruginosa* of basic and neutral macrolide antibiotics, vancomycin and enramycin. The synergistic action of SP127 with an antibiotic may be associated with some structure of an antibiotic, but details are still unknown.

Specificities of antibiotics and target organisms in the synergistic action of SP127 with an antibiotic might suggest the binding of SP127 to the membrane having a specific structure. The binding would cause a consequent change in the structure of the membrane, through which only antibiotics having specific structures are permitted to be incorporated. These problems have not been answered, however, the elucidation of the mode of action of SP127 may give new clues for the study of the mechanism of drug resistance of *P. aeruginosa*.

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