SAFRACINS, NEW ANTITUMOR ANTIBIOTICS

III. BIOLOGICAL ACTIVITY

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Safracins A and B have antibacterial activity against Gram-positive and Gram-negative bacteria *in vitro* but no therapeutic activity in mice infected with *Staphylococcus aureus*. Safracins A and B induce abnormal morphological changes in *Echerichia coli* cells. Tests with transplantable mice tumors demonstrate that safracins A and B inhibit the growth of P388 leukemia and IMC carcinoma.

Although there are many ways of finding antitumor antibiotics, the method using the morphological response of drug-exposed *Echerichia coli* in hypertonic medium seems to be a useful and unique technique for finding substances which bind to nucleic acid. Using this method, safracins A and B were discovered in a culture broth of *Pseudomonas fluorescens* A2-2[1]. Structural analysis indicated that safracins A and B were similar to saframycins produced by Streptomyces[2,3].

This paper describes morphological changes of *E. coli* exposed to safracins A and B and the antibacterial and antitumor activities of safracins A and B.

Materials and Methods

Antibiotics

Safracins A and B were prepared in our laboratories as a yellow crystalline powder. Ampicillin sodium (ABPC), cycloserine (CS), fosfomycin (FOM), kanamycin (KM) and doxorubicin were obtained from Meiji Seika Kaisha Co. Mecillinam (MPC), tetracycline (TC) and chromomycin A_3 were obtained from Takeda Chemical Industries, Ltd. Streptomycin and actinomycin D were obtained from Banyu Pharmaceutical Co., Ltd. Cefazolin (CEZ), mitomycin C and bleomycin were obtained from Fujisawa Phamaceutical Co., Ltd., Kyowa Hakko Kogyo Co., Ltd. and Nippon Kayaku Co., Ltd. respectively.

Organisms

Standard strains stored in our laboratories were used in this study.

In Vitro Antibacterial Activity

The MIC was determined by the agar dilution method using Trypto-soy agar (TSA, Eiken Chem. Co., Ltd.)[4]. Unless otherwise specified, an overnight culture ($10^8 \sim 10^9$ cells/ml) of each test organism in Trypto-soy broth (TSB, Eiken Chem. Co., Ltd.) diluted with physiological saline down to 10^6 cells/ml were used as inoculum source. A loopful (loop: 1 mm in diameter) of the inoculum source was inoculated on agar plates containing antibiotic.

MIC values were determined after 20 hours incubation at 37°C. For *Streptococcus* sp. and *Corynebacterium diphtheriae*, defibrinated horse blood was added to the medium to give a final cencentration of 10%.

Morphological Change of Antibiotic-exposed Bacteria

The overnight culture of *E. coli* NIHJ JC-2 were diluted 100 fold in TSB and incubated for 2~3

hours at 37°C. 0.1 ml of the bacterial suspension was inoculated in 0.9 ml of heart infusion broth (HIB) containing double dilutions of antibiotics with or without 20% sucrose and 0.2% $MgSO_4 \cdot 7H_2O$. After incubation at 37°C for 16~18 hours, microscopic observations were made by phase contrast microscopy.

Therapeutic Effect on Experimental Infection in Mice

Female ICR strain mice, aged 4 weeks, weighing 19~21 g were used in groups of 5 mice each. Challenge organisms were cultured overnight in brain heart infusion broth (BHI) at 37°C and suspended in 5% gastric mucin (Wako Pure Chem. Co.). The mice were infected to fifty times the LD_{50}. 0.5 ml cell suspensions were injected intraperitoneally and the test antibiotics were given subcutaneously. The fifty % effective dose (ED_{50}) was calculated by the REED & MUENCH method from the survival rate on the 5th day[5].

Antitumor Tests

Safracins A and B were tested against P388 leukemia and IMC carcinoma in female CDF_1 (BALB/c×DBA/2 F_1) mice, weighing 19~24 g. P388 leukemia (10^6 cells) and IMC carcinoma (10^6 cells) were implanted intraperitoneally. Treatments were initiated one day after implantation and continued once daily for 4 days. Antitumor activity was evaluated as the increase in mean life span of treated mice over control (ILS %=T/C×100−100).

Results and Discussions

Antibacterial Spectrum

Table 1 shows the *in vitro* antibacterial activity of safracins A and B against a variety of Gram-positive and Gram-negative bacteria. Safracin B was superior to safracin A in antibacterial activity against all organisms tested. The test spectra differ from those of saframycins in activity against most Gram-negative bacteria. Unlike the saframycins, safracins A and B were effective against *Pseudomonas aeruginosa* and *Serratia marcescens*[3].

Morphological Changes of *E. coli* Cells

The morphological changes of *E. coli* NIHJ JC-2 exposed to two fold dilutions of safracins A and B were observed under phase contrast microscopy. When *E. coli* cells were incubated for 4 hours in hypertonic medium with safracins A and B at concentrations varying from 25 to 100 μg/ml and from 0.20 to 3.12 μg/ml respectively, various abnormal forms such as an tadpole or starfish appeared (Fig. 1).

Table 1. *In vitro* antibacterial activity of safracins A and B.

Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	Safracin A	Safracin B		Safracin A	Safracin B
Staphylococcus aureus FDA 209P	0.20	6.25	*E. coli* O-111	0.78	12.5
S. aureus FDA 209P*	0.20	3.13	*E. coli* T-7	0.78	25
S. aureus 308A-1	0.20	3.13	*Salmonella typhosa* Boxhill-58	0.20	3.13
Streptococcus pyogenes E-14*	<0.20	0.20	*Shigella flexneri* EW-10	0.20	1.56
S. pyogenes S-8*	<0.20	0.20	*S. sonnei* EW-33	0.20	1.56
S. viridans America*	0.20	0.78	*Klebsiella pneumoniae* DT	0.20	1.56
S. faecium IFO 3128*	0.39	3.13	*Proteus vulgaris* IFO 3988	3.13	50
S. pneumoniae Type-I*	<0.20	0.20	*P. morganii* IFO 3168	100	100
Corynebacterium diphtheriae Tront	<0.20	0.20	*P. mirabilis* IFO 3849	50	100
Bacillus subtilis PCI 219	0.20	3.13	*Pseudomonas aeruginosa* U-31	25	100
Escherichia coli NIHJ JC-2	0.78	25	*Serratia marcescens* IFO 12648	0.78	25

*: TSA+10% defibrinated horse serum.

Fig. 1. The abnormal forms of *E. coli* NIHJ JC-2 after 16 hours of exposure with safracin B 0.39 μg/ml. (a) The tadpole, (b) the starfish. Bar 10 μm.

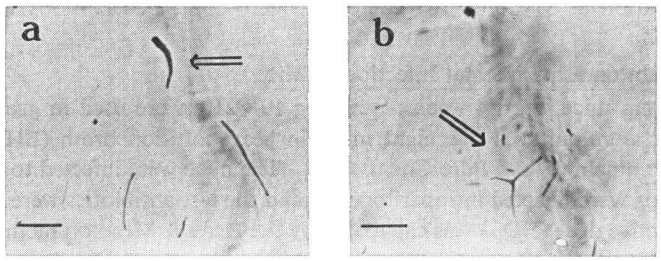

Fig. 2. Morphological changes induced by safracins A and B in HIB supplemented with sucrose and $MgSO_4 \cdot 7H_2O$.

Drug	Concentration (μg/ml)									
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.20
Safracin A	GI		AF				N			
Safracin B				GI				AF		N

GI : Growth inhibition, AF : Abnormal form, N : Normal growth

Fig. 3. Morphological changes induced by safracins A and B in HIB.

Drug	Concentration (μg/ml)									
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20
Safracin A	GI			F			N			
Safracin B				GI					F	

GI : Growth inhibition, F : Filament form, N : Normal growth

With safracin A concentrations over 100 μg/ml and safracin B concentrations over 6.25 μg/ml, cell growth was completely inhibited (Fig. 2) and abnormal forms were not observed. However, in HIB without supplementation of sucrose and $MgSO_4 \cdot 7H_2O$ with safracins A and B, *E. coli* cells changed to filamentous forms rather than the abnormal forms.

Many reports have already been published on the morphological changes induced by antibiotics on growing *E. coli* cultures in hypotonic media. MATSUMAE *et al.*[6] have classified several antibiotics into 3 groups based on the kind of morphological changes induced in *E. coli* cells and have concluded that the majority of antitumor agents belong to their group 1 defined by the ability to cause filament formation or elongation. As is well known, penicillins and cephalosporins also cause filament formation and elongation so that it is impossible to distinguish the group 1 antitumor agents from them just on the basis of these changes. On the other hand, in hypertonic medium only antitumor agents which bind to nucleic acid of bacteria induce the abnormal changes described above, so that these changes may be a useful tool for screening new substances which bind to nucleic acid.

In Vivo Antibacterial Activity

The therapeutic efficacies of safracins A and B against intraperitoneal infection of *Staphylococcus*

Fig. 4. Morphological changes induced by antibacterial and antitumor antibiotics in HIB supplemented with sucrose and $MgSO_4 \cdot 7H_2O$.

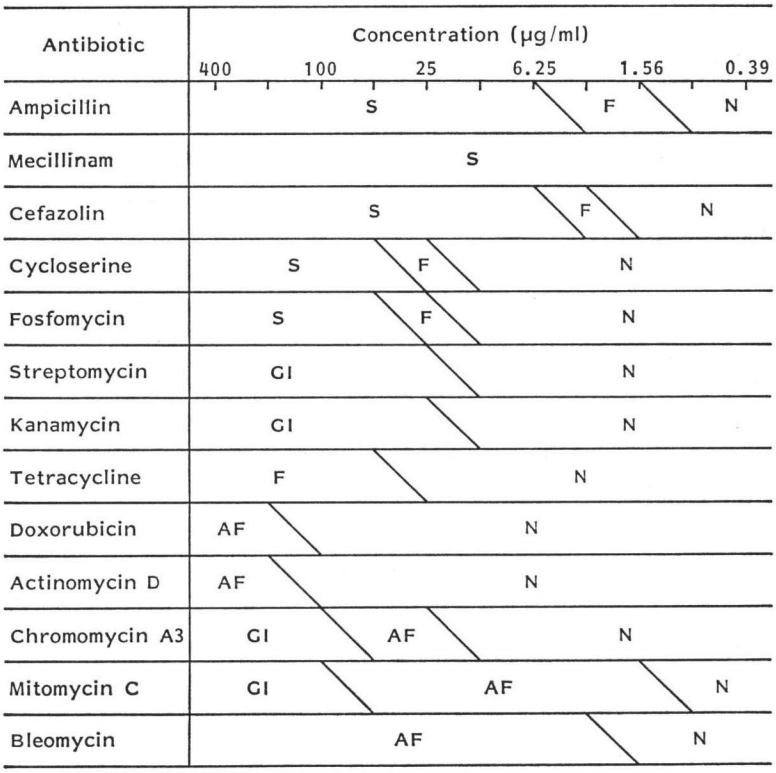

Table 2. Effects of safracins A and B on the life span of mice bearing P388 leukemia.

Drug	Dose (mg/kg/day, i.p.)	MST (days)	ILS (%)	Survivors on day 30
Safracin A	0	11.0		0/5
	1	13.8	25	0/5
	2.5	14.6	33	0/5
	5	14.6	33	0/5
	10	15.2	38	0/5
	25	15.4	40	0/5
	50	16.5	50	0/5
Safracin B	0	10.6		0/5
	0.1	14.4	36	0/5
	0.25	14.8	40	0/5
	0.5	16.6	57	0/5
	1.0	18.0	70	0/5
	2.5	4.6	Toxic	

MST: Mean survival time.
ILS: Increase in MST over control.

Table 3. Effects of safracins A and B on the life span of mice bearing IMC carcinoma.

Drug	Dose (mg/kg/day, i.p.)	MST (days)	ILS (%)	Survivors on day 30
Safracin A	0	11.0		0/5
	1	10.8		0/5
	2.5	12.6	15	0/5
	5	15.4	40	0/5
	10	24.4	120	0/5
	25	28.4	158	3/5
	50	22.5	104	1/5
Safracin B	0	10.6		0/5
	0.1	17.0	60	0/5
	0.25	17.2	62	0/5
	0.5	25.0	136	1/5
	1.0	28.0	164	3/5
	2.5	7.2	Toxic	

MST: Mean survival time.
ILS: Increase inMST over control.

aureus 308A-1 in mice were studied. ED_{50} values were indeterminable since survival ratios were not proportionate to concentrations of safracins A and B injected. Protective effects were observed only at 100 mg/kg for safracin A and 15 mg/kg for safracin B. These results suggest that the ED_{50} values are very close to the LD_{50} values.

Antitumor Activity

The antitumor activities of safracins A and B were examined against two different tumors: P388 leukemia and IMC carcinoma. As shown in Table 2, safracins A and B were found to be active against P388 leukemia (ILS 25%).

By administration of these two antibiotics, survival times of mice with IMC carcinoma were also increased, and at their optimal doses, 50 mg/kg/day for safracin A and 1 mg/kg/day for safracin B, 60% of the mice survived over 30 days.

The present studies demonstrate that newly isolated antibiotics safracins A and B possess antitumor and antibacterial activities, but no activity in therapy of mice infected with microorganisms. Plans are being made for further studies on activities in a variety of tumors and mechanisms of cytotoxic actions of these two antibiotics.

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