STUDIES ON ANTIMICROBIAL SUBSTANCE B44P (STREPTOVARICIN) PRODUCED BY A STRAIN OF ACTINOMYCETES. III

CHEMOTHERAPEUTIC EFFECT ON STAPHYLOCOCCAL INFECTION IN MICE

HISAJI YAMAZAKI

National Institute of Health, Tokyo

(Received for publication October 31, 1967)

A chemotherapy study was carried out on the substance B44P against staphylococcal infection in mice and resulted in a demonstration of its moderate therapeutic activity. The substance was active against multi-drug-resistant staphylococcus as well as against drug-sensitive staphylococcus *in vivo*. As a reference, tetracycline exhibited a curative effect in mice infected with drug-sensitive staphylococcus, but was ineffective against multi-drug-resistant staphylococcus infection. From these results and the low toxicity of the substance B44P, it is suggested that the substance B44P might be applicable in chemotherapy of the disease caused by multi-drug-resistant staphylococcus.

In a previous paper[1], the author reported that the substance B44P exhibited strong inhibitory activity *in vitro* against Gram positive bacteria including *Mycobacterium tuberculosis* and some Gram negative bacteria while its toxicity was low in mice, rats, rabbits and dogs. The results obtained in the study led the author to anticipate the usefulness of this antibiotic in chemotherapy. The substance B44P was found to be identical with streptovaricin[2]. Many chemotherapy studies were described in which experimental tuberculosis and murine leprosy were treated by streptovaricin[3,4,5,6], but no papers have been published dealing with chemotherapy of staphylococcal infection. Thus, the author attempted to test the substance B44P for chemotherapeutic effectiveness against staphylococcal infection in mice.

Lately, drug-resistant pathogenic bacteria have given rise to problems in clinic. Of course, there are some antibiotics effective against infections caused by drug-resistant bacteria, *e. g.* kanamycin, cephalosporins, synthetic penicillins, novobiocin and so on. But it is still worth searching for new medicaments which can suppress such infections. From this point of view, the author examined the substance B44P for the therapeutic effect on the infection in mice caused by clinically isolated multi-drug-resistant *Staphylococcus aureus*.

Consequently, the substance B44P was found to have a moderate curative activity *in vivo* against drug-resistant and sensitive staphylococcus infection as well.

Materials and Methods

The substance B44P and tetracycline:
The same samples were used as described in the previous paper[1].

Staphylococcus aureus, strain Smith:

Smith strain used was derived from that maintained in the author's laboratory. A highly virulent clone was selected and used in the experiments. The selection procedure was as follows: extremely diluted short-term culture of strain Smith was dispersed into a medium composed of heart infusion broth Difco, 0.4 % calf serum and 0.15 % agar. Following cultivation, several colonies showing diffuse growth were fished out one by one with micropipettes. Each of them was cultivated for 24 hours at 37°C in double-strengthened brain heart infusion broth. Ten-fold dilution series of each culture were prepared and mixed separately with an equal volume of sterile 8 % mucin suspension. Each mixture was injected intraperitoneally into 10 male *dd* mice weighing 20 g at a dose of 0.5 ml/mouse. The minimum lethal doses (M. L. D.) in each series was determined through 24-hour observation. Thus, the most virulent clone was selected and preserved as a stock in sterile 20 % skim milk at −20°C.

Staphylococcus aureus, multi-drug-resistant strain:

This strain was clinically isolated by Dr. K. YANO, Yamanouchi Pharmaceutical Co., Ltd., from a patient suffering from pyothorax in Kiyose Sanatorium, Kiyose, Japan and named Tanaka strain. This strain was allegedly resistant to penicillin, streptomycin, tetracycline, chloramphenicol and sulfonamides. The virulence of this strain was enhanced by the author by animal passage of 8 transfers through the peritoneal cavity of mice. After the passage, this strain showed resistance to penicillin (>100 units/ml), streptomycin (>100 mcg/ml), tetracycline (>100 mcg/ml), capreomycin (100 mcg/ml) and homosulfamine (>100 mcg/ml) but not to chloramphenicol (6.25 mcg/ml) *in vitro*. The method for stock preservation was the same as for the strain Smith.

Mucin suspension:

A batch of "Granular Mucin Type 1701-W" manufactured by the Wilson Laboratories, Chicago, Ill., U.S.A. was moistened thoroughly in a mortar with a portion of distilled water, let stand about 30 minutes and rubbed until free from lumps, adding another portion of distilled water to make a uniform suspension. The suspension was intermittently sterilized by heating at 80°C for 30 minutes once a day for 3 successive days. Immediately before use, the suspension was adjusted with sterile 10 % dibasic sodium phosphate to pH 6.8 and made to a concentration of 8 % mucin.

Challenge of mice with staphylococcus:

The *Staph. aureus* was inoculated from the stock described above into double-strengthened brain heart infusion broth and cultivated at 37°C for 24 hours. With the Smith strain, the 24-hour culture was diluted 10^5 times with physiological saline and the diluent was mixed with an equal volume of the 8 % mucin suspension with thorough stirring. A volume of 0.5 ml of the mixture was injected intraperitoneally to male *dd* mice weighing 20 g; this amount by this method contained 50 M.L.D. With the Tanaka strain, the 24-hour culture was mixed with an equal volume of the mucin suspension without dilution. The rest of the procedure was the same as for the Smith strain. When injected intraperitoneally, 0.5 ml of the mixture contained 10 M.L.D. In both cases, with Smith strain and Tanaka strain, all the mice challenged with the dose described died between 10 and 17 hours after infection.

Treatment:

Three hours after infection, the substance B44P or tetracycline was administered to mice once intraperitoneally, intravenously, subcutaneously or orally. In another experiment, the antibiotics were injected 6 times subcutaneously 3, 6, 9, 12, 18 and 24 hours after the infection. Observations were made through 7 days.

Results

The therapeutic effects of the substance B44P and tetracycline were examined against staphylococcal infection in mice caused by drug-sensitive and multi-drug-

Table 1. Therapeutic effects of the substance B44P and tetracycline in mice by intraperitoneal administration against staphylococcal infections caused by drug-sensitive (Smith) and multi-drug-resistant (Tanaka) strains

Dose (mg/kg)	Numben of survivors/Number treated			
	Staph. aureus Smith		*Staph. aureus* Tanaka	
	B44P	Tetra-cycline	B44P	Tetra-cycline
10	5/5		5/5	0/5
5	5/5		5/5	0/5
2.5	2/5		3/5	0/5
1.25	2/5		2/5	0/5
0.63	0/5	5/5	1/5	0/5
0.31	0/5	4/5	0/5	0/5
0.16		3/5		
0.08		0/5		
0.04		0/5		
Control	0/5	0/5	0/5	0/5

Strain Tanaka was clinically isolated and is resistant to penicillin, streptomycin, tetracycline, capreomycin and sulfamine.

The mice were treated once intraperitoneally 3 hours after intraperitoneal infection.

Table 2. Therapeutic effects of the substance B44P and tetracycline in mice by intravenous administration against staphylococcal infections caused by drug-sensitive (Smith) and multi-drug-resistant (Tanaka) strains

Dose (mg/kg)	Number of survivors/Number treated			
	Staph. aureus Smith		*Staph. aureus* Tanaka	
	B44P	Tetra-cycline	B44P	Tetra-cycline
80	5/5		5/5	0/5
40	2/5		3/5	0/5
20	0/5		0/5	0/5
10	0/5		0/5	0/5
5	0/5		0/5	0/5
2.5	0/5	5/5	0/5	0/5
1.25		5/5		
0.63		4/5		
0.31		3/5		
0.16		0/5		
Control	0/5	0/5	0/5	0/5

The mice were treated once intravenously 3 hours aftar intraperitoneal infection.

Table 3. Therapeutic effects of the substance B44P and tetracycline in mice by subcutaneous administration against staphylococcal infections caused by drug-sensitive (Smith) and multi-drug-resistant (Tanaka) strains

Dose (mg/kg)	Number of survivors/Number treated			
	Staph. aureus Smith		*Staph. aureus* Tanaka	
	B44P	Tetra-cycline	B44P	Tetra-cycline
100	5/5		5/5	0/5
50	5/5		5/5	0/5
25	4/5		3/5	0/5
12.5	0/5		1/5	0/5
6.25	0/5		0/5	0/5
3.13	0/5		0/5	0/5
1.56		5/5		
0.78		5/5		
0.39		5/5		
0.2		0/5		
0.1		0/5		
Control	0/5	0/5	0/5	0/5

The mice were treated once subcutaneously 3 hours after intraperitoneal infection.

Table 4. Therapeutic effects of the substance B44P and tetracycline in mice by oral administration against staphylococcal infection caused by drug-sensitive (Smith) and multi-drug-resistant (Tanaka) strains

Dose (mg/kg)	Number of survivors/Number treated			
	Staph. aureus Smith		*Staph. aureus* Tanaka	
	B44P	Tetra-cycline	B44P	Tetra-cycline
400	5/5		5/5	0/5
200	4/5		5/5	0/5
100	3/5		3/5	0/5
50	0/5		2/5	0/5
25	0/5		0/5	0/5
12.5	0/5	5/5	0/5	0/5
6.25		3/5		
3.13		2/5		
1.56		1/5		
0.78		1/5		
Control	0/5	0/5	0/5	0/5

The mice were treated once orally 3 hours after intraperitoneal infection.

resistant strains. Tables 1, 2, 3 and 4 show the therapeutic effects of these two antibiotics with single administration by intraperitoneal (I.P.), intravenous (I.V.), subcutaneous (S.C.) and oral (P.O.) routes, respectively. As can be easily seen from the tables, the minimum 100 % curative doses against drug-sensitive Smith strain infection were 5 mg/kg I.P., 80 mg/kg I.V., 50 mg/kg S.C. and 400 mg/kg P.O. for the substance B44P, and 0.63 mg/kg I.P., 1.25 mg/kg I.V., 0.39 mg/kg S.C. and 12.5 mg/kg P.O. for tetracycline. With tetracycline, there was little to choose between the curative activity

by I.P. treatment and that by S.C. treatment; the effectiveness of I.V. therapy was slightly inferior to that of I.P. or S.C. therapy. On the other hand, with the substance B44P, the highest effectiveness was exhibited by I.P. treatment and there was a marked difference between the activities by I.P. and S.C. treatments.

As can be seen from the tables, tetracycline, as expected, was quite ineffective against infection with Tanaka strain resistant to penicillin, streptomycin, tetracycline, capreomycin and sulfonamides. On the contrary, the substance B44P was, as might also be expected, effective against Tanaka strain infection as well as against Smith strain infection.

Table 5. Therapeutic effects of the substance B44P and tetracycline in mice by repeated subcutaneous injections against staphylococcal infections caused by drug-sensitive (Smith) and multi-drug-resistant (Tanaka) strains

Dose (mg/kg)	Number of survivors/Number treated			
	Staph. aureus Smith		*Staph. aureus* Tanaka	
	B44P	Tetracycline	B44P	Tetracycline
50 ×6	5/5		5/5	0/5
25 ×6	5/5		5/5	0/5
12.5 ×6	3/5		4/5	0/5
6.25×6	3/5		1/5	0/5
3.13×6	0/5		0/5	0/5
1.56×6	0/5		0/5	0/5
0.78×6		5/5		
0.39×6		5/5		
0.2 ×6		0/5		
0.1 ×6		0/5		
Control	0/5	0/5	0/5	0/5

The mice were treated subcutaneously 3, 6, 9, 12, 18 and 24 hours after intraperitoneal infection.

In another experiment, mice were treated repeatedly 6 times by subcutaneous injections of the antibiotics 3, 6, 9, 12, 18 and 24 hours after the staphylococcal challenge. The results are shown in Table 5. Comparison of the results given in Table 5 with those in Table 3, demonstrates that divided administration of the substance B44P can reduce the effective dose to about an half of that effective in undivided treatment.

Discussion

From the previous experimental results[1] indicating that the substance B44P had a strong antimicrobial activity *in vitro* unaffected by serum and also had low toxicity, the substance B44P was expected to show therapeutic effect against staphylococcal infection in mice. In fact, this substance was found in the present study to cure mice infected with *Staph. aureus* Smith. The curative effects, however, were not as strong as those of tetracycline, particularly, with intravenous, subcutaneous and oral treatments. The minimum *in vitro* growth-inhibitory concentration of the substance B44P and tetracycline against *Staph. aureus* Smith in heart infusion broth was 0.63 mcg/ml and 0.16 mcg/ml, respectively (Table 6). Had the substance B44P shown the *in vivo* behavior similar to that of tetracycline, its minimum curative doses in intravenous, subcutaneous and oral treatments should have been about one-tenth of its real minimum curative doses seen in Tables 2, 3 and 4. This discrepancy might be attributed to the difference in the mode of absorption, distribution, excretion, stability, solubility, *etc.* Actually, the concentration of the substance B44P in the blood was rather low, although it was absorbed from the digestive tract and appeared rapidly in the blood. Neither its stability nor solubility are high. However, the substance B44P exhibited a curative effect against staphylococcal infection in mice.

Table 6. Minimum inhibitory concentration of the substance B44P and tetracycline against strains Smith and Tanaka in heart infusion broth

	Staph. aureus Smith	*Staph. aureus* Tanaka
Substance B44P	0.63 mcg/ml	0.78 mcg/ml
Tetracycline	0.16 mcg/ml	>100 mcg/ml

A similar experiment was performed in which the mice were infected with Tanaka

strain instead sf Smith strain. Tanaka strain had been isolated from a patient and was resistant to 5 kinds of chemotherapeutics. As seen in Table 6, this strain was resistant to tetracycline but sensitive to the substance B44P *in vitro*. From this fact, one could anticipate that tetracycline might be ineffective but the substance B44P might be effective in curing mice infected with Tanaka strain. The experimental results came up to the expectations.

Although the activity is not as high as that of tetracycline, the substance B44P should be considered for chemotherapy of staphylococcal infection, particularly, for those caused by multi-drug-resistant strains.

Acknowledgement

The author wishes to express his deep thanks to Dr. H. UMEZAWA and Dr. K. NITTA for guidances of this study and to Dr. K. YANO for his kind supply of *Staphylococcus aureus* Tanaka.

References

1) YAMAZAKI, H. : Studies on antimicrobial substance B44P (streptovaricin) produced by a strain of actinomycetes. II. Microbiological and pharmacological studies. J. Antibiotics 21 : 209～221, 1968
2) SIMINOFF, P.; R. M. SMITH, W. T. SOKOLSKI & G. M. SAVAGE : Streptovaricin. I. Discovery and biologic activity. Am. Rev. Tuberc. Pulm. Dis. 75 : 576～583, 1957
3) RHULAND, L. E.; K. F. STERN & H. R. REAMES : Streptovaricin. III. *In vivo* studies in the tuberculous mouse. Am. Rev. Tuberc. Pulm. Dis. 75 : 588～593, 1957
4) STERN, K. F.: J. E. GRAY & L. E. RHULAND : Studies with streptovaricin in the tuberculous guinea pig. Am. Rev. Tuberc. Pulm. Dis. 77 : 976～982, 1958.
5) MCCUNE, R. M.; K. DEUSCHLE, C. JORDAHL, R. D. PREZ, C. MUSCHENHEIM & W. MCDERMOTT : The influence of streptovaricin used alone and with isoniazid in an experimental tuberculous infection in animals and some clinical observations. Am. Rev. Tuberc. Pulm. Dis. 75 : 659～666, 1957
6) CHANG, Y. T. : Effects of kanamycin, streptovaricin, paromomycin, novobiocin, and ristocetin on murine leprosy. Am. Rev. Tuberc. Pulm. Dis. 79 : 673～676, 1959