IN VITRO AND *IN VIVO* EVALUATION OF ENDURACIDIN, A NEW PEPTIDE ANTIBIOTIC SUBSTANCE

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A study was undertaken on the *in vitro* and *in vivo* antibacterial activities of enduracidin, a basic, peptide antibiotic substance extracted from *Streptomyces fungicidicus* No. B 5477, and the following was observed.

1. Enduracidin was found to have a high antibacterial activity against Gram-positive cocci and bacilli, but no activity was observed against Gram-negative bacilli. No cross-resistance with other antibiotics was observed.

2. The antibacterial activity of enduracidin was not affected by the pH in a range of 6~8 and nor was influenced by the addition of serum; the activity, however, was considerably affected by the size of inoculum.

3. Enduracidin was found to be therapeutically more effective against experimental *Staphylococcus aureus* or *Streptococcus hemolyticus* infection in mice than penicillin G, and most effective by the intraperitoneal route of administration. Further, the therapeutic activity of enduracidin varied with the route of inoculation in the case of staphylococcal infection.

Enduracidin is a basic, peptide antibiotic substance extracted from *Steptomyces fungicidicus* No. B 5477, and has been shown by hydrolysis to contain more than 7 amino acids, including α-amino-3,5-dichloro-4-hydroxyphenyl acetic acid[1,2]. It has been shown by TSUCHIYA *et al.*[3] that this antibiotic substance possesses potent antibacterial activity against Gram-positive bacteria, and is far less toxic than the heretofore available peptide antibiotics such as bacitracin and gramicidin S.

In the present paper are described the findings of its antibacterial activity on freshly isolated strains of Gram-positive pathogenic bacteria as well as of its therapeutic effect on the mice experimentally infected with *Staphylococcus aureus* and Group A *Streptococcus hemolyticus*.

Experimental Methods

1. Determination of sensitivity: The agar plate dilution method was employed in all the tests for determination of sensitivity. Enduracidin hydrochloride and other test agents were dissolved in sterile distilled water, and serial two step dilutions were prepared, starting with a concentration of 1,000 mcg/ml. Separately, aliquots of agar medium were placed in flasks, sterilized, the above-mentioned agents added in a volume of 1/9; mixed well, and plates prepared. Each of the plates so prepared was divided into 16 sections by marking the bottom of the Petri dish with the glass pencil; and each section was streaked for a length of 1~1.5 mm with a loopful of each test strain cultured overnight

in nutrient broth, using a platinum loop of about 1 mm inside diameter. The inoculated plates were incubated for 24 hours (*Cl. perfringens* anaerobically), and the sensitivity was expressed in terms of the minimal inhibitory concentration.

2. Strains and media used: The test strains of *Staphylococcus aureus*, Group A *Streptococcus hemolyticus* and *Corynebacterium diphtheriae* were isolated from clinical meterial, respectively, and the test strains of *Cl. perfringens* from material of food poisoning. *Streptococcus*, *Diplococcus pneumoniae*, *Corynebacterium diphtheriae* and *Brucella* cultured overnight in 10 % blood supplemented trypticase-soy broth were used as the inoculum, and test medium was 10 % horse blood supplemented trypticase-soy agar. Heart-infusion medium containing 1 % of glucose was used for *Cl. perfringens*. Nutrient broth or nutrient agar was used for all other strains.

3. Agents used: Enduracidin hydrochloride, bacitracin, gramicidin S and penicillin G.

4. Method of experimental infection of mice: STP-182 strain of *Staphylococcus aureus* was cultured in a brain-heart infusion broth overnight, centrifuged, and washed with a casamino acid medium 3 times; it was then suspended in an equal volume of casamino acid medium, and 0.5 ml of the suspension injected into the mouse tail vein, or an equivalent volume of 5 % gastric mucin was added and 0.5 ml of the mixture inoculated intraperitoneally.

For streptococcal infection Group A type 1 strain $T_1/125$ was used. It had been incubated overnight in the trypticase-soy broth, centrifuged, washed with a casamino acid medium 3 times, and suspended in an equal volume of casamino acid medium; 0.5 ml of this suspension was inoculated intraperitoneally into mice.

Results

1. Antibacterial Spectrum of Enduracidin

In Table 1 is summarized the antibacterial activity of enduracidin in comparison with those of penicillin G, bacitracin and gramicidin S. Enduracidin shows extremely potent antibacterial activity on Gram-positive cocci and bacilli, and it is noteworthy that the antibiotic is highly active even against group D streptococci. On the other hand, the agent is inactive against Gram-negative bacilli.

The antibacterial activity of enduracidin is much higher than that of peptide antibiotics such as bacitracin and gramicidin S against Gram-positive bacteria; the

Table 1. Antibacterial spectrum of enduracidin (Minimal inhibitory concentration)

Test organism	No. of strains used	Enduracidin mcg/ml	Gramicidin S mcg/ml	Bacitracin mcg/ml	Penicillin G u/ml
Staphylococcus aureus	2	0.39～0.78	3.12～25	12.5～25	<0.19～>100
Streptococcus pyogenes	1	<0.19	12.5	0.78	<0.19
Streptococcus mitis	1	0.78	50	12.5	0.39
Streptococcus faecalis	2	<0.19	3.12～12.5	12.5～25	
Diplococcus pneumoniae	4	<0.19	12.5 ～25	0.78	<0.19
Corynebacterium diphtheriae	3	<0.19～1.56	12.5	0.39～0.78	<0.19～0.39
Bacillus anthracis	1	<0.19	6.25	100	
Bacillus subtilis	1	<0.19	3.12	>100	
Brucella melitensis	1	>100	50	>100	100
Brucella abortus	1	>100	25	>100	1.56
Brucella suis	1	>100	100	>100	0.78
Salmonella paratyphi A	1	>100	>100	>100	
Salmonella paratyphi B	1	>100	>100	>100	
Salmonella pullorum	3	>100	25～>100	>100	
Serratia sp.	1	>100	>100	>100	

substance is highly active even against bacteria resistant to other antibiotics; no cross-resistance was observed.

2. Sensitivity to Enduracidin of the Strains Freshly Isolated from Clinical Materials

(1) *Staphylococcus aureus*:

Eighty seven strains of *Staphylococcus aureus* isolated from clinical material, including many strains resistant to other antibiotics, were employed. In this experiments, the inoculum suspensions of 10^6/ml viable cell units were tested for the sensitivity to enduracidin, bacitracin, gramicidin S and penicillin G. The findings are given in Table 2.

Table 2. Sensitivity of freshly isolated strains of *Staphylococcus aureus* to enduracidin.

Test antibiotic	Number of strains inhibited by concentration (mcg/ml)										
	<0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100
Enduracidin	86		1								
Gramicidin S					67	20					
Bacitracin		1	1	3	4	4	23	23	19	9	
Penicillin G	17	2	3	2	4	7	6	15	9	5	17

The suspension of 10^6/ml viable cell units was used as the inoculum.

Enduracidin, in a 0.2 mcg/ml concentration, totally inhibited the growth of these strains except one; however, the antibacterial activity of the other three agents was poorer than that of enduracidin, and many of the strains were resistant to penicillin G.

(2) Group A Streptococci:

The sensitivity of the 52 strains tested was found to be in a range of 0.1~0.4 mcg/ml of enduracidin, which is slightly lower than the sensitivity to penicillin G which is in a range of 0.013~0.1 mcg/ml, but is considerably higher than that of

Table 3. Sensitivity of Group A *Streptococcus hemolyticus* (52 strains)

Addition of blood to medium	Enduracidin concentration (mcg/ml)											
	<0.05	0.10	0.20	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100
With blood		3	36	13									
Without blood	21	9	19	3									

Addition of blood to medium	Bacitracin concentration (mcg/ml)											
	<0.05	0.10	0.20	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100
With blood			12	8	21	1	1	2	2	2	3		
Without blood		3	9	12	12		2	10		4			

Addition of blood to medium	Penicillin G concentration (u/ml)									
	<0.0032	0.0063	0.0125	0.025	0.05	0.10	0.20	0.39	0.78	1.55	>3.12
With blood			1	34	12	5					
Without blood			1	36	10	1	4				

bacitracin (Table 3). It appeared that the addition of blood at a rate of 10 % had little effect on the bacterial activity of enduracidin.

(3) *Corynebacterium diphtheriae* and *Clostridium perfringens*

The sensitivity of the 32 strains of *Corynebacterium diphtheriae* was found to be mostly in a range of 0.2~0.4 mcg/ml (or unit/ml) of enduracidin, bacitracin and penicillin G, respectively, with little difference between the three (Table 4).

Thirty strains of *Cl. perfringens* used showed a similar sensitivity to the three antibiotic substances (Table 5).

Table 4. Sensitivity of *Corynebacterim diphtheriae* to enduracidin

Test antibiotic	Concentration (mcg/ml)			
	0.10	0.20	0.39	0.78
Enduracidin		16	16	
Bacitracin		2	29	1
Penicillin G	3	26	3	

Organism : 32 strains supplied by the National Institute of Health, Tokyo.
Medium : Trypticase-soy agar containing 10 % of horse serum.

Table 5. Sensitivity of *Clostridium perfringens* to enduracidin

Test antibiotic	Concentration (mcg/ml)			
	<0.39	0.78	1.56	3.12
Enduracidin	28	1	1	
Bacitracin	9	13	5	2
Penicillin G	30			

Organism : 30 strains isolated from material of food poisoning.
Medium : Heart infusion agar containing 1 % of glucose.

3. Environmental Conditions Affecting Sensitivity

(1) Size of inoculum :

Table 6 shows the distributions of sensitivity when a 10^8 cells/ml inoculum suspension and a 10^6 cells/ml suspension of *Staphylococcus aureus* were used. In the case of 10^8/ml inoculum suspension, the minimal inhibitory concentration was found to be in a range of 0.4~0.8 mcg/ml with most strains, but in the case of 10^6 cells/ml, 0.2 mcg/ml concentration totally inhibited the growth of almost all strains.

A similar trend was also noted with *Bacillus subtilis*.

Table 6. Effect of size of inoculum on sensitivity

Size of inoculum	Concentration (mcg/ml)			
	<0.19	0.39	0.78	Total strains
10^8	6	49	5	60
10^6	86		1	87

Organism : *Staphylococcus aureus*

(2) pH value of the medium :

The nutrient agar was adjusted to various pH values in a range of 6.0~8.0, and

Table 7. Effect of pH of medium on sensitivity

Strain	Sensitivity (mcg/ml)				
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
B 1	2.5	1.25	1.25	1.25	1.25
B 2	2.5	1.25	1.25	1.25	1.25
B 3	1.25	1.25	1.25	1.25	1.25
B 4	1.25	1.25	1.25	1.25	1.25

Organism : *Staphylococcus aureus*. Size of inoculum : 10^8/ml.

Table 8. Effect of addition of serum on sensitivity

Strain	Amount of serum added			
	0	10 %	20 %	40 %
B 1	1.25 mcg/ml	2.5	5	1.25
B 2	1.25	1.25	2.5	2.5
B 3	1.25	1.25	2.5	1.25
B 4	1.25	2.5	2.5	5

Organism : *Staphylococcus aureus*. Size of inoculum : 10^8/ml. pH of medium : pH 7.0.

the sensitivity of 4 strains of *Staphylococcus aureus* was determined at the respective pH values. The findings are shown in Table 7. Fluctuations in sensitivity were scarcely found in a range of pH 6.0~8.0.

(3) Addition of serum:

To the nutrient agar was added horse serum at a rate of 10, 20 and 40 %, and the effect on sensitivity was determined. The addition of horse serum scarcely influenced the sensitivity (Table 8).

4. Therapeutic Effect of Enduracidin on Experimental Infections in Mice

(1) *Staphylococcus aureus*:

Table 9. Therapeutic effect of enduracidin on intravenous *Staphylococcus* infection

Route of medication	Dose		Survival/Challenge	
	mcg/mouse	mg/kg	Enduracidin	Penicillin G
Subcutaneous	200	12.8	9/10	1/10
	100	6.4	5/10	0/10
	50	3.2	8/10	0/10
	25	1.6	1/10	0/10
	12.5	0.8	0/10	0/10
Intraperitoneal	200	12.8	9/10	0/10
	100	6.4	7/10	0/10
	50	3.2	8/10	0/10
	25	1.6	6/10	0/10
	12.5	0.8	0/10	0/10
Intravenous	200	12.8	8/10	
	100	6.4	9/10	
	50	3.2	7/10	
	25	1.6	5/10	
	12.5	0.8	0/10	

Organism: STP-182. Challenge: 1.4×10^8/mouse. Period of observation: 2 weeks.

Table 10. Therapeutic effect of enduracidin on intraperitoneal *Staphylococcus* infection

Route of medication	Dose		Survival/Challenge	
	mcg/mouse	mg/kg	Enduracidin	Penicillin G
Subcutaneous	500	32.0	6/10	1/10
	250	16.0	0/10	3/10
	125	8.0	1/10	3/10
	62.5	4.0	0/10	0/10
	31.3	2.0	0/10	0/10
Intraperitoneal	100	6.4	9/10	1/10
	50	3.2	10/10	0/10
	25	1.6	7/10	0/10
	12.5	0.8	8/10	0/10
	6.25	0.4	0/10	0/10
Intravenous	200	12.8	0/10	
	100	6.4	0/10	
	50	3.2	2/10	
	25	1.6	1/10	
	12.5	0.8	0/10	

Organism: STP-182. Challenge: 7×10^7/mouse with 5 % gastric mucin. Period of observation: 2 weeks.

Table 11. Intravenous *Staphylococcus* infection-ED_{50} of enduracidin intraperitoneally administered.

Dose of enduracidin		Survival/Challenge
mcg/mouse	mg/kg	
100	6.60	8/10
80	5.28	8/10
70	4.62	6/10
60	3.96	9/10
50	3.30	6/10
40	2.64	6/10
30	1.98	5/10
20	1.32	1/10
10	0.66	0/10

Organism: STP-182. Challenge: 8×10^8/mouse i. v. Period of observation: 2 weeks.

Table 12. Therapeutic effect of enduracidin on intraperitoneal *Streptococcus* infection

Route of medication	Dose		Survival/Challenge		
	mcg/mouse	mg/kg	Enduracidin	Penicillin G	Bacitracin
Subcutaneous	5.00	0.33	8/10	3/10	0/10
	2.50	0.16	3/20	0/10	0/10
	1.25	0.08	2/10	0/10	0/10
Intraperitoneal	1.25	0,083	6/10	0/10	0/10
	0.63	0.042	2/10	0/10	0/10
	0.32	0.021	1/10	0/10	0/10
Intravenous	5.00	0.33	3/10		
	2.50	0.16	4/10		
	1.25	0.083	0/10		

Organism: T_1/125 Gr-A Ty-1 (M-5). Challenge: 5×10^6/mouse. Period of observation: 1 week.

Enduracidin was administered to mice challenged intravenously with the STP-182 strain of *Staphylococcus aureus*. Positive effect was obtained with a dose of not less than 50 mcg/mouse (3.2 mg/kg) subcutaneously, not less than 25 mcg/mouse (1.6 mg/kg) intraperitoneally, and not less than 25 mcg/mouse intravenously. Penicillin G, in a subcutaneous or intraperitoneal dose of 200 mcg/mouse, was found to be therapeutically ineffective (Table 9).

In mice challenged intraperitoneally with the mucin-added bacterial suspension, enduracidin was effective in an intraperitoneal dose of not less than 12.5 mcg/mouse, but was barely effective in a subcutaneous dose of 500 mcg/mouse and an intravenous dose of 200 mcg/mouse. Penicillin G, used as the control, also showed a very poor effect (Table 10).

In the intravenously challenged animal, the intraperitoneal ED_{50} of enduracidin was about 30 mcg/mouse or 1.98 mg/kg (Table 11).

(2) *Streptococcus hemolyticus*

In mice challenged intraperitoneally with the T_1/125 strain of Group A Type 1 *Streptococcus*, enduracidin was effective by a subcutaneous or intravenous route at a dose of 5 mcg/mouse (0.33 mg/kg), and an intraperitoneal dose of not less than 1.25 mcg/mouse (0.083 mg/kg). In a separate experiment where the test agent was intraperitoneally administered, the ED_{50} of enduracidin was found to be not more than 0.25 mcg/mouse (0.016 mg/kg). Penicillin G and bacitracin, at the same dose, were found totally ineffective (Tables 12 and 13).

Table 13. Intraperitoneal *Streptococcus* infection-ED_{50} of intraperitonally administered enduracidin.

Dose of enduracidin		Survival/Challenge
mcg/mouse	mg/kg	
4.0	0.264	10/10
3.0	0.190	9/10
2.0	0.132	10/10
1.0	0.066	6/10
0.5	0.033	6/10
0.25	0.016	5/10

Organism : T_1-125 Gr-A Ty-1 (M-5). Challenge : 6×10^6/mouse i. p. Period of observation : 2 weeks.

Discussion

The *in vitro* antibacterial activity of enduracidin against Gram-positive bacteria was found to be far higher than that of other peptide antibiotics such as bacitracin or gramicidin S, which are effective against Gram-positive bacteria. Also the staphylococci which were resistant to other antibiotics including penicillin were found to be highly sensitive to enduracidin. No cross-resistance was found among the tested antibiotics.

The antibacterial activity of enduracidin was not influenced by the pH value in a range of pH 6 and 8, nor was it affected by the addition of serum. Further, the minimal inhibitory concentration of enduracidin did not vary with the kind of medium (such as nutrient agar, heart infusion agar, brain heart infusion agar and trypticase-soy agar). The minimal inhibitory concentration, however, varied considerably with the size of inoculum; it was shown that when inoculum suspension of *Staphylococcus* and *Bacillus subtilis* is diluted 100-fold, the minimal inhibitory concentration of enduracidin was reduced to less the 1/4.

In mice experimentally infected with *Staphylococcus aureus* and *Streptococcus hemolyticus*, enduracidin was found to be therapeutically more effective than penicillin G, and in both cases, the agent was most effective by intraperitoneal route of administration. In mice intraperitoneally infected with *Staphylococcus aureus*, enduracidin by the intraperitoneal route was quite effective, but ineffective by the subcutaneous or intravenous

route of administration. As intraperitoneal infection with the mucin-added inoculum usually progresses rapidly, it may be assumed that enduracidin is distributed slowly to the tissues so that therapeutic activity is comparatively low.

From the *in vitro* and *in vivo* findings, enduracidin may be expected to be clinically effective against infections by Gram-positive bacteria.

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