ENDURACIDIN, A NEW ANTIBIOTIC. III

IN VITRO AND *IN VIVO* ANTIMICROBIAL ACTIVITY*

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Enduracidin showed antibacterial activity *in vitro* against Gram-positive bacteria and *N. gonorrhoeae*, but not against Gram-negative bacteria. The antibacterial activity of enduracidin was not greatly influenced by the pH of the test medium. Enduracidin was more stable at pH 4 and 7 than at pH 9. This antibiotic demonstrated bactericidal as well as bacteriostatic activity *in vitro* at a similar concentration against *Staph. aureus*. The development of resistance to enduracidin was slow and no cross resistance was observed between enduracidin and other known antibiotics. Enduracidin was effective against experimental infections produced in mice by strains of *Staph. aureus*, *Sterept. pyogenes* and *D. pneumoniae* type I. Enduracidin was most effective against streptococcal infection. This antibiotic was effective by the subcutaneous, intraperitoneal and intravenous routes, but not orally. A single intramuscular dose of 2 mg/kg of enduracidin in the rabbit produced significant plasma levels extending even to 24 hours.

Enduracidin, a new basic polypeptide antibiotic, is produced by *Streptomyces fungicidicus* No. B-5477. Biological characterization of this organism was studied by HIGASHIDE *et al.*[1], and the isolation and physico-chemical characterization of the antibiotic have been described by ASAI *et al.*[2]

The present report is concerned with the antimicrobial activity *in vitro*, such as antimicrobial spectrum, influence of pH of the medium, stability, bactericidal activity, development of resistance and cross resistance. It is also concerned with the therapeutic effect *in vivo* against experimental Gram-positive bacterial infections and blood levels of the antibiotic.

Materials and Methods

Antibiotics: Enduracidin hydrochloride was dissolved in sterile distilled water for *in vitro* study or in appropriate media for *in vivo* study.

Antimicrobial test: The minimal inhibitory concentration of the antibiotic was determined by the two-fold serial agar dilution assay method. Trypticase soy agar (BBL) or agar medium plus 10 % beef blood were used as the assay medium. The test organism was grown for 18 to 24 hours on Trypticase soy agar, and one loopful of a suspension containing about 1 mg per ml of test organism was inoculated on each assay plate. The plates were incubated at 37°C and readings were made routinely at 18 hours. The minimum

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inhibitory concentration (MIC) was defined as the lowest concentration of the antibiotic preventing the visible growth.

Development of resistance: The development of resistance for the antibiotic was studied in Trypticase soy broth (BBL) using *Staph. aureus* 209P, *Strept. pyogenes* E-14 and *D. pneumoniae*. Transfer was made every 48 hours from the tube containing the highest concentration of the antibiotic permitting growth into the next series of broth tubes containing the same and several higher concentrations of the antibiotic.

Bactericidal activity: The viability of *Staph. aureus* in the presence of enduracidin was determined by the plate count technique. An 18 hours broth culture of the *Staph. aureus* 209P was diluted 10^3 times in Trypticase soy broth and the antibiotic was added to give concentrations of 1.0, 0.1 and 0.01 μg per ml. Aliquots were withdrawn from each tube prior to incubation and at intervals of 2, 4, 6, 8, 24 and 48 hours during incubation at 37°C. Platings were made in duplicate at several dilutions to ensure meaningful count. Colony counts were made after 48 hours.

Therapeutic effect in mice: Male CF 1-JCL mice weighing 18~22 g were used. Intraperitoneal infections were made with 0.5 ml of 5 % mucin containing 1/10 its volume of *Staph. aureus* 308 A-1* culture (Trypticase soy broth), or *Strept. pyogenes* E-14 suspension (2×10^{-4} mg per ml, blood Trypticase soy agar culture); or 0.5 ml of broth containing *D. pneumoniae* type I (2×10^{-6} mg per ml, blood Trypticase soy agar culture). Intravenous infections were made with 0.2 ml of Trypticase soy broth culture (2 fold dilution) of *Staph. aureus* 308 A-1. Treatment was given either by single subcutaneous, intraperitoneal, or intravenous injection or by oral administration. The antibiotic was injected immediately after challenge. The 50 per cent effective dose (ED_{50}) was calculated from the survival rate after 7 days by the method of REED and MUENCH.

Plasma concentration: Venous blood samples were collected from rabbit ear into phosphate buffer containing 5 % sodium citrate (1 : 1) at intervals of 1/12, 1/2, 1, 2, 4, 8, 24 and 48 hours after intramuscular injection of the antibiotic. Plasma was separated by centrifugation. Concentration of the antibiotic in plasma was determined by a modification of the KANAZAWA's method using *B. subtilis* PCI-219 as the test organism.

Results

Antimicrobial Test *in Vitro*

Antibacterial spectrum:

The antibacterial activity of enduracidin against Gram-positive and Gram-negative organisms was summarized in Table 1.

Enduracidin was effective against Gram-positive bacteria and *Neisseria gonorrhoeae*. Even *Staph. aureus* 1840**, which is resistant to some of the known antibiotics, was sensitive to this antibiotic. However, enduracidin was not effective against Gram-negative bacteria.

Influences of medium pH, serum and inoculum size upon the antibacterial activity of enduracidin:

The minimum inhibitory concentration of enduracidin upon *Staph. aureus* 209P, Heatley, 308 A-1 and 1840 was observed under various conditions of medium or inoculum size. As indicated in Table 2, the activity was slightly higher at pH 8.0 than that at pH 7.5 to 6.0.

* *Staph. aureus* 308 A-1 was obtained from Department of Animal Microbiology, College of Agriculture, University of Osaka Prefecture.

** *Staph. aureus* 1840 was previously reported by ARAKI *et al.* in Annual Report of the Takeda Research Laboratories, 22, 140, 1963. This organism was clinically isolated, and it is resistant to penicillin, tetracycline, streptomycin and sulfonamide.

Table 3 indicated that the addition of 10 % horse serum to the medium showed no influences upon the activity, but that the activity was slightly lower at 20~50 % concentration of the serum. Table 4 indicated clearly that the antibacterial activity increased as the inoculum size decreased.

Distribution of sensitivity of clinically isolated staphylococcal strains:

The data in Table 5 indicated that enduracidin was effective against clinically isolated staphylococci* at the concentration of 0.5~2.5 mcg/ml and that 71 strains among 78 showed its MIC at 1 mcg/ml, whereas the MIC against laboratory maintained staphylococci was 0.78 mcg/ml as indicated in Table 1. This pattern showed a sharp contrast in that the other antibiotics presented relatively wide range of MIC against clinically isolated staphylococcus.

Table 1. Antibacterial spectrum of enduracidin

Organism	Medium	Enduracidin mcg/ml
Staphylococcus aureus 209 P	Trypticase soy agar	0.78
Staphylococcus aureus Heatley	〃	0.78
Staphylococcus aureus 1840	〃	0.78
Streptococcus pyogenes E-14	Trypticase soy agar +10 % beef blood	0.39
Streptococcus pyogenes Dick	〃	0.39
Streptococcus pyogenes S-8	〃	0.39
Streptococcus pyogenes NY-5	〃	0.39
Streptococcus viridans sp.	〃	0.78
Diplococcus pneumoniae type I	〃	0.78
Diplococcus pneumoniae type II	〃	0.78
Diplococcus pneumoniae type III	〃	0.78
Corynebacterium diphtheriae	〃	0,78
Bacillus subtilis PCI-219	Trypticase soy agar	1.56
Neisseria gonorrhoeae	Trypticase soy agar +10 % beef blood	6.25
Shigella flexneri EW-10	Trypticase soy agar	>100
Shigella sonnei EW-33	〃	>100
Salmonella typhosa Boxhill-58	〃	>100
Escherichia coli UMEZAWA	〃	>100
Vibrio cholerae INABA	〃	>100
Klebsiella pneumoniae	〃	>100
Pseudomonas aeruginosa	〃	>100
Proteus vulgaris	〃	>100

Inoculum size : One loopful of bacterial suspension (1 mg/ml).

Table 2. Effect of medium pH on antibacterial activity of enduracidin

Organisms	MIC in mcg/ml				
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Staph. aureus 209P	1.56	0.78	0.78	0.78	0.78
〃 Heatley	1.56	1.56	1.56	0.78	0.78
〃 308 A-1	1.56	1.56	1.56	1.56	0.78
〃 1840	1.56	1.56	1.56	1.56	0.78

Inoculum size : One loopful of bacterial suspension (1 mg/ml).

Table 3. Effect of horse serum concentrations in medium on antibacterial activity of enduracidin

Organisms	MIC in mcg/ml			
	0 %	10 %	20 %	50 %
Staph. aureus 209P	0.78	0.78	1.56	1.56
〃 Heatley	0.78	0.78	1.56	1.56
〃 308 A-1	0.78	0.78	1.56	1.56
〃 1840	0.78	0.78	1.56	1.56

Inoculum size : One loopful of bacterial suspension (1 mg/ml).

Stability of enduracidin measured by antibacterial activity:

Enduracidin solutions in phosphate buffers at pH 5, 7 and 9 were kept at 4°, 37° and 100°C for 14 days, and the growth inhibiting activity against *Staph. aureus* 209P on agar medium was observed after 0, 1, 2, 3, 4, 5, 6, 7 and 14 days. As shown in Table 6, enduracidin solutions at pH 5, 7 and 9 were stable more than 14 days at 4° and 37°C. Table 7 shows the results at 100°C indicating that

* The cultures were kindly supplied by Miss SHIMIZU of Central Clinical Laboratory, Osaka University Hospital.

Table 4. Effect of inoculum size on antibacterial activity of enduracidin

Organisms	MIC in mcg/ml					
	3×10^3	3×10^4	3×10^5	3×10^6	3×10^7	3×10^8
Staph. aureus 209P	0.18	0.39	0.78	0.78	0.78	1.56
// Heatley	0.39	0.39	0.78	0.78	0.78	0.78
// 308 A-1	0.78	0.78	0.78	0.78	1.56	1.56
// 1840	0.78	0.78	0,78	0.78	0.78	1.56

the activity of the solution at pH 9 diminished rapidly but the activity of antibiotic at pH 5 and 7 showed no changes at least for 300 minutes.

Bactericidal activity:

The viability of *Staph. aureus* 209P, determined by plate count after incubation in Trypticase soy broth with various concentrations of enduracidin is demonstrated in Fig. 1. The logarithm of the viable count is plotted against time of exposure to the antibiotic. The concentration of 1 mcg/ml of the antibiotic clearly demonstrated bactericidal action, and viability was not seen even after 48-hour incubation. This concentration of enduracidin was equal to the minimal inhibitory concentration by agar dilution method against *Staph. aureus*. Considerable killing was obtained at the 0.1 mcg/ml level, but the culture recovered after 8-hour incubation. Viable count did not differ from

Table 5. Distribution of sensitivity of *Staphylococcus* strains against enduracidin and other antibiotics

Minimal inhibitory concentration (mcg/ml)	Distribution (number of strains)						
	Enduracidin	Erythromycin	Penicillin G	Cephaloridine	Chlortetracycline	Chloramphenicol	Dihydrostreptomycin
>100		29	37		26		33
100		1	4		5	7	2
50		2	2		3	7	4
25		1	7		1	4	2
12.5			5	4	7	50	19
5			3	5	19	9	12
2.5	4	3	2	18	15	1	4
1	71	5	2	9	2		2
0.5	3	24	3	11			
0.25		13	3	13			
<0.25			10	18			

Table 6. Stability of enduracidin in solution at various pH at 4° and 37°C

Temperature	pH	MIC in mcg/ml								
		0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	14 days
4°C	5	0.78*	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56
	6	0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56
	7	0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56
37°C	5	0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56
	7	0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56
	9	0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56	1.56

* Test organism: *Staph. aureus* 209P

Table 7. Stability of enduracidin in solution at various pH at 100°C

pH	MIC in mcg/ml*					
	0 min.	5 min.	30 min.	60 min.	120 min.	300 min.
5	0.78	0.78	0.78	0.78	0.78	0.78
7	0.78	0.78	0.78	0.78	0.78	0.78
9	0.78	3.125	12.5	12.5	12.5	12.5

* Test organism: *Staph. aureus* 209P

that of control at the lowest level tested, 0.01 mcg/ml. These results indicate the bactericidal nature of the antibiotic.

Development of resistance:

The patterns of development of resistance to enduracidin, penicillin G, cephaloridine and chlortetracycline were compared using *Staph. aureus* 209P, *Strept. pyogenes* E-14 and *D. pneumoniae* type I. The rapidity and degree of resistance to various antibiotics developed in *Staph. aureus* 209P is shown in Fig. 2.

Against enduracidin, staphylococci showed an early low-grade rise in resistance then gradually increased between the 5th and 24th transfers, and reached 64 times resistant after 24th transfer. The pattern of development of resistance to enduracidin was similar to that of chlortetracycline. The development of resistance against penicillin G was similar as that against cephaloridine.

Strept. pyogenes E-14 and *D. pneumoniae* type I did not develop resistance to the antibiotics tested during the entire transfer.

Fig. 1. Bactericidal activity of enduracidin on *Staph. aureus* 209P

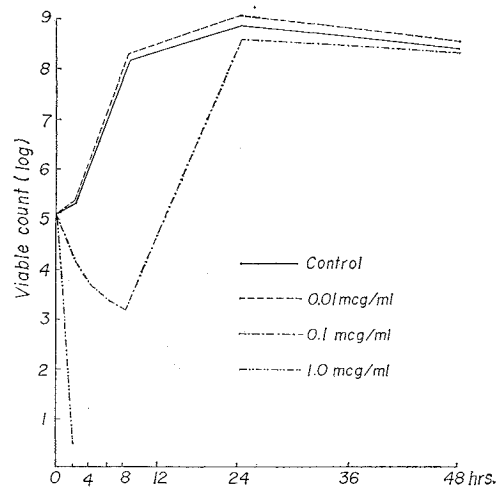
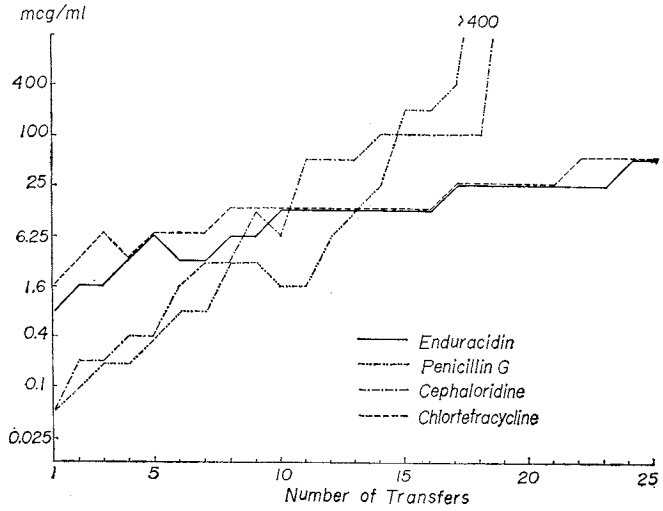


Fig. 2. Patterns of development of resistance of *Staphylococcus aureus* 209P to enduracidin, penicillin G, cephaloridine and chlortetracycline



Cross resistance:

Cross resistance between enduracidin and other antibiotics was studied with *Staph. aureus* 209P which was made resistant respectively to enduracidin, erythromycin, penicillin G, cephaloridine

Table 8. Cross resistance test among enduracidin, erythromycin, penicillin G, cephaloridine and chlortetracycline

Organism	MIC in mcg/ml				
	Enduracidin	Erythromycin	Penicillin G	Cephaloridine	Chlortetracycline
Staph. aureus 209P (parent)	0.78	0.39	<0.09	<0.09	3.12
R-Enduracidin	50	0.39	<0.09	<0.09	3.12
R-Erythromycin	0.39	>400	<0.09	<0.09	3.12
R-Penicillin G	0.78	0.39	100	25	0.78
R-Cephaloridine	0.39	0.39	400	>400	0.39
R-Chlortetracycline	0.78	0.39	<0.09	<0.09	25

or chlortetracycline by serial subcultures in Trypticase soy broth containing increasing concentrations of each antibiotic. The data given in Table 8 were obtained by the agar dilution method.

Enduracidin maintained its full activity against microorganisms resistant to other antibiotics, and the enduracidin-resistant organism was sensitive to other antibiotics.

Antimicrobial Test *in Vivo*

Therapeutic effect against experimental Gram-positive bacterial infection:

Therapeutic effect of enduracidin against experimental infections produced by strains of *Staph. aureus* 308 A-1, *Strept. pyogenes* E-14 and *D. pneumoniae* type I in mice were shown in Table 9. Against Gram-positive bacterial infection, enduracidin was effective by subcutaneous, intraperitoneal and intravenous administrations, but ineffective when given orally.

Table 9. Effect of enduracidin against Gram-positive bacterial infections in CF-1 mice

	Staph. aureus 308 A-1		*Strept. pyogenes* E-14	*D. pneumoniae* type 1
In vitro sensitivity (mcg/ml)	0.1		0.025	0.0015
Challenge route	IP	IV	IP	IP
Challenge dose ($\times LD_{50}$)	17.8	5.5	316	560
Administration route and ED_{50} (mg/kg) SC	4.82	1.76	0.088	0.71
IP	0.193	1.25	0.022	0.22
IV	1.62	1.11	0.081	0.65
Oral	>100	>100	>100	>100

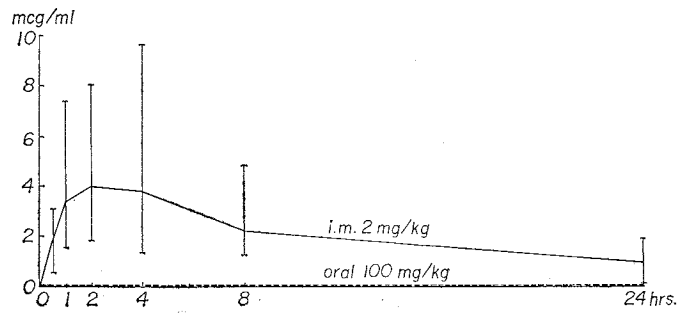
Against intraperitoneal staphylococcal infection, the ED_{50} of enduracidin was 4.82 (sc), 0.193 (ip) and 1.65 (iv) mg/kg. However, in intravenous staphylococcal infection, the ED_{50} of this antibiotic was 1.76, 1.25 and 1.11 mg/kg, respectively. The protective activity of enduracidin against staphylococcal infections was greater than that of other known antibiotics.

Against streptococcal infection, the ED_{50} of enduracidin was 0.088, 0.022 and 0.081 mg/kg, respectively. Against diplococcal infection, the ED_{50} of this antibiotic was 0.71, 0.22 and 0.65 mg/kg, respectively. The therapeutic effect of this antibiotic in streptococcal and diplococcal infection was between 10 and 100 time stronger than that of other known antibiotics.

Plasma level:

To determine the plasma concentration obtained with a single dose of enduracidin, seven rabbits were given 2 mg/kg intramuscularly. Results are demonstrated in Fig. 3. Plasma samples were collected at 1/12, 1/2, 1, 2, 4, 8 and 24 hours after the

Fig. 3. Enduracidin plasma level after single administration



administration. The peak of the plasma levels was shown to be 2.9~8.0 mcg/ml and these levels were lower than those of the other known antibiotics. However, the level in 6 of the 7 rabbits was still measurable even after 24 hours. This finding suggested a prolonged activity of this antibiotic.

On the other hand, plasma level was not detected throughout the observation period in three rabbits given 100 mg/kg orally.

Discussion

The antibacterial spectrum of enduracidin was determined by the usual agar dilution method. The result showed that enduracidin was active against Gram-positive bacteria and *N. gonorrhoeae*. Although the activity of this antibiotic against Gram-positive bacteria was equivalent to that of tetracycline, it was also effective against a strain of *Staph. aureus* 1840, which is resistant to several antibiotics. It was suggested that enduracidin appears to be useful against several antibiotic-resistant staphylococcal infection.

The development of resistance to enduracidin was slow. Even at 25 th transfer, *Staph. aureus* 209P did not increase more than 64 time resistance. The streptococci and diplococci developed no resistance to enduracidin. The degree of development of resistance was similar to that of chlortetracycline.

Enduracidin has bactericidal action and further biochemical studies on the interaction between enduracidin and microorganism are in progress, and will be reported in a forthcoming communication.

Remarkable effect was observed in the therapeutic activity against Gram-positive bacterial infections. Enduracidin showed greater therapeutic activity in mice experimentally infected with *Strept. pyogenes* than that against staphylococcal and diplococcal infections. The therapeutic activity of the antibiotic against experimental infections produced by strains of *Staph. aureus*, *Strept. pyogenes* and *D. pneumoniae* was 10~100 times greater than that of other known antibiotics. One of the cause for this therapeutic effect was probably that the plasma level was maintained longer than that of other antibiotics.

The plasma concentration of enduracidin differed from that of the other known antibiotics in the following points: very high concentrations were not attained during the observation period, but detectable levels were noted in the plasma even after 24 hours in 6 of 7 rabbits given an intramuscular dose of 2 mg/kg.

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