Studies on Tobicillin*, a New Antibiotic Drug for Enterococciosis in Yellowtail *Seriola quinqueradiata*

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Tobicillin (TBPC), an ester derivative of penicillin G (PCG), was examined as a treatment for enterococciosis in yellowtail.

After 6 h at 25°C at pH 3, the residual rate of TBPC was over 80%, but that of PCG was only 20%. TBPC was stable in an acidic solution (pH 3).

The area under the blood concentration-time curve (AUC) between 0 and 10 h after oral administration was about 7 times higher for TBPC (35.2 µg/ml·h) than that for PCG (5.2 µg/ml·h).

In drug sensitivity tests using 156 strains of field-isolated *Enterococcus seriolicida*, the MICs of PCG were 0.39–0.78 µg/ml, and no strains were resistant to PCG. On the other hand, the MICs of erythromycin, lincomycin and oxytetracycline showed biphasic distributions that included both resistant and sensitive strains.

In a preliminary evaluation of the efficacy of TBPC against experimental enterococciosis in yellowtail, the cumulative death rate 5 days after infection was 56% for the non-treated group and 0% for the group treated with 100 mg (potency)/kg/day.

These results indicate that TBPC will be one of the effective antibiotics for the treatment of enterococciosis in yellowtail.

Key words: tobicillin, PCG, enterococciosis, *Enterococcus seriolicida*, treatment, yellowtail, absorption

Culture of yellowtail *Seriola quinqueradiata* accounts for most of the production of Japanese marine fish farms. As with any intensively cultured fish, fish disease is a major problem. Thus, treatment of cultured yellowtail with antibiotics against bacterial infection is essential for profitability.¹⁾ In particular, enterococciosis, a bacterial infection that was first observed in yellowtail in Japan in 1974,²⁾ accounted for 67.9% of the losses of cultured yellowtail in 1991.¹⁾

Macrolides such as erythromycin (EM) and spiramycin (SP), and lincomycin (LCM) have been used as chemotherapeutic agents for the treatment of enterococciosis.^{3–6)} However, in recent years, these antibiotics have become ineffective against *Enterococcus seriolicida* (*syn. Lactococcus garvieae*),⁷⁾ the bacterium causing this disease. Their extensive uses have led to an increase in multiple drug-resistant strains of *E. seriolicida*.^{8–10)}

Therefore, the development of a new drug having a different mechanism of drug action from that of macrolides has been awaited. On the other hand, there have been scarcely any reports on the use of penicillins, which are effective against gram-positive organisms, for the treatment of enterococciosis.^{11,12)} The only exception is a report on the drug sensitivity of *E. seriolicida* isolated between 1974 and 1981 by Aoki *et al.*¹³⁾ Penicillins, such as penicillin G (PCG) and methicillin, are given parenterally.

Because they are not resistant to acid, they are largely decomposed by gastric acid and inactivated.¹⁴⁾ Another penicillin, ampicillin (ABPC), which is comparatively stable in acid, did not show any efficacy against enterococciosis in a clinical study performed by Shiomitsu *et al.*¹⁵⁾

In general, drugs are often modified chemically to enhance their physicochemical properties and antibacterial activities for a particular clinical application. One such modification improves the absorption of drugs that are not easily absorbed in the digestive tract. Drugs that show an in vivo pharmacological effect after absorption are called prodrugs.

Bicozamycin (BCM) is poorly absorbed in yellowtail after oral administration. Nakano *et al.*¹⁶⁾ improved the oral absorption of BCM by creating a benzoic ester of BCM, i.e., a prodrug of BCM.

To produce a prodrug of PCG for the treatment of enterococciosis, we synthesized tobicillin (TBPC), a new antibiotic, by making a derivative of PCG with 3-isobutryryl-hydroxymethyl ether.

We investigated the stability of TBPC in acid, and fed yellowtail a TBPC-containing diet to (i) confirm the transfer of PCG to the blood and (ii) to evaluate the efficacy of the drug against enterococciosis in a preliminary clinical study. To assess the efficacy of TBPC against enterococciosis, we also investigated the sensitivity of 156 isolates of

* A patent application of TBPC was filed and published as WO93/08196.

E. seriolocida to the drug *in vitro*. These strains were isolated from enterococciosis-affected yellowtail taken from Japanese fish farms between 1992 and 1996.

Materials and Methods

Tobicillin

Tobicillin (TBPC) is an ester of PCG synthesized at Chemical Products Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. The chemical formula, molecular weight and chemical structure are shown in Fig. 1.

pH Stability

TBPC and PCG solutions were prepared as follows: For the TBPC solution, about 20 mg (39.2 μ M) of TBPC was accurately weighed and dissolved in acetonitrile to make 20 ml. Five milliliter of this solution was diluted with acetonitrile to make 50 ml. The diluted solution, 2.5 ml, was diluted with Walpole buffer solutions at pHs 3, 4, 5 and 6 to make 25 ml, corresponding to a final concentration of about 65 μ g (potency)/ml (Walpole buffers consisted of various mixtures of 1 M hydrochloric acid and 1 M sodium acetate). For the PCG solution, about 15 mg (39.2 μ M) of PCG potassium was accurately weighed and dissolved in water to make 20 ml. A portion of this solution (exactly 0.25 ml) was diluted with 2.5 ml of acetonitrile. This was diluted with Walpole buffer solutions to make 25 ml (about 65 μ g (potency)/ml). The solutions were stored in a constant temperature bath at 25°C for 2, 4 and 6 h. The concentrations of TBPC and PCG in the solutions were then measured by reverse phase high performance liquid chromatography (RP-HPLC),¹⁷⁾ using a UV detector. Sodium thiosulfate was added to the PCG solutions to prevent the decomposition of PCG during the determination by HPLC. The detector was set at 220 nm for TBPC and 215 nm for PCG.

Fish

The yellowtail used in this study were reared at Tsukuba Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. These fish had not been treated with any antibiotics for at least 4 weeks prior to the study.

Changes in Blood Concentration of PCG

Twelve yellowtail, approximately 200 g, were kept in

each of two 100-l circulating water tanks made of polycarbonate (48 cm in diameter $\times$ 80 cm in depth). Water temperature was maintained at 24.5–25.0°C.

The fish food was prepared as follows. A commercially available feed not containing antibiotics (Marine 6: Maruha) was granulated and mixed with TBPC or PCG potassium salt. These concentrations were such that the feed equivalent to 1.7% of fish weight contained an amount of the drug that would provide 100 mg (potency)/kg fish body weight. The mixture was then mixed with an equal mass of water and pelleted.

The feed was given to the fish once ad libitum. Three yellowtail were taken for blood samples at 3, 5, 7 and 10 h post administration. The blood was collected from the heart with a heparinized syringe and stored at –20°C until analyzed.

PCG was analyzed by a microbiological assay method with *Micrococcus luteus* ATCC 9341. The test strain was inoculated into nutrient broth (Kyokuto) and pre-incubated at 37°C for 20 h. One milliliter of the culture broth was inoculated into 100 ml of nutrient agar medium (nutrient broth containing 1.5% agar (Difco)). The mixture was shaken well, poured into sterilized petri dishes (10 ml/dish) and allowed to gel. Paper discs (8 mm in diameter, thin, Toyo Rosi Kaisya) containing 35 μ l of blood samples were placed on the agar, and the dishes were incubated for 20 h at 37°C. The diameters of inhibitory zones were measured, and PCG was assayed by the standard curve method. The area under the blood concentration-time curve (AUC) was calculated by the trapezoidal method.

MICs of Drugs against *E. seriolocida*

A total of 156 isolates of *E. seriolocida* were collected from cultured yellowtail with enterococciosis in 1992, 1994 and 1996. Fifty-one of the isolates were obtained in Ehime Prefecture, 42 in Ohita, 18 in Kagoshima, 8 in Kumamoto and 37 in Nagasaki, and identified as *E. seriolocida* by morphology and slide agglutination with an anti-*E. seriolocida* rabbit serum.

Six antibiotics were used: PCG, ABPC, EM, LCM, oxytetracycline (OTC) and florfenicol (FFC). The minimal inhibitory concentrations (MICs) of *E. seriolocida* for the test antibiotics were determined by the agar plate dilution method specified by the Japan Society of Chemotherapy.¹⁸⁾ Each isolate was inoculated into heart infusion broth (Difco) supplemented with 0.2% glucose. The broth was allowed to stand at 25°C for 20 h and diluted 100-fold to prepare the inoculation liquid (about 10^6 cfu/ml). This liquid was inoculated by the stamp method onto heart infusion agar supplemented with 0.2% glucose that contained one of the antibiotics and was incubated at 25°C for 24 h to measure the MICs of these antibiotics for each isolate.

Preliminary Study of Therapeutic Effect of TBPC on Enterococciosis in Yellowtail

Nine yellowtail, approximately 300 g, were kept in each of two 100-l tanks (described above). Water temperature was kept at about 20.0°C. All 18 yellowtail were inoculated intramuscularly with 1.1×10^6 cfu of *E. seriolocida* strain HY89038rP4-3, which had been incubated overnight on heart infusion agar supplemented with 0.2% glucose, suspended in physiological saline. One hour after

Molecular formula: $C_{27}H_{30}N_2O_6S$

Molecular weight: 540.61

(+)-3-isobutyryloxymethylphenyl (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate

Fig. 1. Diagram showing the chemical structure of tobicillin.

inoculation, the fish in one tank (the TBPC group) were forcibly given 100 mg (potency)/kg of TBPC orally in the form of a suspension of homogenized fish meal in an equal mass of water. This was repeated for the following 4 days. Fish in the second tank received no treatment (non-treated group).

Results

pH Stability of TBPC and PCG

After 6 h at 25°C, the residual rates of TBPC and PCG (Figs. 2 and 3, respectively) were 90% or more in the solutions at pHs 5 and 6. TBPC was clearly more stable than PCG at pH 3. As for the time dependence of the stability, the residual rates of TBPC were 90% or more until 4 h after preparation and 80% or more 6 h after preparation. In contrast, the residual rate of PCG was 50% 2 h after preparation, and only 20% 6 h after preparation.

Changes in Blood Concentrations of PCG

Changes in blood concentrations of PCG in yellowtail are shown in Fig. 4. The maximum blood concentration (T_{max}) of TBPC occurred 3 h after administration. The blood concentration had a mean maximum (C_{max}) of 5.11 $\mu\text{g/ml}$ and gradually decreased to 0.98 $\mu\text{g/ml}$ 10 h after administration.

T_{max} of PCG also occurred 3 h after administration. However, the concentration of PCG reached a maximum of only 0.75 $\mu\text{g/ml}$ and gradually decreased to 0.32 $\mu\text{g/ml}$ 10 h after administration. The area under the blood con-

Fig. 4. Mean concentration of penicillin G in yellowtail *Seriola quinqueradiata* after a single oral dose of 100 mg (potency)/kg of tobramycin or penicillin G. *Error bars show standard errors of the mean.

centration-time curve (AUC) between 0 and 10 h after oral administration was about 7 times higher for TBPC (35.2 $\mu\text{g/ml}\cdot\text{h}$) than that for PCG (5.2 $\mu\text{g/ml}\cdot\text{h}$).

MICs of Drugs against *E. seriolicida*

The distribution of MICs of each chemotherapeutic agent for the 156 tested isolates is shown in Table 1.

Since TBPC is a prodrug having ester bonding, it had no antibacterial activity.

Biphasic MIC distributions were observed for antibiotics other than PCG and FFC. Some isolates were resistant to some of the drugs. The MIC distributions of PCG, ABPC and FFC were very similar. The MICs were 0.39–0.78 $\mu\text{g/ml}$ for PCG, 0.78–1.56 $\mu\text{g/ml}$ for ABPC and 1.56–3.13 $\mu\text{g/ml}$ for FFC. None of the isolates were resistant to any of these drugs.

The MICs of PCG were slightly lower than those of ABPC.

For EM, the MICs of the sensitive isolates were mostly in the range from ≤ 0.05 to 0.2 $\mu\text{g/ml}$ while those of the resistant isolates were mostly ≥ 100 $\mu\text{g/ml}$. Some isolates were highly resistant to EM.

Similarly, the MICs of LCM were mainly 0.2 $\mu\text{g/ml}$ for sensitive strains but 50– ≥ 100 $\mu\text{g/ml}$ for most of the resistant strains. Some isolates were highly resistant to LCM.

The MICs of OTC were mainly 0.78 $\mu\text{g/ml}$ for sensitive strains, 6.25 $\mu\text{g/ml}$ for moderately resistant strains and ≥ 100 $\mu\text{g/ml}$ for highly resistant strains. Some isolates were highly resistant to OTC.

The MIC₉₀ values of EM, LCM and OTC for some strains increased markedly with the year of collection, indicating an increasing resistance of these strains to these drugs.

The MIC₉₀ of FFC changed slightly for some strains in some years, but no changes were observed in MIC₉₀ values of PCG and ABPC.

Preliminary Results of Therapeutic Effect of TBPC

Five of the nine *E. seriolicida*-infected yellowtail in the non-treated control group died within 5 days (Table 2). No deaths occurred in the TBPC group.

Fig. 2. Effect of pH stability of tobramycin solution.

Fig. 3. Effect of pH stability of penicillin G solution.

Table 1. Distribution of minimal inhibitory concentrations (MIC) of 7 antimicrobial drugs against 156 strains of *Enterococcus seriolocida* isolated from 1992 to 1996

ANTIBIOTIC	YEAR	Number of strains showing each MIC ($\mu\text{g/ml}$)												STRAINS	MIC ₉₀ *
		≤ 0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	≥ 100		
TBPC	1992												156	156	≥ 100
	1994												156	156	≥ 100
	1996												156	156	≥ 100
PCG	1992				1	26								27	0.78
	1994				14	51								65	0.78
	1996				11	53								64	0.78
ABPC	1992					27								27	0.78
	1994					63	2							65	0.78
	1996					58	6							64	0.78
EM	1992	5	20	1									1	27	0.1
	1994	4	3	21									37	65	≥ 100
	1996	10	19	3									32	64	≥ 100
LCM	1992			23						2				27	12.5
	1994			4						4	16		41	65	≥ 100
	1996			7			3			10	17		27	64	≥ 100
OTC	1992				2	20	5							27	1.56
	1994				2	6	1	15	12			3	26	65	≥ 100
	1996					14	20	5	12				13	64	≥ 100
FFC	1992						27							27	1.56
	1994						14	49	2					65	3.13
	1996							64						64	3.13

*: MIC₉₀ means the MIC value for inhibition of 90% of the isolates.

Table 2. Therapeutic effect of tobramycin in yellowtail with enterococcosis

Group	Number of fish	Number of dead fish Days after infection						Mortality (%)
		0	1	2	3	4	5	
Non-treated control	9	0	1	0	0	1	3	56
tobramycin	9	0	0	0	0	0	0	0

Infection: Yellowtail, weighing about 300 g, were infected intramuscularly with *Enterococcus seriolocida* HY89038rP4-3 at 1.1×10^6 CFU/fish.

Medication: Oral administration for 5 consecutive days after infection.

Discussion

Penicillins are widely used for the treatment of bacterial infection in humans and livestock but not in fish.^{19,20} The only penicillins used in fish are ABPC and amoxicillin, which are used for the treatment of pseudotuberculosis in yellowtail.²¹

Penicillins have not been actively used for the treatment of diseases in fish because, in general, they are unstable in heat and acid, and because the desired level can not be obtained by practical oral route.¹⁴

Since the antibacterial activity of penicillins against organisms is exerted by inhibiting biosynthesis of the bacterial cell walls, these drugs are very safe and are more active against gram-positive organisms than gram-negative organisms. Therefore, these drugs are expected to be effective against enterococcosis from the viewpoint of their bac-

tericidal activity.

In the present study, we found that the decomposition of PCG at pH 3 (which is equivalent to the pH of gastric acid), was faster than that of its derivative, TBPC. The retention time of feed in the stomach is not clear, but in our study, 80% or more of the TBPC, even at pH 3, was retained 6 h after administration.

The AUC between 0 and 10 h of TBPC after administration was about 7 times greater than that of PCG.

From the results of the stability of TBPC and PCG in the acidic solution and the blood concentration of PCG in yellowtail after oral administration, TBPC appears to have a greater resistance to gastric acid than PCG.

The drug sensitivities of *E. seriolocida* isolated from five areas of Japan between 1992 and 1996 were determined. There were strains resistant to EM, LCM and OTC, but the MICs of PCG or ABPC were concentrated in a narrow range. None of the strains were resistant to PCG or ABPC.

Since PCG is the same kind of drug as ABPC, the possibility of development of cross-resistance was considered. However, since no resistance to these drugs was observed in the present survey, the resistance of *E. seriolocida* to penicillins appeared to be very low because of the difference of the season when pseudotuberculosis and enterococcosis are most prevalent.

It was shown that PCG concentrations sufficient to inhibit the growth of *E. seriolocida* can be maintained for several hours after oral administration of TBPC. Furthermore, a preliminary clinical study of TBPC in enterococcosis-affected yellowtail appears to show that TBPC has an antibacterial activity against *E. seriolocida*.

The present results suggest that TBPC has promise as a drug for treating enterococciosis and that further investigations will be worthwhile. We have recently found that TBPC has an excellent field efficacy to natural infection of *E. seriolicida* in yellowtail and will report these results in a subsequent paper.

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