THIOLACTOMYCIN, A NEW ANTIBIOTIC

III. *IN VITRO* ANTIBACTERIAL ACTIVITY

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Thiolactomycin is a new broad-spectrum antibiotic, active *in vitro* against many species of Gram-positive cocci, *Enterobacteriaceae*, *Haemophilus*, acid-fast bacteria and anaerobic bacteria. However, the activity is generally moderate and bacteriostatic in action. This antibiotic eludes cross resistance with any of the known antibacterial drugs such as ampicillin, carbenicillin or cycloserine. The effect on Gram-negative bacilli *in vitro* is little affected by the inoculum size, the presence of horse serum, the pH of the medium or the type of medium.

When thiolactomycin was added to the assay medium, the minimum inhibitory concentration of ampicillin against inducible β-lactamase producing strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was reduced. Thiolactomycin inhibited the production of inducible β-lactamase in several organisms.

Thiolactomycin (TLM) is a new antibiotic obtained from the fermentation broth of a strain of actinomycetes which was identified as a new species of *Nocardia*.[1] TLM has a unique chemical structure with no chemical relation to any group of known antibiotics.[2]

This paper describes results of *in vitro* tests used to examine some characteristics of the antibacterial effects of TLM. Also some studies on the effects of this antibiotic on β-lactamase induction are presented.

Materials and Methods

Antibiotics

TLM sodium was prepared in our laboratories as a white crystalline solid. The following antibacterial drugs were used for comparisons: carbenicillin (CBPC, Fujisawa Pharmaceutical Co.), ampicillin (ABPC, Meiji Seika Co.), cefazolin (CEZ, Fujisawa Pharmaceutical Co.), ceftezole (CTZ, Chugai Pharmaceutical Co.), cloxacillin (MCIPC, Meiji Seika Co.) and D-cycloserine (CS, Nakarai Chemicals Co.).

Organisms

Standard strains stored in our laboratories were used in this study. The clinical isolates were obtained from several hospitals near Tokyo.

Most of the strains were stab cultured in 0.5% nutrient agar (NA) medium and stored at room temperature with rubber stoppers. Strains of *Haemophilus influenzae*, *Streptococcus pyogenes* and some other organisms were suspended in broth containing 10% glycerol and stored at −70°C.

Determination of *In Vitro* Antibacterial Activity

The *in vitro* antibacterial activity of TLM and other antibiotics was determined mainly by the agar-plate dilution method. Unless otherwise specified, an overnight culture ($10^8 \sim 10^9$ viable cells/ml) of each test strain in heart infusion broth (HIB) was diluted 100 ~ 1000 fold with saline (about 10^6 viable cells/ml). One loopful of this suspension was spotted on each of several heart infusion agar (HIA) plates containing two-fold serially graded concentrations of each antibiotic. The agar plates were incubated

at 37°C for 20 hours, and the minimum inhibitory concentration (MIC) was determined.

The following media, as specified for each species, were used to determine the MIC value. For *S. pyogenes* (original strain and L-form), HIB containing 4.1% NaCl and 0.1% $MgCl_2$ was used as the preculture and the test medium. *H. influenzae* were cultured on HIA containing 4% horse blood digested with pepsin. *Mycobacteria* were cultured in KIRCHNER liquid medium (Eiken). For fungi, SABOURAUD agar was used and incubated at 27°C for 24~72 hours. For anaerobic bacteria, GAM-agar (Nissui, modified formula of Gifu Univ., Japan) or SHAEDLER agar (BBL) containing 5% blood was used and incubated in Gas-Pak jars at 37°C for 48 hours.

Influence of Various Factors on Antibacterial Activity

The changes in the MIC values of TLM and other antibiotics in response to different kinds of test media and pH value of a test medium, to the addition of horse serum and to various inoculum sizes were determined. The test strains used were 4 strains of Gram-positive cocci, 10 strains of *Enterobacteriaceae* and 2 strains of *Pseudomonas aeruginosa*. The MIC values were determined by the serial dilution agar method on HIA as the basal medium.

For examining the influence of test media, Nutrient agar (Eiken), HIA (BBL), MUELLER-HINTON agar (MHA) (Difco) and Antibiotic medium No. 3 agar (Anti 3) (Difco) were chosen. In examining the influence of the pH of the test media, HIA with pH values of 5.0, 7.0 and 8.0 was used. The influence of serum on the MIC values was determined on HIA supplemented with 10 or 50% horse serum inactivated by heating at 60°C for 20 minutes. To determine the influence of inoculum size, an overnight culture of test strains in HIB was diluted 10-fold serially ($10^8 \sim 10^5$ viable cells/ml) and one loopful of each dilution was inoculated and cultured on each of a series of HIA plates containing graded concentrations of each antibiotic.

Effects on the Growth Curves of Bacteria

1) Estimation by photometer: *Klebsiella pneumoniae* 3K25 and *Serratia marcescens* 101 were used as test strains. The growth curves of the test strains were recorded automatically by photometry (Bio-Log 11 photometer JASCO). After subculturing twice in HIB, each culture was diluted 1000-fold (about 10^6 cells/ml) with fresh HIB. The inoculated medium was poured into photometer cells and incubated at 35°C. When the optical density had risen to 70~90 T%, the drugs were added and the incubation was continued for 20 hours.

2) Estimation by viable cell counts: The inoculated media were prepared as described above and incubated at 37°C for 2~3 hours, after which the drugs were added and the incubation was continued for 5 hours. Aliquots were removed at various time intervals and viable cell numbers were determined by counting on plates.

Isolation of Constitutive Mutants with and without β-Lactamase Synthesis from a Strain of *P. aeruginosa* with Inducible β-Lactamase Synthesis

A constitutive β-lactamase synthesis mutant, J-169-C222 and a β-lactamase-less mutant, J-169-C222-L8 were obtained from *P. aeruginosa* J-169, as described by OHMORI *et al.*[3,4] Strain J-169 was first converted to the J-169-C222 mutant which was then changed to the J-169-C222-L8 form. The original strain was grown exponentially in nutrient broth and treated with 100~300 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by ADELBERG *et al.*[5] The isolation procedures were carried out by a combination of the methods described by ROSSELET *et al.*[8] and OHMORI *et al.*[3,4]

Determination of Inhibition of β-Lactamase Formation in the Test Strains

Bacteria were grown in 5 ml T-medium (3.2% trypticase peptone, 2.0% yeast extract, 0.5% NaCl) with shaking at 37°C for 15 hours, and 0.3 ml of the culture was transferred to 4.7 ml fresh T-medium to give an initial bacterial concentration of 10^7 cells/ml. After incubation with shaking for 1~2 hours (logarithmic phase) TLM was added to final concentrations of 12.5~400 μg/ml and the incubation was continued for 1 hour at 30°C. Then 6-APA, 400~500 μg/ml final concentration (*P. aeruginosa*) or carbenicillin (CBPC), 1 μg/ml final concentration (*S. aureus*), was added as inducer and incubation was continued for 3 hours in the same way as above. The cells were harvested by centrifugation, washed twice with 0.1 M phosphate buffer and suspended in 6 ml of the same solution at pH 6.5 (*P. aeruginosa*)

or pH 7.0 (*S. aureus*). The cell suspension was sonicated in a Branson sonicator for 30 seconds at 0°C. The sonicated suspension was centrifuged for 10 minutes at 8,000×*g* and the β-lactamase activity of the supernatant fluid was determined according to the modified SARGENT's method described by SAWAI *et al.*[6] The enzymic activity per 0.1 mg protein of the crude samples is expressed as μ moles of substrate hydrolyzed in 60 minutes at 30°C. Protein determinations were made by the method of LOWRY *et al.*[7]

Results

Antimicrobial Spectrum

The antimicrobial spectrum of TLM is summarized in Table 1. At concentrations of 1.56~100 μg/ml, TLM suppressed the growth of standard strains of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, S. pyogenes, Escherichia coli, Salmonella* sp., *K. pneumoniae, Proteus mirabilis, Shigella* sp., *H. influenzae, Mycobacterium, Bacteroides* and *Fusobacterium* sp.. TLM has a

Table 1. Antimicrobial spectrum of TLM and CS.

Organism	MIC (μg/ml)		Organism	MIC (μg/ml)	
	TLM	CS		TLM	CS
B. subtilis PCI-219	100	12.5	[b]*H. influenzae* 1014-33	3.1	400
B. cereus T-1	200	12.5	[b]*H. influenzae* 1076–48	6.3	25
M. luteus B	25	12.5	[b]*H. influenzae* 764–69[3]	3.1	200
[a]*S. pyogenes* SV	37.5	—	*Ps. aeruginosa* J-272	800	100
[a]*S. pyogenes* SV-L-form[1]	150	—	*Ps. aeruginosa* M-57740[4]	1.6	100
S. epidermidis TO-3	50	50	*Ps. aeruginosa* J-169	800	—
S. aureus 209-P	25	12.5	*Ps. aeruginosa* J-169-C222	400	—
S. aureus 222[2]	100	25	*Ps. aeruginosa* J-169-C222L8[5]	400	—
S. aureus JU-5[2]	50	12.5	[c]*M. tuberculosis* $H_{37}RV$	25	—
S. aureus A-5	50	12.5	[c]*M. tuberculosis* $H_{37}RV$-SMr	25	—
E. coli NIHJ	50	25	[c]*M. tuberculosis* $H_{37}RV$-INHr	25	—
E. coli NO-9	50	12.5	[c]*M. tuberculosis* $H_{37}RV$-PASr	12.5	—
E. coli 11	25	12.5	[e]*B. fragilis* V-6	3.1	—
S. enteritidis T-1	12.5	25	[e]*B. fragilis* V-8	12.5	—
S. typhi TANAKA	12.5	25	[e]*B. fragilis* subsp. *vulgatus* ATCC 8482	50	—
K. pneumoniae 3K25	200	100	[e]*B. distasonis* ATCC 8503	12.5	—
K. pneumoniae 15C	100	100	[e]*F. glutinosum* 1006	100	—
Sh. flexneri 2b T-1	6.3	25	[e]*F. necroforum* S-45	12.5	—
Sh. sonnei T-1	100	12.5	[d]*Candida* sp.	1600	—
Pr. mirabilis 1287	50	50	[d]*Cryptococcus* sp.	1600	—
Pr. mirabilis 9	50	50	[d]*Trichophyton* sp.	1600	—
S. marcescens FU-111	50	400	[d]*Aspergillus* sp.	1600	—
S. marcescens T-50	50	200			

Agar plate dilution method, HIA(BBL), inoculum size: 1 loopful of a suspension of 10^6 cells/ml.

[a] HI broth(BBL)+4.1% NaCl+0.1% $MgCl_2$, broth dilution method.

[b] HIA(BBL)+4% horse blood digest.

[c] KIRCHNER liquid medium (Eiken), broth dilution method.

[d] SABOURAUD agar, 27°C, 24~72 hours.

[e] GAM agar, anaerobic culture.

[1] L-Form, derivate of SV strain. [2] PCase producer.

[3] β-Lactam resistant. [4] β-Lactam supersensitive mutant of J-272.

[5] Inducible β-lactamase-less mutant of J-169.

Table 2. Influence of various factors on the activity of TLM.

	MIC (μg/ml)									
	Media				pH of medium			Horse serum (%)		
	NA	HIA	MHA	Anti-3	5.0	7.0	8.0	0	10	50
B. subtilis PCI-219	50	100	50	50	25	100	200	—	—	—
B. cereus T-1	100	200	100	100	50	400	400	100	400	400
M. luteus B	12.5	25	25	12.5	3.1	25	50	—	—	—
S. aureus 209 P	50	25	12.5	25	6.3	25	50	25	50	800
S. aureus JU-5	400	50	25	100	12.5	50	100	50	50	800
E. coli NIHJ	200	50	50	200	50	50	25	50	50	100
E. coli 11	12.5	25	25	25	50	25	25	12.5	25	50
S. enteritidis T-1	12.5	12.5	6.3	6.3	3.1	12.5	25	12.5	12.5	50
S. typhi TANAKA	12.5	12.5	6.3	12.5	3.1	12.5	12.5	12.5	12.5	12.5
K. pneumoniae 3K25	200	200	100	200	200	200	200	100	100	100
K. pneumoniae 15 C	200	100	50	100	100	100	100	100	50	100
Sh. flexneri 2b TO-1	12.5	6.3	6.3	12.5	1.6	6.3	12.5	—	—	—
Sh. sonnei TO-1	100	100	50	100	50	100	100	50	50	100
S. marcescens FU-111	100	50	25	50	25	50	100	50	50	200
S. marcescens TO-50	100	50	25	50	50	50	100	100	50	200
Ps. aeruginosa J-272	800	800	800	800	200	800	800	800	800	50
Ps. aeruginosa J-169	800	800	800	800	200	800	800	800	400	800

Agar plate dilution method.
Test medium: HIA(BBL), inoculum size: 1 loopful of a suspension of 10^6 cells/ml.
NA: Nutrient agar (Difco), HIA: Heart Infusion agar (BBL), MHA: MUELLER-HINTON agar (Difco), Anti-3: Antibiotic 3 agar (Difco).

wide-range antimicrobial spectrum, except for fungi.

Influence of Various Experimental Conditions on Activity

The influence of the test media, pH values of the test medium, and presence of horse serum on the activity of TLM were examined with 17 strains of Gram-positive cocci and Gram-negative bacilli (Table 2).

Varying the composition of the media caused little variation in the MIC values of the test strains. On HIA and MHA, the MIC values tended to be lower than those obtained on NA and Anti-3 agar.

The effect of varying the pH values on the activity of TLM depended on the bacterial species tested. Gram-positive cocci were more sensitive at acidic pH than at alkaline pH and this tendency was also observed though less markedly, in *P. aeruginosa, Shigella* sp. and *Salmonella* sp.. The sensitivity of *E. coli* and *K. pneumoniae* was unchanged in the range from 6.0 to 8.0.

Addition of 10% horse serum to the test medium did not change the MIC values of TLM against test organisms, but further increases up to 50% tended to increase the MIC values, especially for *S. aureus*.

Influence of inoculum size on the MIC values was examined with 24 strains representing 4 species of bacteria. As shown in Fig. 1, the variation of MIC values of TLM was slight in *K. pneumoniae* and *S. marcescens*, compared with that observed with ABPC and CBPC in the range from 10^5 to 10^3 cells/ml of inoculum size. In the case of *S. aureus*, moderately increasing MIC values were observed at large inoculum size.

Fig. 1. Influence of inoculum size on activity of TLM, ABPC and CBPC.

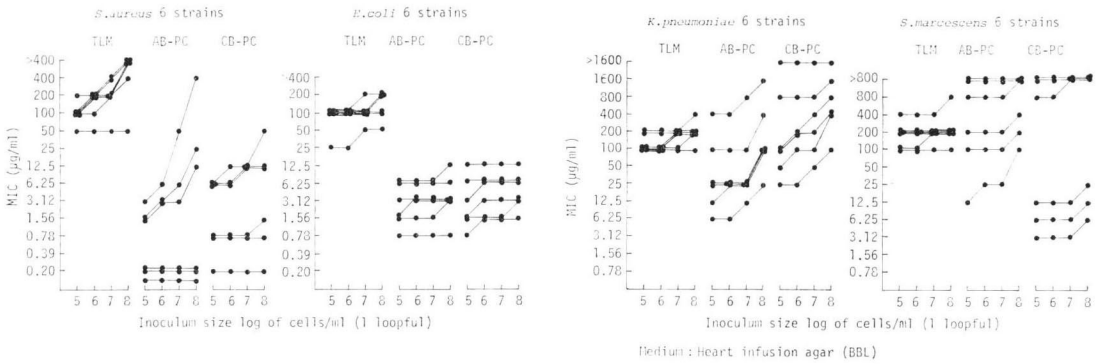


Table 3. Distribution of susceptibilities of clinical isolates to TLM and other antibiotics.

Organism	Anti-biotic	MIC (μg/ml)													
		>400	400	200	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2	0.1
S. aureus (45–39)	TLM	1	2		9	23	5	4	1						
	ABPC				1	2	2	2	4	6		3	1	18	
E. coli (27)	TLM		2	3	19	1	1	1							
	ABPC							1	10	6	7	2	1		
	CBPC							1	7	11	5	2	1		
K. pneumoniae (27)	TLM			15	10	2									
	CBPC	1	3	13	3	5	2								
S. marcescens (49)	TLM			3	32	11	3								
	ABPC	8	7	2	4	6	16	6							
P. aeruginosa (48)	TLM	17	12	16	1				1	1					
	CBPC	1	3	3	19	18	2			2					
**H. influenzae* (46)	TLM								10	27	4	5			
	ABPC						1		1	1			4	29	10
	CS	3	20	12	7	3	1								

Agar plate dilution method, Medium: HIA(BBL), *: HIA+4% horse blood digest, inoculum size: 1 loopful of a suspension of 10^6 cells/ml.

Susceptibilities of Clinical Isolates

The MIC values of TLM for clinical isolates are shown in Table 3. The distribution of MIC values of TLM for 45 clinical isolates of *S. aureus* had a peak at 50 μg/ml and MIC values of 400 μg/ml or more were observed in only 3 strains.

The MIC distribution of 27 strains of *E. coli*, 27 of *K. pneumoniae* and 49 strains of *S. marcescens* showed peaks at 100, 100~200 and 50~100 μg/ml respectively. The growth of all *E. coli* strains was inhibited at 400 μg/ml or below. The activity of TLM against 48 clinical isolates of *P. aeruginosa* was lower than that observed against the other organisms tested and high resistance with a MIC of 400 μg/ml or above was observed in 29 strains.

The MIC distribution for strains of *H. influenzae* had a peak at 3.13 μg/ml and no resistant strains with a MIC above 6.25 μg/ml were observed.

Thus, the activity of TLM against clinical isolates was generally rather weak compared with that of

Fig. 2. Effects of TLM, CBPC and CEZ on growth curves of *K. pneumoniae* 3K25 and *S. marcescens* 101.

—— Turbidity ——

Biophotometer: Bio-Log 11 photometer JASCO.
Medium: HIB (BBL).

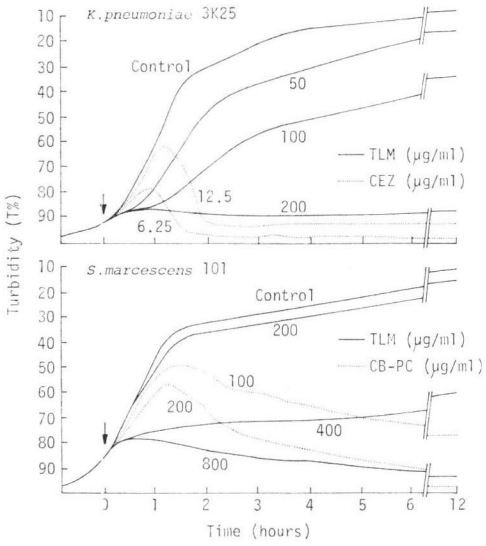
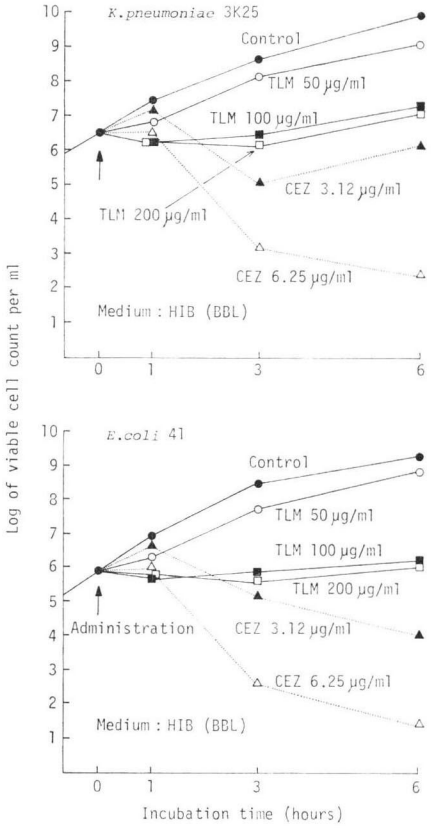


Fig. 3. Effects of TLM and CEZ on the growth curves of *K. pneumoniae* 3K25 and *E. coli* 41.

—— Viable counts ——



ABPC and CBPC. The range of the MIC values of TLM for the isolates was comparatively narrow and no cross resistance was observed between TLM on one side and ABPC, CBPC or CS on the other.

Effects on Growth Curves of Bacteria

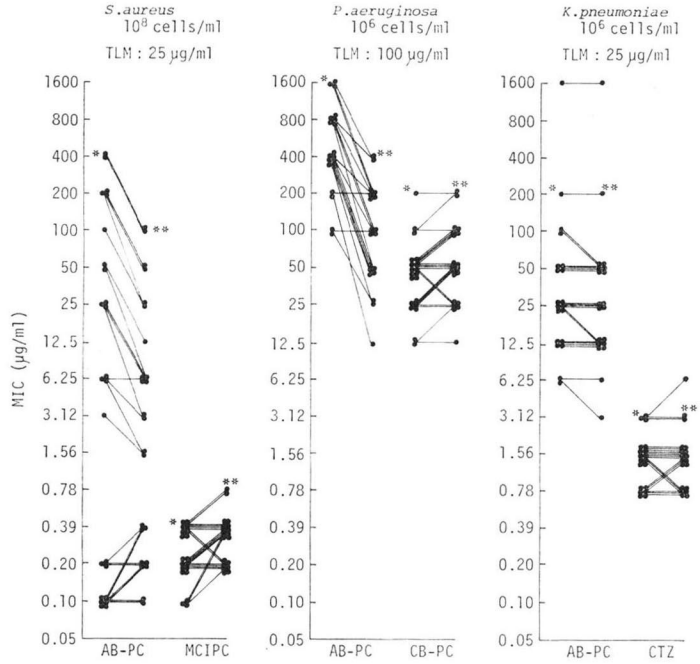
The effects of TLM on the growth curves of *K. pneumoniae* 3K25 and *S. marcescens* 101 were compared with those of CEZ and CBPC respectively by biophotometry. As shown in Fig. 2, by addition of the antibiotic to MIC or above, the growth was suppressed after a very short time, but with TLM no reduction of the cell mass (O.D.) was obtained, indicating that no cell-lysis occurred. Further, the changes in viable counts obtained with TLM and CEZ were measured with the strains *E. coli* 41 and *K. pneumoniae* 3K25. As shown in Fig. 3, these strains survived, even when exposed to concentrations above MIC of TLM. Thus, TLM is bacteriostatic, not bactericidal.

Combined Antibacterial Action of TLM and Some β-Lactam Antibiotics *In Vitro*

Tests with TLM combined with the β-lactam antibiotics ABPC, CBPC, MCIPC and CTZ were made by the agar plate dilution method on 25 strains of *P. aeruginosa*, 25 strains of *K. pneumoniae* and 24 strains of *S. aureus*. The MIC values of the β-lactam antibiotics for these strains were determined by using agar-plates which contained 1/2～1/4 MIC of TLM.

The results are summarized in Fig. 4. The MIC values of ABPC against most strains of *P. aeruginosa* tested were reduced by combination with subinhibitory concentrations of TLM to about one-eighth of those of the ABPC alone. However, no difference in MIC values was observed between CBPC alone

Fig. 4. Combination effects of TLM and β-lactam antibiotics on the MICs against clinical isolates.



MIC values of ABPC, MCIPC, CBPC and CTZ for the clinical isolates were assayed by using plates with 1/2~1/4 the MIC of TLM and no agent respectively.
Assay medium: HIA (BBL)
*: MIC of β-lactam antibiotic alone.
**: MIC of β-lactam antibiotic on agar containing 1/2–1/4 MIC of TLM.

and CBPC in combination with TLM. The β-lactamase producing ability of 25 strains of *P. aeruginosa* was assayed by the agar-overlay method, as described by ROSSELET *et al.*,[8] and it was proved that 24 of the 25 strains were inducible β-lactamase producers.

For clinical isolates of *S. aureus*, the TLM-ABPC combination was synergistic against 15 inducible β-lactamase producing strains but not against 9 β-lactamase negative strains. On the other hand, a combination of TLM and MCIPC was non-synergistic against all strains of *S. aureus* tested.

No combination effects were observed between TLM and ABPC or CTZ against clinical isolates of *K. pneumoniae* which produced constitutive β-lactamase.

Inhibition of β-Lactamase Formation

When TLM was incubated with crude β-lactamase (6~15 μM for 60 minutes), there was no decrease in the effect of the enzyme on CET or PCG. This held true for all β-lactamases prepared from *P. aeruginosa* KAN-4, J-169 (inducible), *S. aureus* JU-5 (inducible) or *K. pneumoniae* 3K25 (constitutive) in 0.1 M phosphate buffer at pH 6.5 or 7.0 and 30°C. Therefore it is concluded that β-lactamase is not directly affected by TLM.

Next, the inhibitory effect of TLM on β-lactamase formation by intact cells was examined using *P. aeruginosa* J-162, J-169 (inducible), *S. aureus* JU-5 (inducible), and *K. pneumoniae* (constitutive).

As shown in Table 4, the inducible β-lactamase production of strains of *P. aeruginosa* and *S. aureus* was inhibited in proportion to the concentration of TLM. The 50% inhibition dose of the antibiotic

was approximately as follows: *P. aeruginosa* J-162, 50~100 μg/ml; *P. aeruginosa* J-169, 200~400 μg/ml and *S. aureus* JU-5, 12.5~25 μg/ml. On the other hand, in *K. pneumoniae* 3K25, a constitutive β-lactamase producer, no reduction of β-lactamase production was observed, even when the amount of the antibiotic was more than 200 μg/ml. Furthermore, the extracellular enzyme activity of the supernatant from the reaction mixture was not increased by the addition of TLM.

To confirm whether the inhibitory activity of TLM on β-lactamase production and its synergistic effect with β-lactam antibiotics is restricted to the inducible enzyme producing strains, tests similar to the above were carried out using some β-lactamase mutants derived from an inducible β-lactamase producing strain of *P. aeruginosa*. The results are summarized in Table 5.

For *P. aeruginosa* J-169, an inducible β-lactamase producer, TLM reduced β-lactamase production and was synergistic with ABPC. However, in J-169-C222, a constitutive β-lactamase producer, which was derived from *P. aeruginosa* J-169, neither reduction of the en-

Table 4. Inhibition by TLM of β-lactamase production by strains of *P. aeruginosa*, *K. pneumoniae* and *S. aureus*.

TLM (μg/ml)	β-Lactamase activity μmole/60 minutes/0.1 mg-protein			
	P. aeruginosa J-162	*P. aeruginosa* J-169	*K. pneumoniae* 3K25	*S. aureus* JU-5
0	6.75	7.76	6.12	6.24
12.5	NT	NT	NT	4.23
25	5.20	NT	5.71	0.89
50	3.85	6.12	5.77	0.15
100	2.42	5.11	5.77	0.03
200	0.79	4.00	4.90	NT
400	0.03	2.12	NT	NT

An over night culture was transferred to 4.7 ml fresh T-medium giving an initial bacterial concentration of 10^7 cells/ml. TLM was added after shaking for 1~2 hours to final concentrations of 12.5~400 μg/ml and shaking was continued at 30°C for 1 hour. Then, 6-APA(400~500 μg/ml final concentration, *Ps. aeruginosa*) or CBPC(1 μg/ml-final concentration, *S. aureus*) was added as inducer and incubation was continued for 3 hours.

The test cultures were harvested and intracellular crude β-lactamase was prepared according to the method described in the text.

β-Lactamase assay: ASRGENT-SAWAI method.

Protein assay: FOLIN-LOWRY method.

Substrate: *P. aeruginosa*, 10~20 μmole of CET.
S. aureus, 10 μmole of PCG.
K. pneumoniae, 10 μmole of PCG.

Table 5. Effect of TLM on β-lactamase production and synergism with β-lactam antibiotics against the original strains of *P. aeruginosa* and its β-lactamase-constitutive and β-lactamase-less mutants.

Strains of *P. aeruginosa*	β-Lactamase activity μmole/60 minutes/0.1 mg-protein					MIC (μg/ml) ABPC		CBPC	
	Without induction	Induction with 6-APA addition of TLM (μg/ml) 0	25	100	400	Alone	Combination with TLM (100 μg/ml)	Alone	Combination with TLM (100 μg/ml)
J-169(Original)	≦0.03	7.76	—	5.1	2.1	1600	400	400	200
J-169-C222	7.64	10.62	11.9	11.9	13.1	1600	1600	400	200
J-169-C222-L8	≦0.03	≦0.03	—	—	—	50	50	100	100
J-162(Original)	≦0.03	6.75	5.2	2.4	0.03	400	25	25	25
J-162-C19	5.46	13.66	—	10.1	5.5	800	800	50	50
GNB-64	≦0.03	≦0.03	—	—	—	100	100	50	100

Inhibiting activity of TLM for β-lactamase production was measured by the same procedure as in Table 4.

J-169-C222: Constitutive β-lactamase producing mutant derived from the J-169 strain.

J-169-C222-L8: β-Lactamase-less mutant derived from the J-169-C222 strain.

J-162-C19: Constitutive β-lactamase producing mutant derived from the J-162 strain.

GNB-64: Naturally β-lactamase-less strain.

zyme activity nor synergistic activity with ABPC was observed on addition of TLM. The susceptibility to ABPC of J-169-C222-L8, a β-lactamase-less mutant derived from J-169-C222, was about 32 times higher than that of the parent strain, and the TLM-ABPC combination was non-synergistic. With *P. aeruginosa* J-162 (original strain) and its β-lactamase-forming mutants, results were obtained similar to those with the strain J-169 and its mutants. Furthermore, as Table 5 shows, there was not much change in the susceptibility to CBPC between the original strain and its β-lactamase forming mutants.

These data suggest that the inhibitory activity of TLM on β-lactamase production may be restricted to inducible enzymes.

Discussion

The distinctive antimicrobial activity of TLM was shown by *in vitro* tests. This antibiotic was found to be a broad spectrum antibiotic active against many species of pathogens including Gram-positive cocci, enteric bacteria, acid-fast bacteria and also anaerobic bacteria. However, its effects are generally moderate and bacteriostatic in action. The range of the MICs of clinical isolates to TLM was comparatively narrow and no cross resistance was observed between TLM and other antibiotics. With its antibacterial activity and unique chemical structure the antibiotic may have a distinctive mechanism for its effect on organisms.

Furthermore, the present investigation revealed that the production of inducible β-lactamase in several microorganisms was inhibited by TLM. As a result of this action, the combination of TLM and β-lactam antibiotics produced a synergistic activity against these inducible β-lactamase-producing microorganisms. No antibiotics other than TLM with such effects against the β-lactamase producing system have been reported yet, although several substances which directly inactivate β-lactamase, such as clavulanic acid,[9,10] have been investigated. Since many species of Gram-negative bacteria including *P. aeruginosa* (but not *K. pneumoniae etc.*) have inducible β-lactamase,[11,12] substances like TLM may be used as therapeutic agents against infections caused by such organisms, in combination with β-lactam antibiotic.

This antibiotic is a very interesting substance with several distinctive biological characteristics as revealed in this paper and by the *in vivo* tests presented in a following report.[13]

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