

THIOLACTOMYCIN, A NEW ANTIBIOTIC
IV. BIOLOGICAL PROPERTIES AND CHEMOTHERAPEUTIC
ACTIVITY IN MICE

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The new thiolactone antibiotic, thiolactomycin, is rapidly absorbed in rats when administered either orally or by intramuscular injection. A peak in concentration of the drug is reached in the blood and in various visceral organs within 15 minutes after administration. The concentration decreases rather rapidly and about 51~69% of the drug is excreted in urine during the first 24 hours.

Though the *in vitro* effect of thiolactomycin is moderate, it effectively protected mice challenged intraperitoneally with several strains of *S. marcescens* and *K. pneumoniae* and was more effective than carbenicillin in treating experimental acute urinary tracts infected with *S. marcescens*.

Also, in mice whose immunodefense was decreased by treatment with cyclophosphamide, thiolactomycin was more effective than carbenicillin against *S. marcescens* challenge.

The new antibiotic thiolactomycin has a thiolactone structure²⁾ and is produced by a species of *No-cardia*.¹⁾ In *in vitro* studies thiolactomycin was found to have broad-spectrum activity with rapid bacteriostatic action.³⁾

This paper describes the absorption, distribution, excretion and chemotherapeutic activity of thiolactomycin in mice.

Materials and Methods

Antibiotics

Thiolactomycin (TLM; purity above 95%) was made by our Research Laboratories.

Other antibiotics were purchased commercially in Japan; carbenicillin (CBPC, Fujisawa Pharmaceutical Co.), ampicillin (ABPC, Meiji Seika Co.), ceftazidime (CTZ, Chugai Pharmaceutical Co.), gentamicin (GM, Shionogi Pharmaceutical Co.) and piperacillin (PIPC, Toyama Chemical Co.).

Bacterial Strains

The bacterial strains used in this study were as follows: 3 strains of *Serratia marcescens*, 2 strains of *Klebsiella pneumoniae* and 1 strain of *Escherichia coli*. These strains were originally isolated from human but they are also virulent in mice.

Before use the strains were passed through animals several times to obtain virulent and well adapted strains. Infected tissues containing the different strains were stored in a freezer at -20°C to maintain virulence.

Each strain was cultured on Nutrient agar (NA) plates (Eiken Chemical Co.) immediately before use and the typical colonies formed were used for the challenges.

Animals

Mice: ddY/s (SPF), male and female, 5 weeks old, 20~30 g

Rats: Sprague-Dawley, male, 250~300 g

Rabbits: JC-CSK, male, 2.5~3.2 kg

Determination of Absorption, Distribution and Excretion of TLM in Experimental Animals

Groups of 10 rats were used to determine serum levels and tissue distribution. The rats were given 20 mg/kg of TLM intramuscularly or orally and sacrificed 15 to 120 minutes later. From each rat, blood was collected and allowed to clot and the serum was separated.

TLM was given to groups of 4 rats in the above manner and urine samples were collected, using "metabolic cages", at selected intervals for 24 hours after administration.

Biliary levels were determined in 4 rats. The animals were anesthetized with ether and a polyethylene tube was introduced into the bile duct by the ordinary procedure. TLM was administered intramuscularly or orally as a single dose of 20 mg/kg. Bile samples were collected at suitable intervals for 24 hours after administration.

Assays were carried out by both high performance liquid chromatography (HPLC) and microbioassay. Chromatograms were obtained using a Hitachi High Performance Liquid Chromatography (model 635; Hitachi Co., Ltd.). The chromatography was carried out under the following conditions: UV range, 238 nm; flow rate, 1.0 ml/minute; column size, $4\phi \times 150$ mm; packing, LiChrosorb RP-18 5μ ; solvent, $\text{CH}_3\text{CN} - \text{H}_2\text{O} - \text{H}_3\text{PO}_4$ (550: 450: 1).

To prepare samples for the HPLC assay, one volume of serum, urine or bile was mixed with 1~5 volumes of acetonitrile and stirred vigorously. Lung, liver, kidney, spleen and heart samples were homogenized with 1~4 volumes of acetonitrile in a Waring blender. These homogenate were then centrifuged at $1,000 \times g$ for 10 minutes and the supernatants were used for the HPLC assay.

Bioassay was carried out by the agar plate diffusion method using *P. aeruginosa* GNB-75-M50^{ss}-3, a mutant super-sensitive to TLM, as the test organism. The mutant was derived from strain GNB-75 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment.

For bioassay, the serum, urine and bile samples were used directly. The organs were homogenized with 3 volumes of 1/15 M phosphate buffered saline (pH 7.0) in a Waring blender and then centrifuged at $1,000 \times g$ for 10 minutes and the supernatants were used for bioassay.

Experimental Infections in Mice

1) Protection against systemic infection: For the challenge, 3 strains of *S. marcescens*, 2 strains of *K. pneumoniae* and 1 strain of *E. coli* were used. The strains were cultured overnight at 37°C on NA plates. The cells were then harvested and suspended in saline. The suspensions were diluted with 0.4 ml of saline or 5% gastric mucin (Difco) and 0.2 ml of the diluted suspensions were injected intraperitoneally into mice. TLM and other antibiotics were diluted 3-fold serially with saline and each dilution was administered to mice subcutaneously or orally at doses of 0.05~13.5 mg/mouse at 1 and 3 hours after the challenge injections. Each experimental group consisted of 5~10 animals.

The dose in mg/mouse required to protect 50% of the animals from death (ED_{50}) for 7 days was calculated by the BEHRENS-KÄRBER method.⁴⁾

2) Protection against experimental pyelonephritis in mice: Experimental pyelonephritis was induced by the method described by NISHI *et al.*^{5,6)} The mice were anesthetized with ether after urination had been effected by digital pressure on the bladder. Thereafter the mice bladders were inoculated transurethrally with 0.2 ml of *S. marcescens* or *K. pneumoniae* suspensions, adjusted to 2.5×10^9 and 4.6×10^9 cells/ml respectively by using injection-needles with rounded points. Immediately afterwards the orificium-urethrae-externum was closed with a KOCHER's forceps for 4 hours to inhibit urination.

Antibiotics were administered at doses of 0.5~2 mg/mouse 4 times subcutaneously 5, 7, 12 and 24 hours after the challenge. Each group consisted of 7 infected animals, with 7 untreated animals as controls.

The mice were killed 5 days after the challenge and the kidneys were removed. After the gross abscesses had been graded, the organs were homogenized with saline in a Waring blender. The number of bacteria in the kidney homogenate was assessed on agar plates.

3) Protection against systemic infections in mice with decreased host-defense: Mice with decreased host-defense were produced by treatment with cyclophosphamide (CPA) or hydrocortisone (HC). The mice were injected with 2 mg/mouse of CPA or HC intraperitoneally once a day for 4 (CPA) or 7 (HC)

consecutive days. In the CPA group the number of white blood cells (WBC) decreased during the treatment from 6,500 cells/mm³ to 1,400 cells/mm³. After 2 days the WBC count was stabilized at 64.6% of the original level. In the HC-treated group there was little change in the number of WBC and the body weights of the mice increased slowly.

Systemic infection was induced in the mice by inoculating them intraperitoneally with a test strain of *S. marcescens* 101.

The LD₅₀ of this strain for mice with experimentally decreased host defense was decreased to 1/3 to 1/6 of that in normal mice.

Protection tests with TLM, CBPC and GM in this infection system were carried out by the same procedure. TLM and other antibiotics were given subcutaneous at doses of 0.3~9 mg/mouse at 1 and 3 hours after infection.

Results

Absorption, Distribution and Excretion of TLM in Rats

Blood Levels and Tissue Distribution

Serum levels and tissue distribution in rats of intramuscularly and orally administered TLM after a single dose of 20 mg/kg are shown in Figs. 1 and 2 (HPLC assay). The highest levels in serum were found 15 minutes after intramuscular injection (49 µg/ml). During the next 30 minutes drug concentrations in serum declined at a relatively rapid rate and after 60 minutes the concentrations fell to about one-tenth of the peak level.

Distribution of TLM into tissues was also very rapid and, as in serum, the highest levels were observed after 15 minutes in all organs. The highest concentrations were found in the kidney, followed by the liver, lung, heart and spleen in that order.

It may be noted that relatively high serum levels and rapid distribution into the tissues were observed also when TLM was given orally.

There was no significant difference in results between HPLC assay and bioassay.

Fig. 1. Tissue and urinary levels of intramuscularly administered thiolactomycin in rats.

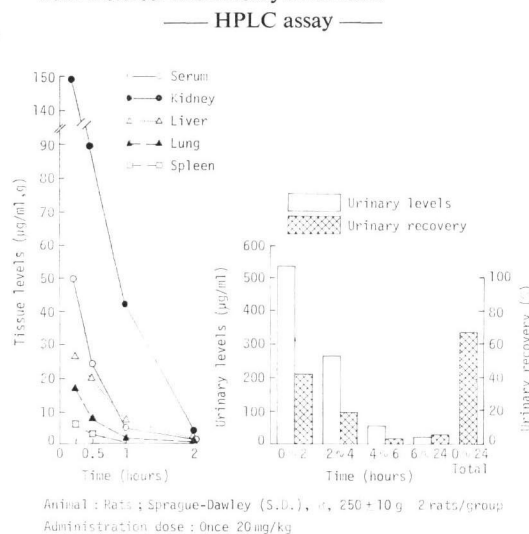
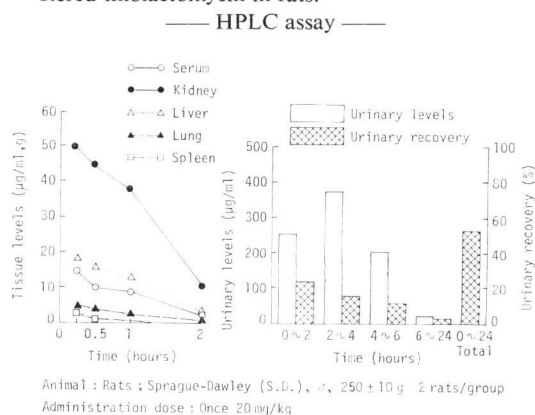


Fig. 2. Tissue and urinary levels of orally administered thiolactomycin in rats.



Urinary Excretion

Urinary excretion of the antibiotic was examined in rats administered TLM intramuscularly or orally in a single dose of 20 mg/kg.

The results are shown in Figs. 1 and 2 (HPLC assay). With intramuscular injection the highest concentrations were observed soon after administration and most of the antibiotic was excreted within

Fig. 3. Biliary excretion of thiolactomycin in rats.

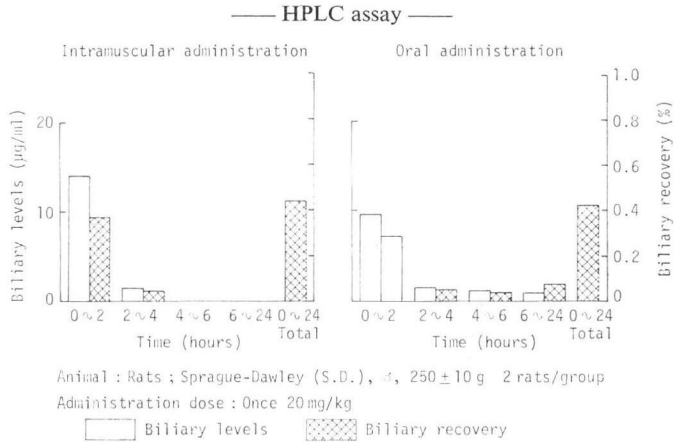


Fig. 4. The relationship between the protective effects of thiolactomycin and treatment time in mice infected intraperitoneally with *K. pneumoniae* 3K25.

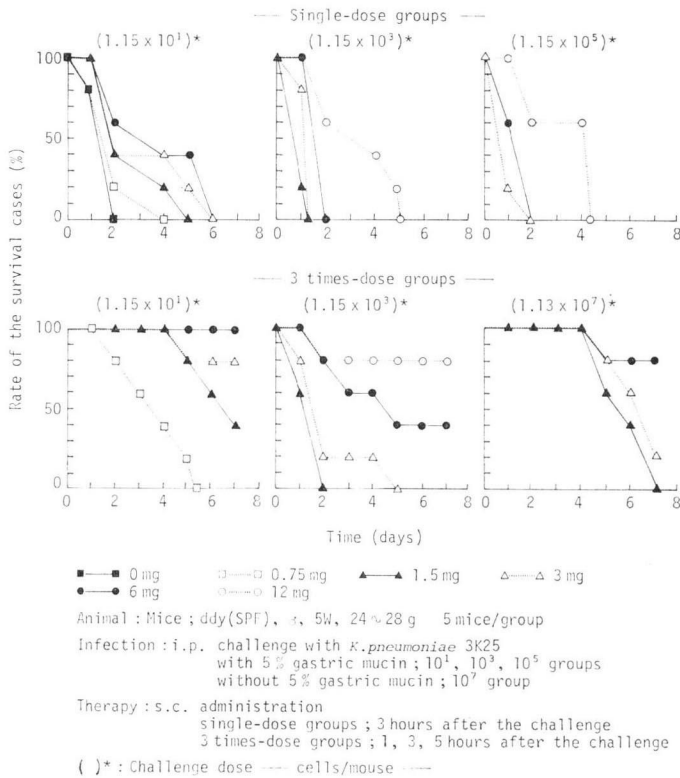


Table 1. Protective effect of thiolactomycin and some other antibiotics against systemic infection with several strains in mice.

Strains	MIC ($\mu\text{g/ml}$)				Challenge dose/mouse		5% Gastric mucin	Number of doses	ED ₅₀ (mg/mouse)				
	TLM	CBPC	ABPC	CTZ	cells/mouse	$\times \text{LD}_{50}$			TLM		CBPC	ABPC	CTZ
									s.c.	p.o.			
<i>S. marcescens</i> 101	(10 ⁶) 100 (10 ⁸) 100	12.5 25	/	/	6.8 \times 10 ⁷	13	Without	1	4.29	12.8	>8.00	/	/
<i>S. marcescens</i> 101	(10 ⁶) 100 (10 ⁸) 100	12.5 25	200 400	/	5.0 \times 10 ⁵	50	With	2	1.40*	2.80*	1.80*	5.50*	/
<i>S. marcescens</i> 92	(10 ⁶) 200 (10 ⁸) 200	800 800	800 800	/	5.0 \times 10 ³	50	With	2	\leq 1.50	4.88	>12.0	8.68	/
<i>S. marcescens</i> 5	(10 ⁶) 200 (10 ⁸) 200	3.12 6.25	12.5 50	/	3.0 \times 10 ⁴	370	With	2	2.21	6.00	0.38	0.38	/
<i>K. pneumoniae</i> 3K25	(10 ⁶) 200	/	25.0	1.56	2.0 \times 10 ⁷	10	Without	2	3.85	/	/	>27.0	1.73
<i>K. pneumoniae</i> 3K25	(10 ⁶) 200	/	25.0	1.56	10 ³	1000	With	2	7.38	12.8	>27.0	15.6	/
<i>K. pneumoniae</i> 15C	(10 ⁶) 100	/	/	/	10 ⁵	20	With	2	4.23	/	19.3	2.14	/
<i>E. coli</i> 11	(10 ⁶) 25	/	0.39	1.56	5.0 \times 10 ³	3000	With	2	4.00	/	0.13	<0.06	0.19

Animal: Mice: *ddY* (SPF), ♂, 5W, 22~27 g, 5 mice/group (*: 10 mice/group).

Infection: i.p. challenge.

Therapy: s.c. or p.o. once: 1 hour after the challenge.

twice: 1 and 3 hours after the challenge.

4 hours. It was found that 66.5% of the antibiotic was excreted in the urine in 24 hours.

When TLM was administered orally, somewhat lower urinary levels and more prolonged excretion time were observed compared to those found after intramuscular injection. The total excretion in 24 hours was 52.8% by HPLC assay.

There was no significant difference in results between HPLC assay and bioassay.

Biliary Excretion

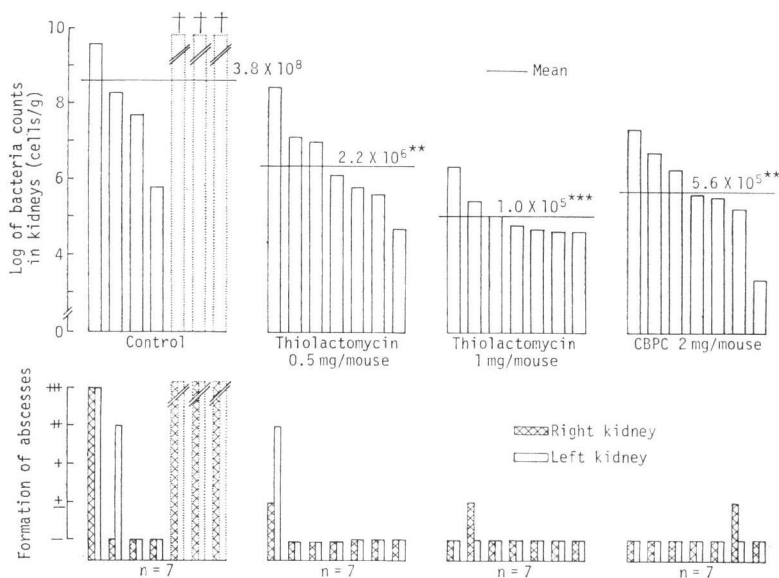
Biliary total recovery averaged only 0.42% with intramuscular and 0.41% with oral administration. Fig. 3 (HPLC assay) summarizes the results found for biliary excretion in rats receiving TLM intramuscularly or orally in a single dose of 20 mg/kg. Trace amounts of antibiotic were found in the bile soon after administration, but 4 hours later it could not be detected.

Experimental Infections in Mice

Protective Effect against Systemic Infections

The relationship between the protective effect of TLM and the time of treatment in mice infected intraperitoneally with *K. pneumoniae* 3K25 is shown in Fig. 4. The groups given TLM 3 times had

Fig. 5-a. Protective effects of thiolactomyacin and CBPC against experimental pyelonephritis induced with *S. marcescens* 101 in mice.



Animal: Mice: *ddY* (SPF). ♀, 5 W, 21 ~ 25 g, 7 mice/group.

Infection: Challenged via the urethra with *S. marcescens* 101.

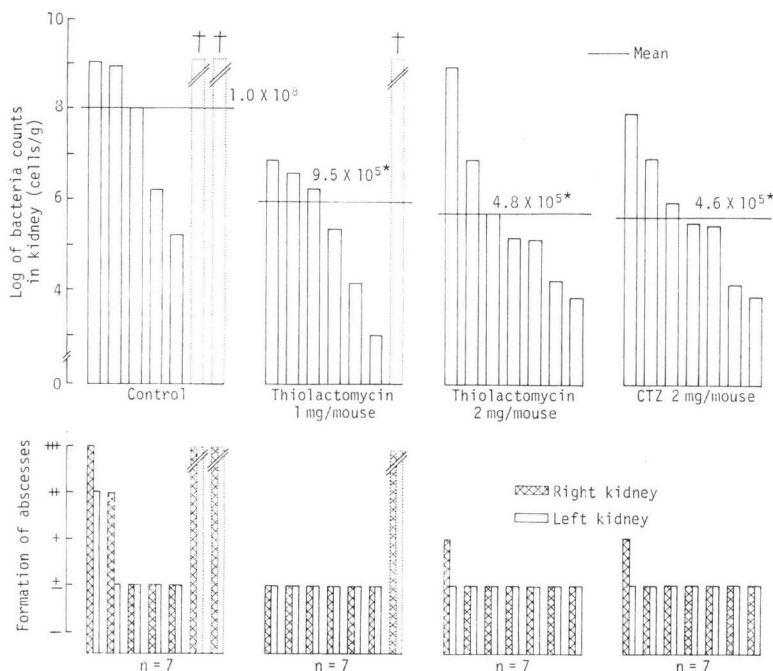
Challenge dose: 5.0×10^4 cells/mouse.

Therapy: s.c. administration four times: 5, 7, 12 and 24 hours after the challenge.

Abscess counts on a kidney	Degree of abscess formation
Normal	—
Turbid or swelling	±
1 ~ 4	+
5 ~ 10	++
> 10	+++

** : $p < 0.01$. *** : $p < 0.001$

Fig. 5-b. Protective effects of thiolactomycin and CTZ against experimental pyelonephritis induced with *K. pneumoniae* 3K25 in mice.



Animal: Mice *ddY* (SPF). ♀, 5 W, 22~24 g, 7 mice/group.
 Infection: Challenged via the urethra with *K. pneumoniae* 3K25.
 Challenge dose: 9.1×10^4 cells/mouse.
 Therapy: s.c. administration four times: 5, 7, 12 and 24 hours after the challenge.

Abscess counts on a kidney	Degree of abscess formation
Normal	—
Turbid or swelling	±
1~4	+
5~10	++
>10	+++

*: $p < 0.05$.

higher survival rates for each challenge dose than had the single-dose groups. In later protection tests the drugs were given at 1 and 3 hours.

The protective effects of TLM and the other antibiotics against systemic infections with several strains of *S. marcescens*, *K. pneumoniae* and *E. coli* are summarized in Table 1.

The ED_{50} of TLM for infection with *S. marcescens* 101 (resistant to TLM and ABPC and susceptible to CBPC) was 4.29 mg/mouse in single dose administrations and 1.40 mg/mouse in experiments with two subcutaneous doses, indicating higher therapeutic efficacy than obtained with ABPC and CBPC. Furthermore, in infections with *S. marcescens* 92, which shows resistance to all of the drugs *in vitro*, TLM was more effective than the other two antibiotics.

In mice infected with *S. marcescens* 5 (resistant to TLM and susceptible to CBPC and ABPC), the ED_{50} of TLM was 2.21 mg/mouse with subcutaneous administration, which meant somewhat less effec-

tivity than obtained with the other two antibiotics.

Although the *in vitro* activity of TLM was 1/4~1/8 of that of ABPC and CBPC for two strains of *K. pneumoniae* (3K25 and 15C) serving as challenge strains, the therapeutic effect of TLM was superior to that of CBPC and almost comparable to that of ABPC.

In the case of *E. coli* 11, a strain highly sensitive to ABPC and CTZ, the ED₅₀ varied with the difference in MIC, so that the *in vitro* activity of TLM was inferior to that of the two control antibiotics.

From the Table 1 it is evident, moreover, that there is a clear therapeutic effect on infections with several strains when the antibiotic is administered orally, and a somewhat weaker effect of subcutaneous administration.

Protective Effect against Experimental Pyelonephritis in Mice Challenged via the Urethra

Protective effects of TLM and CBPC or CTZ against experimental pyelonephritis in mice induced by *S. marcescens* 101 and *K. pneumoniae* 3K25 are shown in Figs. 5-a and 5-b.

The figures show that in the kidneys of untreated control animals there was a large number of bacteria averaging 3.8×10^7 cells/g of renal tissue for *S. marcescens* 101 and 1.0×10^8 cells/g for *K. pneumoniae* 3K25. Macroscopic examination revealed enlargement of the organs and the formation of abscesses in the renal pelvis of parenchyma.

The figures clearly indicate the inhibition of the growth of pathogens and of the formation of abscesses by treatment with TLM. After 4 administrations of the antibiotic in doses of 1 mg/mouse, for example, number of viable cells in the kidneys decreased to about 10^4 cells/g in infections with *S. marcescens* 101 and to about 10^6 cells/g in infections with *K. pneumoniae* 3K25.

The therapeutic potency of TLM was greater than that of ABPC in the case of mice infected with *S. marcescens* 101 and also in infection with *K. pneumoniae* 3K25. The effectiveness of TLM in these tests was comparable with that of CTZ.

Protective Effect against Systemic Infections in Mice with Decreased Host-defense

The protective effects of TLM, CBPC and GM against systemic infection with *S. marcescens* 101 in CPA or HC pretreated mice were investigated and the results are shown in Table 2.

The ED₅₀ of TLM in mice pre-treated with CPA was 3.25 mg/mouse, while that of CBPC was 6.96 mg/mouse. Thus, TLM was about twice as effective as CBPC. But in the case of HC pre-treatment, the ED₅₀ of TLM was equivalent to that of CBPC.

Table 2. Protective effects of thiolactomycin, CBPC and GM against systemic infection with *S. marcescens* 101 in CPA or HC pretreated mice.

Pretreatment	Challenge dose/mouse		ED ₅₀ (mg/mouse)			MIC (μg/ml)		
	cells/mouse	× LD ₅₀	TLM	CBPC	GM	TLM	CBPC	GM
CPA	1.3×10^7	50	>4.0	>4.0	0.12	(10 ⁶) 100	12.5	/
CPA	7.2×10^6	30	3.25	6.96	0.05	(10 ⁵) 100	25	/
HC	8.3×10^6	12	2.83	2.83	0.02			

Animals: Mice, ddY (SPF), ♂, 5W, 21.1~23.1 g, 5 mice/group.

Pretreatment: i.p. administration

CPA (2 mg/mouse); once a day for 4 consecutive days.

HC (2 mg/mouse); once a day for 7 consecutive days.

Infection: i.p. challenge with *S. marcescens* 101.

Therapy: s.c. administration twice; 1 and 3 hours after the challenge.

Discussion

The present study revealed that TLM is widely distributed at relatively high concentrations after intramuscular or oral administration in mice. The elimination of this compound from body fluids is relatively rapid and within a short time most of it is excreted into the urine in its active form.

Relatively good therapeutic effects of TLM were observed in mice challenged intraperitoneally and in urinary tract infections with several strains of *S. marcescens* and *K. pneumoniae*, even though its *in vitro* antibacterial activity is moderate.³⁾ Also in mice whose defense was reduced by cyclophosphamide treatment, TLM was more effective than CBPC against systemic infection.

These observations clearly show that the relative potency of TLM in severely infected mice is achieved by its rapid absorption and wide distribution into infected sites where an effective concentration of the drug is needed.

Acknowledgment

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