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 structure and is produced by a species of Nocardia. In vitro studies thiolactomycin was found to have broad-spectrum activity with rapid bacteriostatic action.

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.), ceftezole (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, 4.5 x 150 mm; packing, LiChrosorb RP-18 5 μm, solvent, CH3CN - H2O - H3PO4 (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 x 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-M50~3, 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 x 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 (ED50) 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 Nishii 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 x 108 and 4.6 x 105 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/mm\(^3\) to 1,400 cells/mm\(^3\). 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 LD\(_{50}\) 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.

--- HPLC assay ---

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

--- HPLC assay ---
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.

--- HPLC assay ---

![Graph showing biliary levels over time for intramuscular and oral administration]

Animal: Rats; Sprague-Dawley (S.D.), 250 ± 10 g, 2 rats/group
Administration dose: Once 20 mg/kg

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

--- Single-dose groups ---

(1.15 x 10^3)*

--- 3 times-dose groups ---

(1.15 x 10^5)*

Animal: Mice; ddY (SPF), 8, 8W, 24 ± 28 g, 5 mice/group
Infection: i.p. challenge with K. pneumoniae 3K25 with 5% gastric mucin; 10^3, 10^5, 10^6 groups without 5% gastric mucin; 10^7 group
Therapy: s.c. administration

( )* : Challenge dose --- cells/mouse ---

--- Oral administration ---

--- Intramuscular administration ---

Biliary levels

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (µg/ml)</th>
<th>Challenge dose/mouse</th>
<th>5% Gastric mucin</th>
<th>Number of doses</th>
<th>ED₈₀ (mg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLM</td>
<td>CBPC</td>
<td>ABPC</td>
<td>CTZ</td>
<td>cells/mouse x LD₉₀</td>
</tr>
<tr>
<td>S. marcescens 101</td>
<td>(10⁶) 100 12.5 25 / /</td>
<td>6.8 x 10⁷</td>
<td>13 Without</td>
<td>1</td>
<td>4.29 12.8 &gt;8.00 / /</td>
</tr>
<tr>
<td>S. marcescens 101</td>
<td>(10⁶) 100 12.5 200 / /</td>
<td>5.0 x 10⁵</td>
<td>50 With</td>
<td>2</td>
<td>1.40* 2.80* 1.80* 5.50* / /</td>
</tr>
<tr>
<td>S. marcescens 92</td>
<td>(10⁶) 200 800 800 / /</td>
<td>5.0 x 10⁵</td>
<td>50 With</td>
<td>2</td>
<td>=1.50 4.88 &gt;12.0 8.68 /</td>
</tr>
<tr>
<td>S. marcescens 5</td>
<td>(10⁶) 200 3.12 6.25 12.5 50 / /</td>
<td>3.0 x 10⁴</td>
<td>370 With</td>
<td>2</td>
<td>2.21 6.00 0.38 0.38 /</td>
</tr>
<tr>
<td>K. pneumoniae 3K25</td>
<td>(10⁶) 200 / 25.0 1.56</td>
<td>2.0 x 10⁷</td>
<td>10 Without</td>
<td>2</td>
<td>3.85 / / &gt;27.0 1.73</td>
</tr>
<tr>
<td>K. pneumoniae 3K25</td>
<td>(10⁶) 200 / 25.0 1.56</td>
<td>10⁸</td>
<td>1000 With</td>
<td>2</td>
<td>7.38 12.8 &gt;27.0 15.6 /</td>
</tr>
<tr>
<td>K. pneumoniae 15C</td>
<td>(10⁶) 100 / / /</td>
<td>10⁸</td>
<td>20 With</td>
<td>2</td>
<td>4.23 / / 19.3 2.14 /</td>
</tr>
<tr>
<td>E. coli 11</td>
<td>(10⁶) 25 / 0.39 1.56</td>
<td>5.0 x 10⁵</td>
<td>3000 With</td>
<td>2</td>
<td>4.00 / / 0.13 &lt;0.06 0.19</td>
</tr>
</tbody>
</table>

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

![Graph showing protective effects of thiolactomycin and CBPC against experimental pyelonephritis induced with *S. marcescens* 101 in mice.](image)

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 × 10⁴ cells/mouse.
Therapy: s.c. administration four times: 5, 7, 12 and 24 hours after the challenge.

<table>
<thead>
<tr>
<th>Abscess counts on a kidney</th>
<th>Degree of abscess formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>Turbid or swelling</td>
<td>±</td>
</tr>
<tr>
<td>1~4</td>
<td>+</td>
</tr>
<tr>
<td>5~10</td>
<td>++</td>
</tr>
<tr>
<td>&gt;10</td>
<td>+++</td>
</tr>
</tbody>
</table>

**, p<0.01. ***: p<0.001
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 ED_{50} 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 $3.8 \times 10^6$ cells/g of renal tissue for S. marcescens 101 and $1.0 \times 10^8$ 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 ED_{50} 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 ED_{50} 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.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Challenge dose/mouse</th>
<th>ED_{50} (mg/mouse)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells/mouse $\times$ LD_{50}</td>
<td>TLM</td>
<td>CBPC</td>
</tr>
<tr>
<td>CPA</td>
<td>$1.3 \times 10^7$ 50</td>
<td>$&gt;4.0$</td>
<td>$&gt;4.0$</td>
</tr>
<tr>
<td>CPA</td>
<td>$7.2 \times 10^6$ 30</td>
<td>3.25</td>
<td>6.96</td>
</tr>
<tr>
<td>HC</td>
<td>$8.3 \times 10^6$ 12</td>
<td>2.83</td>
<td>2.83</td>
</tr>
</tbody>
</table>

Animals: Mice, ddY (SPF), $\delta$, 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.
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. 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

We wish to thank Dr. S. Goto, Professor of the Department of Microbiology, School of Medicine, Toho University for supplying the clinical isolates.

References


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