# ANTIBACTERIAL ACTIVITY OF PALMITOYLTUBERACTINAMINE N AND DI-β-LYSYLCAPREOMYCIN IIA

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Palmitoyltuberactinamine N (Pal-Tua N) and di-β-lysylcapreomycin IIA (di-β-Lys-Cpm IIA), which are synthetic derivatives of the antituberculous agent tuberactinomycin (Tum) and capreomycin (Cpm) respectively, were tested for anti-bacterial activity.

Pal-Tua N inhibited not only tuberactinomycin-resistant *Mycobacterium smegmatis* but also *Escherichia coli*, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, although it has lost activity against *Mycobacterium tuberculosis*. Di-β-Lys-Cpm IIA inhibited the growth of laboratory-derived Tum-resistant *M. smegmatis* and *M. tuberculosis* as well as Tum-resistant *M. tuberculosis* from patients with one exceptional case.

Tuberactinomycin (Tum) and capreomycin (Cpm) families (Fig. 1) are known as antituberculous antibiotics. Although Pal-Tua N was easily prepared semisynthetically by the palmitoylation of tuberactinamine (Tua N), di-β-Lys-Cpm IIA had to be obtained by total synthesis. Recently, the procedure for the semisynthesis of di-β-Lys-Cpm IIA starting from commercially available Cpm has been reported[9].

Since viomycin (Vim)-resistance of mutants isolated from *Mycobacterium smegmatis* was due to altered ribosomes, it can be concluded that this antibiotic is a ribosome inhibitor[1]. Based on minimal inhibitory concentration values of more than one hundred derivatives of Tum and Cpm, the correlation between the structure and activity of these antibiotics has been discussed in previous papers[2~7]. The analogs were also tested for their inhibitory activities both on the growth of the drug-resistant *M. smegmatis* and on ribosomes derived from drug-sensitive and drug-resistant *M. smegmatis*. Pal-Tua N inhibited the growth of Vim-resistant mutants, and it was not a ribosome inhibitor. Di-β-Lys-Cpm IIA was more effective than the natural product[8]. Further study on the biological activity of Pal-Tua N and di-β-Lys-Cpm IIA was made in the work now presented.

## Materials and Reagents

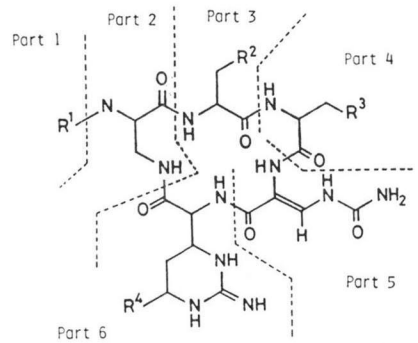
### Antibiotics and Reagents

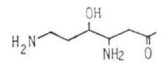
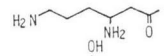
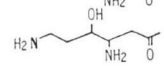
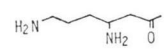
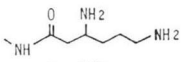
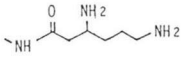
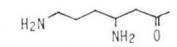
Viomycin was a product of Pfizer Ltd. (Japan), rifampicin (Rif) was purchased from Lederle Jap. Co., Ltd. (Japan). Transfer ribonucleic acid (*Escherichia coli* B) and poly(U) were obtained from Miles Laboratories, Inc., and pyruvate kinase and phosphoenolpyruvate monopotassium salt from Sigma Chemical Co. [$^{14}$C]Phenylalanine was purchased from Daiichi Pure Chemical Co., Ltd. (Japan) and [$^{3}$H]UTP (38.5 Ci/mmol) from New England Nuclear, USA.

### Synthesis of Pal-Tua N

To a suspension of Tua N·2HCl (300 mg, 0.488 mmol) in *N,N*-dimethylformamide (10 ml) were

Fig. 1. Structures of the natural Tum antibiotics.




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| Tum A |  | OH | OH | OH |
| Tum B ( Vim ) |  | OH | OH | OH |
| Tum N |  | OH | OH | H |
| Tum O |  | OH | OH | H |
| Tua N | H | OH | OH | H |
| Cpm IA | H | OH |  | H |
| Cpm IB | H | H |  | H |
| Cpm IIA | H | OH | $NH_2$ | H |
| Cpm IIB | H | H | $NH_2$ | H |
| Pseudo Cpm IB |  | H | $NH_2$ | H |

added triethylamine (54 mg, 0.537 mmol) and 1-succinimidyl palmitate (189 mg, 0.537 mmol) at room temperature. After stirring overnight, the reaction mixture was concentrated *in vacuo*. The residue obtained was then triturated with ethyl acetate to give a powder, which was reprecipitated from water - ethanol - ether: yield 356 mg (89.4%), mp 264°C (dec.).

*Anal.* Found: C 46.93, H 7.64, N 16.93, Cl 4.19.
Calcd for $C_{35}H_{61}N_{11}O_9 \cdot HCl \cdot 4.5H_2O$: C 46.84, H 7.97, N 17.17, Cl 3.95.

## Synthesis of Di-β-Lys-Cpm IIA

The semisynthesis of di-β-Lys-Cpm IIA was performed as described previously[9].

## Strains and Culture

*M. smegmatis* R15 and the Vim-resistant *M. smegmatis* R33 were used in this study[10]. *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG were obtained from ATCC and maintained in our laboratory. A Vim-resistant mutant of *M. tuberculosis* $H_{37}Rv$ was obtained by serial transfers of the culture to media containing increasing amounts of Vim. Vim-sensitive and -resistant strains of *M. tuberculosis* derived from patients were obtained through Drs. H. MAEDA and Y. YAMAMURA from Toneyama National Hospital, Osaka, Japan. *Corynebacterium diphtheriae* PW 8 was obtained from T. UCHIDA in our institute. *Escherichia coli* HAK10 was generously given by Dr. M. KUWANO (Ooita Medical School). The

other microbial strains were obtained from the laboratory culture collection in our institute. The culture medium for *E. coli* HAK 10, *M. smegmatis, Shigella dysenteriae, Klebsiella pneumoniae, Vibrio cholerae, Bacillus subtilis* and *Staphylococcus aureus* contained the following ingredients per liter of distilled water: nutrient broth 10 g, NaCl 2 g, and glycerol 40 ml. *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG were grown in Kirchner medium containing potassium phosphate (monobasic) 4 g, sodium phosphate (dibasic $12H_2O$) 3 g, citric acid 2.5 g, L-asparagine 5 g, magnesium sulfate 0.6 g, glycerol 20 ml, and calf serum albumin (Eiken Chem.) 50 ml, per liter pH, 7.0. *C. diphtheriae* was grown in CY medium as described in the reference[11]. *Vibrio parahaemolyticus* was cultured in heart infusion broth (Difco 0038) supplemented with sodium chloride at 3% final concentration.

*M. tuberculosis* and *M. bovis* BCG were cultured at 37°C without shaking for 14 days. An aliquot of the culture was taken and homogenized with glass beads by shaking. The optical density at 590 nm of the suspension was adjusted to 0.02 by adding fresh medium. Of these suspensions, 0.5 ml was added to 4.5 ml of the fresh medium containing 2.5, 5.0, 7.5, 10, 20, 40, 80, and 160 $\mu$g per ml of the drugs. The minimum concentrations of the drugs which gave no visible growth after incubation at 37°C without shaking for 14 days were noted (MIC).

*E. coli, M. smegmatis, S. dysenteriae, K. pneumoniae, V. cholerae, V. parahaemolyticus, B. subtilis, S. aureus,* and *C. diphtheriae* were grown at 37°C for 1 ~ 2 days. When optical density at 590 nm reached 1.0, 0.1 ml of the culture was added to 1.0 ml of fresh medium containing 2.5, 5.0, 7.5, 20, 40, and 80 $\mu$g per ml of the drugs. The minimum concentrations which gave no visible growth after incubation at 37°C for 2 days were noted.

Cell-free System

Ribosomes and supernatant fluid were prepared and poly(U)-directed polyphenylalanine synthesis was carried out as described previously[1].

*In Vitro* Transcription System

For the *in vitro* assay of transcription catalyzed by purified RNA polymerase, the reaction mixture (0.105 ml) contained 40 mM Tris-HCl buffer (pH 7.8), 6 mM $\beta$-mercaptoethanol, 60 mM $NH_4Cl$, 10 mM MgOAc, 0.2 mM $Na_2EDTA$, 2.2 mM ATP, 0.3 mM each of GTP, CTP, and [$^3$H]UTP (0.4 mCi/$\mu$mol), 1 mM $K_2HPO_4$, 4 $\mu$g of plasmid colicine E1 DNA and 1 $\mu$g of RNA polymerase. After incubation of the reaction mixture at 37°C for 20 minutes, the reaction was halted by treatment with 5% TCA solution at 0°C for 30 minutes and the reaction mixture was filtered slowly through a glass microfiber filter GF/C (Whatman Biochemicals, Ltd.). The residue on the filter was washed with 5% TCA solution, dried and counted in toluene-based scintillation fluid. RNA polymerase was purified from *M. smegmatis* and *M. bovis* BCG described by GROSS *et al*[12].

For the preparation of plasmid DNA, cleared lysate was prepared according to the method of CLEWELL and HELINSKI[13]. The plasmid DNA was extracted with chloroform - *iso*-amyl alcohol (24: 1), precipitated with ethanol, and purified by ethidium bromide-CsCl density gradient centrifugation. The purified DNA was dialyzed against 10 mM Tris-HCl (pH 7.8) containing 1 mM $Na_2EDTA$.

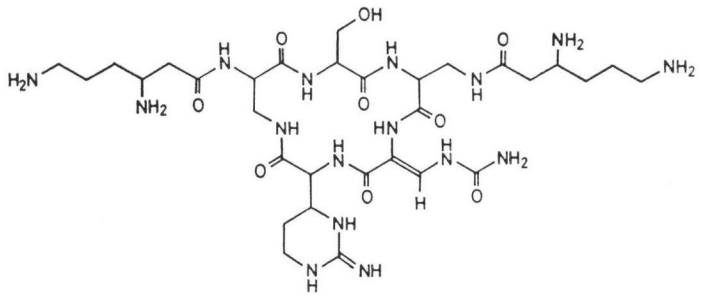
## Results and Discussion

### The Inhibitory Action of Pal-Tua N against Various Microorganisms

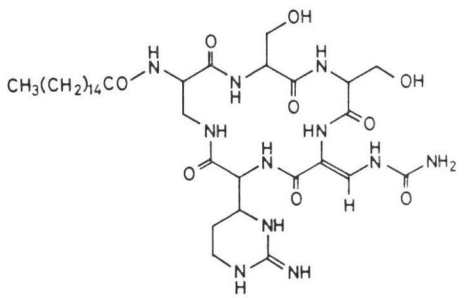
The structures of Tum, Pal-Tua N and di-$\beta$-Lys-Cpm IIA are presented in Figs. 1 and 2. Minimal inhibitory concentration values for Pal-Tua N and Vim against a variety of microorganisms are compared in Table 1. It has been known that Vim is more active against mycobacteria than against other bacterial species. Indeed, it can be seen in Table 1 that Vim is especially active against *M. smegmatis* and *M. tuberculosis*. Vim was also considerably active against *B. subtilis* 6633 and *K. pneumoniae* Sl-101. However, it was not active against any other bacterial species tested in this study (Table 1).

In contrast, Pal-Tua N, which was prepared semisynthetically by replacement the $\beta$-lysine moiety of Tum N with palmitic acid, turned out to be more active against most of the other bacterial species tested

Fig. 2. Structures of di-β-Lys-Cpm IIA and Pal-Tua N.



Di-β-Lys-Cpm II A



Pal-Tua N

Table 1. The antimicrobial spectra of Pal-Tua N.

| Test organisms | MICs (μg/ml) | |
|---|---|---|
| | Pal-Tua N | Vim |
| *Shigella dysenteriae* RIMD 3101010 | 10 | ND |
| *Escherichia coli* HAK 10 | 7.5 | >40 |
| *Klebsiella pneumoniae* RIMD 1102001 | 20 | 7.5 |
| *Vibrio cholerae* T19767 | 10 | 20 |
| *Vibrio parahaemolyticus* RIMD 2210048 | 20 | >40 |
| *Corynebacterium diphtheriae* PW 8 | 5 | 40 |
| *Bacillus subtilis* RIMD 0225015 | 7.5 | 7.5 |
| *Staphylococcus aureus* RIMD 3109007 | 7.5 | 20 |
| *Streptococcus pyogenes* M-type RIMD 3126010 | 7.5 | >80 |
| *Mycobacterium smegmatis* Rabinowitchi 15 | 5 | 5 |
| *Mycobacterium smegmatis* Rabinowitchi 33 | 5 | 40 |
| *Mycobacterium bovis* BCG | >20 | 5 |
| *Mycobacterium tuberculosis* $H_{37}Rv$ | >40 | 5 |

Table 2. Effects on polypeptide synthesis and RNA synthesis of Tum derivatives in cell-free system.

| Drugs | Polypeptide synthesis[a] % inhibition | | RNA synthesis[b] % inhibition |
|---|---|---|---|
| | *M. smegmatis* R15 | *M. smegmatis* R33 | *M. bovis* BCG |
| Tum N | 95 | 53 | 0 |
| Tua N | 90 | 18 | 0 |
| Pal-Tua N | 2 | 0 | 0 |
| Di-β-Lys-Cpm IIA | 96 | 89 | 0 |
| Rifampicin | ND | ND | 94 |

[a] The part of the results are taken from YAMADA *et al.*[12]
Percent inhibition was determined at 1 μg and 5 μg per ml of each drug for *M. smegmatis* R15 and *M. smegmatis* R33 respectively.

[b] Percent inhibition was determined at 10 μg per ml of each drug.

in this study but not against *M. bovis* BCG and *M. tuberculosis* $H_{37}Rv$. The reason for this observation is not clear at the present time. A possibility that palmitic acid formed from Pal-Tua N through hydrolysis by *N*-acylamino acid acylase might effect the growth of *M. smegmatis*[14] is not likely, because palmitic acid inhibited the growth of *M. smegmatis* only at more than 20 $\mu$g/ml, so that the molar ratio of MICs of palmitic acid and Pal-Tua N should be approximately 20. The mode of action of Pal-Tua N was investigated (Table 2). Interestingly, Pal-Tua N could not affect poly(U)-directed polyphenylalanine synthesis, whereas Vim did so as reported previously[8]. Experiments *in vitro* with purified RNA polymerase from *M. bovis* BCG and *M. smegmatis* indicated that RNA synthesis was not affected with Pal-Tua N. Electron-microscopic analysis did not show any difference between the drug-treated cells and controls without the drug. Therefore, the mechanism whereby Pal-Tua N treatment results in inhibition of bacterial growth remains to be elucidated.

### The Inhibitory Action of Di-$\beta$-Lys-Cpm IIA against Mycobacteria

Since it was observed that di-$\beta$-Lys-Cpm IIA was active against Vim-resistant non-pathogenic *M. smegmatis*[8], the Vim-resistant mutant isolated from pathogenic *M. tuberculosis* was tested for susceptibility to the former drug.

As shown in post 1 of Table 3, the drug-resistant *M. tuberculosis* was susceptible to di-$\beta$-Lys-Cpm IIA as well as was the sensitive parental strain. It can be concluded that di-$\beta$-Lys-Cpm IIA is active even against the Vim-resistant mutant isolated from *M. tuberculosis* in the laboratory. In the cell-free system derived from *M. smegmatis*, poly(U)-directed polyphenylalanine synthesis was affected more by di-$\beta$-Lys-Cpm IIA than by any natural Tum, in contrast to Pal-Tua N (Table 2).

Since the mechanism of the drug resistance at the molecular level could be different between laboratory-derived mutants and clinical isolates of *M. tuberculosis*, an attempt to examine the latter strains for their di-$\beta$-Lys-Cpm IIA sensitivity was carried out. Vim-resistant strains were purified either on plates containing Vim or by isolation of one colony on plates without the drug. The MICs were examined and presented in part 2 of Table 3. It became clear that Vim-resistant *M. tuberculosis* from patients were also susceptible to di-$\beta$-Lys-Cpm IIA with only one exception. The observation prompted us to explore further the biochemical mechanism of drug-resistance of such mutants from patients. The work along this line is now in progress.

As to structural and functional relationship of Tum, the $\beta$-Lys moiety is not essential for inhibiting polypeptide synthesis but is involved in its antibacterial activity. Thus, Pal-Tua N, which has palmitic

Table 3. MICs of di-$\beta$-Lys-Cpm IIA for *Mycobacterium tuberculosis*.

| Strains | | | MICs ($\mu$g/ml) | | |
|---|---|---|---|---|---|
| | | | Di-$\beta$-Lys-Cpm IIA | Vim | Tua N |
| Experiment 1 | *M. tuberculosis* $H_{37}Rv$ | Parental | 5 | 5 | 5 |
| | | Vim-resistant | 5 | >40 | |
| Experiment 2 | *M. tuberculosis* (clinical isolates) | Vim-sensitive (1) | 5 | 5 | |
| | | Vim-resistant (2) | | | |
| | | Purified with VM (2–1) | 20 | ≥20 | |
| | | Purified without VM (2–2) | 20 | 20 | |
| | | Vim-resistant (3) | 5 | 20 | |
| | | Vim-resistant (4) | 20 | >40 | |
| | | Vim-resistant (5) | 20 | >40 | |
| | | Vim-resistant (6) | 5 | 10 | |

acid replaced at the position of β-lysine, seems to have antibacterial activity in a manner different from natural Tum. Addition of one more β-lysyl moiety to $R^3$ of Tum O in Fig. 1, strengthens the ability to inhibit polypeptide synthesis, resulting in prohibiting the mycobacterial growth. The molecular manipulation of the β-lysyl residue, therefore, might open the way for preparing drugs with more activity or different activity.

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