Formation of an Antibacterial Metabolite from a New Macrolide Compound 23-O-Benzyl-5-mycaminsosyl-tylonolide (TMC-101), by a Hepatic Microsomal Drug-Metabolizing Enzyme System

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Upon incubation of 23-O-benzyl-5-mycaminsosyl-tylonolide (TMC-101) with liver microsomes in the presence of an nicotinamide adenine dinucleotide phosphate-generating system, at least four metabolites were formed: two of them were also formed by an enzyme(s) in rat serum. One of the metabolites formed by liver microsomes possessed antibacterial activity comparable to TMC-101 as examined by bioautography using Micrococcus luteus ATCC 9341 as a tester strain. Incubation of TMC-101 with rat serum degraded most of the parent compound and did not form the active metabolite. The capacity of liver microsomes to produce the active metabolite was increased by pretreatment of rats with 3-methylcholanthrene, phenobarbital and polychlorinated biphenyl. The metabolite with the antibacterial activity was not estimated to be formed by the N-demethylation of TMC-101, and was chemically unstable.

Keywords — mycaminsosyl-tylonolide; microsome; cytochrome P-450; active metabolite; bioautography; antibacterial activity

Introduction

In general, chemical compounds including drugs are metabolized by a number of hepatic enzymes to yield pharmacologically inactive metabolites, which are subsequently excreted in urine and feces. Metabolites formed by the same enzyme system sometimes show pharmacological activities, which are more potent and less toxic.¹ The concept of the formation of active metabolites is applicable to new drug development.

Because of a characteristic bactericidal or bacteriostatic spectrum, many macrolide antibiotics have been developed and are still in medical use. Some of the antibiotics, including macrolide antibiotics, have been known to undergo biotransformation in the liver. In some cases, the metabolites are believed to be the cause of their toxicity. Triacytylelamycin and erythromycin, 14-membered ring macrolides, are activated by a specific form(s) of cytochrome P-450 in liver microsomes and bind to the cytochrome to form an inactive complex.²-⁵ This complex formation may also be the cause of the toxicity of the antibiotics involving hepatitis. Such toxicity is not known in a macrolide possessing a 16-membered ring. Of derivatives of 16-membered ring macrolides currently developed in Tokyo-jyozzo Research Institute, 23-O-benzyl-5-mycaminsosyl-tylonolide (TMC-101) (Fig. 1) shows high antibacterial activities. Despite the high antibacterial activities of TMC-101, this compound could not be developed for medical

Fig. 1. Chemical Structure of Macrolide Antibiotics TMC-101

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use as a new macrolide antibiotic because of its toxicity. The intraperitoneal injection of TMC-101 to mice at a dose of 100 mg/kg raised the activities of serum transaminases and caused the death of all mice within 6 h.

This study was thus initiated to develop an easy method which allows us to find active metabolites employing TMC-101 as an example. We show here that TMC-101 is metabolized to yield an active metabolite by liver microsomes in the presence of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system.

Materials and Methods

TMC-101 was a gift from Dr. Saito of Toyojyozo Research Lab. Nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast, Tokyo. Nutrient agar, agar granulated and nutrient broth were purchased form BBL Maryland. Heart infusion broth was purchased from Difco Lab., Detroit, Michigan. 3-Methylcholantherene (3-MC) and phenobarbital (PB) were purchased from Sigma. Polychloricbiphenyl (PCB, Kaneclor KC-500) was a gift from Kanegafuchi Chemicals, Osaka. Male Sprague-Dawley rats (10—12 weeks) were purchased from Sankyo Laboratories, Tokyo. Micrococcus luteus ATCC 9341 was kindly provided by Dr. Nakajima of the Hokkaido Institute of Pharmacy.

When necessary, male Sprague-Dawley rats were given an intraperitoneal injection of PCB in corn oil at a dose of 300 mg/kg and sacrificed 5 d later. PB was given in drinking water at a concentration of 0.8% for 3 d and the rats killed on the 5th day. 3-MC in corn oil was injected intraperitoneally once every two days for 5 d at a dose of 25 mg/kg. The rats were killed 2 d after the last injection. All rats were killed by a blow on the head and decapitated. Livers were immediately removed and homogenized with 1.15% potassium chloride. Liver microsomes were prepared by sequential centrifugation at 9000 g for 20 min and subsequently at 105000 g for 60 min. The amounts of microsomal protein were determined by the method of Lowry et al., using bovine serum albumin as the standard.

A typical incubation mixture consisted of 3 mg of microsomal protein, 0.1 M sodium, potassium phosphate buffer (pH 7.4), 0.5 mM TMC-101 (dissolved in 5 μl acetonitrile), and an NADPH-generating system (5 mM magnesium chloride, 0.5 mM NADP, 5.0 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase) in a final volume of 1 ml. The mixture was incubated at 37 °C for 15 min aerobically and then stopped by addition of 1 ml of methanol. This solution was centrifuged at 3000 rpm for 10 min. The 10 μl portion of the supernatant was subjected to high-performance liquide chromatography (HPLC, Toyo soda HLC 803D) equipped with a UV-8 model II spectrophotometer and an Inertil ODS column (i.d. 5 μm, 4.6 × 250 mm, Gasukuro Kogyou Inc.). A solvent system of 0.05 M NaH₂PO₄ (pH 2.0): CH₃CN (3:2) was used at a flow rate of 1.0 ml/min. The eluate was monitored at 280 nm. The retention times were as follows: metabolite a (7.5 min), b (8.7 min), c (16.3 min), and d (18.5 min), respectively. The retention time of TMC-101 was 20.0 min. A preparative separa-

![Fig. 2. HPLC Analysis of the Metabolites of TMC-101 Produced by Liver Microsomes from Untreated Rats](image-url)
tion of TMC-101 and the metabolites was performed using the same HPLC system, except that a preparative column (Lobar column, Lichroprep RP-8, i.d. 40—63 µm, 15 x 250 mm, Merck) was used. A flow gradient was attained from 2.5 to 4.5 ml over 15 min using 100 mM HCl—CH₃CN (1:1) as the solvent. The 10 µl portion of the remaining extract was spotted on a silica gel thin layer plate (DC-Alufolien Kieselgel 60 F254 0.25 mm thick, Merck), which was developed with a solvent system consisting of dichloromethane—methanol—30% ammonia water (10:1:0.1 v/v). The thin layer plate was subjected to bioautography using Micrococcus luteus ATCC 9341 as a tester strain. For bioautography, the thin layer plate was

Fig. 3. HPLC Analyses of TMC-101 and Its Metabolites Incubated in Various Conditions

Liver microsomes from PCB-treated rats and serum from untreated rats were used in these experiments. A: Incubated with microsomes in the presence of the NADPH-generating system. B: Incubated with microsomes in the absence of the NADPH-generating system. C: Incubated with microsomes in the presence of the NADPH-generating system but stopped immediately after addition of the NADPH-generating system. D: Incubated with rat serum (100 µl) in the presence of the NADPH-generating system. E: Incubated with rat serum without the NADPH-generating system. F: Incubated with serum and the NADPH-generating system but stopped immediately after addition of the NADPH-generating system. a, b, c, d: metabolites, e: TMC-101.
placed upside down on the surface of a nutrient broth plate seeded with *Micrococcus luteus* ATCC 9341. After 30 min's contact, the layer plate was stripped off and the nutrient broth plate was incubated overnight at 37 °C. Control experiments were conducted with silica gel thin layer plates without spotting samples or with spotting TMC-101 as a positive control.

The incubation mixture (1 ml) for examination of the N-demethylation of TMC-101 and erythromycin consisted of 100 mM sodium, potassium phosphate buffer (pH 7.4), 0.05 mM ethylenediaminetetraacetic acid (EDTA), 3 mg of microsomal protein, and NADPH-generating system as described above, and a substrate (0.5 mM erythromycin or TMC-101). The reaction was initiated by addition of the NADPH-generating system, and terminated by the addition of trichloroacetic acid after incubation for 15 min at 37 °C. The N-demethylation was estimated by measuring formaldehyde formed by the method of Nash. 8)

The amino and formyl groups of TMC-101 and the metabolites were detected by spraying the thin-layer plate with ninhydrin reagent and 2,4-dinitrophenyl hydrazine, respectively, with the heating.

**Results**

When TMC-101 was incubated with liver microsomes from untreated rats, four metabolites were formed as detected by HPLC (Fig. 2). When TMC-101 was incubated with rat liver microsomes in the absence of an NADPH-generating system, metabolites a, b, and c were formed but d was not formed (Figs. 3A and B). Addition of methanol immediately after the start of incubation resulted in forming only trace amounts of metabolites (Fig. 3C). When TMC-101 was incubated with rat serum, metabolites a and b were formed (Fig. 3D). The metabolites appeared to be formed by hydrolysis of TMC-101, but the exact mechanisms are not known at present. These metabolites were formed by rat serum without aid of the NADPH-generating system (Fig. 3E), and were probably not formed as the result of spontaneous degradation of TMC-101 since these metabolites were detectable after incubation with rat serum (Fig. 3F) and were not detectable without addition of the serum (not shown).

Judging form data shown in Fig. 3, it was assumed that only the metabolite d was produced by a microsomal NADPH-dependent enzyme(s).

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Fig. 4. Effect of the Inducer of Drug-Metabolizing Enzymes on the Metabolism of TMC-101 by Liver Microsomes
A: Incubated with liver microsomes from untreated rats. B: Incubated with liver microsomes from 3-MC-treated rats.
C: Incubated with liver microsome from PCB-treated rats. D: Incubated with liver microsomes from PB-treated rats.
Among enzymes known so far, cytochrome P-450 is the best known which catalyzes oxidation of drugs. Cytochrome P-450 is also known as one which can be induced by xenobiotics such as PB and 3-MC.\textsuperscript{9,10} Therefore, the effects of pretreatment of rats with PB, 3-MC and PCB on the formation of metabolite d were examined. As shown in Fig. 4, the activities of liver microsomes to form the metabolite d were increased by all inducers examined, while the extent of the activity was greater when rats were pretreated with PB or PCB.

The antibacterial activities of these metabolites produced by liver microsomes from PCB-treated rats were measured by bioautography using \textit{Micrococcus luteus} ATCC 9341 as a tester strain. The resulting autogram is shown in Fig. 5. The spots, A and B, were extracted separately from the silica gel plate, and analyzed by HPLC. The A and B were confirmed as TMC-101 and the metabolite d, respectively, and also showed no contamination with each other. Metabolites a, b and c were not recognized to possess antibacterial activities as shown in Fig. 5. The sizes of the inhibitory ring of TMC-101 and the metabolite d in the bioautography assay were almost equal. When calculated from the ratio of peak area in the chromatogram of HPLC, the metabolite showed an antibacterial activity similar to TMC-101. Although not shown, we did not find any detectable antibacterial activities for metabolites a, b and c, which were isolated by HPLC.

To ascertain the structure of metabolite d, we tried to isolate the metabolite d using preparative thin-layer chromatography and alumina column chromatography. However, because of the extreme instability of the metabolite d after isolation, we could not obtain any definite information on the chemical structure of the metabolite by instrumental analysis, including nuclear magnetic resonance (NMR) and mass spectrometry. The sites in the structure of TMC-101 susceptible to metabolic reaction may be the dimethylamino group on the sugar and the formylmethyl group on the macrolide ring.

\begin{table}
\centering
\caption{N-Demethylation of Erythromycin and TMC-101 by Rat Liver Microsomes}
\begin{tabular}{l|l}
\hline
N-Demethylation (nmol/min/mg protein) & Erythromycin & TMC-101 \\
\hline
12.4 & 2.8 \\
\hline
\end{tabular}
\end{table}

Each value represents the average of duplication determination.

Fig. 5. Bioautogram of TMC-101 and Its Metabolite A, TMC-101; B, metabolite d.
Erythromycin is reported to be demethylated by a form of cytochrome P-450 which is inducible by glucocorticoids. Thus, the demethylation of TMC-101 by liver microsomes was examined to determine if the metabolite d was formed by the demethylation. The results shown in Table I indicated that TMC-101 was N-demethylated but to a much lower extent than was expected. TMC-101 was metabolized to form the metabolite d fairly rapidly as can be assumed by the results shown in Fig. 4. In addition, from a ninhydrin test it was confirmed that the amino group was not changed (not shown). Oxidation of the formyl group did not occur since the reactivity of this group with 2,4-dinitrophenylhydrazine remained unchanged in the metabolite. Therefore, it seemed that biotransformation of TMC-101 occurred at a site other than the dimethyl group of TMC-101.

Discussion

Regarding in vivo as compared to in vitro situations, the concentrations of drugs at the active site of drug metabolizing enzymes are remarkably low, thus possibly leading to the formation of a limited number of metabolites. In other words, one may detect a larger number of metabolites upon incubation of liver microsomes with a high concentration of a drug, while the same number of metabolites, except those formed by the phase II reactions, may not be detected in urine or feces after administration of the same drug. In addition, most of the metabolites in excreta are conjugates which are generally pharmacologically inactive. In the present study, we incubated TMC-101 with liver microsomes and the NADPH-generating system. The incubation resulted in producing the metabolite d, which showed potent antibacterial activity. Thus, we can recommend the use of liver microsomes as an enzyme preparation if one wants to find any active metabolites in a newly developed compound.

In the present study, the formation of active metabolite d was shown to be catalyzed by enzymes in liver microsomes. The enzymes were strongly induced by treatment of rats with PB and PCB. The enzymes required NADPH. These lines of evidence suggest that the enzyme involved in the formation of metabolite d is cytochrome P-450, although we have not obtained exact evidence on the involvement of cytochrome P-450 in the reaction.

Metabolites a and b were formed by enzymes in liver microsomes but more efficiently by enzymes in rat serum. Since the enzymes, in catalyzing the formation of a and b in liver microsomes, did not require NADPH for their activities, the reaction must not be catalyzed by cytochrome P-450.

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