Note

Comparative Metabolism of Milbemycin A₃ and A₄ in Rat Liver Microsomes

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(Received November 25, 1991;

Accepted April 9, 1992)

INTRODUCTION

A class of milbemycins possessing a 16-membered macrolide structure have been isolated and identified as products of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*.^{1–5)} This series of products have high insecticidal and acaricidal activities, but lack antifungal or antimicrobial activities.

A mixture of milbemycin A₃ (M.A₃) and milbemycin A₄ (M.A₄) has been developed for agricultural use as milbemectin (M.A₃/M.A₄=3/7), which has high acaricidal activity.

In vivo metabolism studies of M.A₃ and M.A₄ in rats using ³H-labeled preparations have been reported.⁶⁾ Although no essential difference was observed in the metabolic fates, dissipation of M.A₃ in the rat tissues appeared to be faster than that of M.A₄. This result suggested that M.A₃ was metabolized faster than M.A₄, leading us to *in vitro* metabolism studies to confirm whether the metabolic rates of M.A₃ and M.A₄ were different. The studies also involved the identification of main metabolites of M.A₃ and M.A₄, and tentative characterization of an enzyme responsible for metabolizing them in rat liver microsomes.

MATERIALS AND METHODS

1. Chemicals

M.A₃ and M.A₄²⁾ were purified by High-performance Liquid Chromatography (HPLC) to be more than 98%.

2. Preparation of Rat Liver Microsomes

Male Wistar rats weighing ca. 180 g were

sacrificed by decapitation. The liver was removed immediately, washed with saline and weighed. The procedures described below were carried out at 0 to 5°C. After homogenization to 25% in 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl in a Potter Elvehjem homogenizer with a Teflon pestle, the homogenate was centrifuged at 9000 × *g* for 20 min. The 9000 × *g* supernatant was centrifuged at 105,000 × *g* for 60 min to obtain a microsomal fraction, which was resuspended in the same buffer. Protein concentration of the microsomal fraction was determined spectrometrically at 750 nm according to the method of Lowry *et al.*⁷⁾

3. Incubation

A mixture consisting of M.A₃ or M.A₄ dissolved in 40 μl of methanol, 2 ml of 0.2 M phosphate buffer (pH 7.4) containing 2% bovine serum albumin (BSA), 0.5 ml of 2.4 mM NADP, 0.5 ml of 29 mM glucose-6-phosphate, 0.5 ml of glucose-6-phosphate dehydrogenase (10 unit/ml) and 40 μl of 0.1 M MgCl₂ was preincubated for 2 min. After addition of 0.5 ml of the microsomal fraction, a sample was aerobically incubated with shaking at 37°C for 10 and 15 min for M.A₃ and M.A₄, respectively. The reaction was terminated by addition of 3 ml of acetone. The incubation mixture was extracted with ethyl acetate/hexane (1/1, v/v) after evaporation of acetone. The ethyl acetate/hexane extracts were evaporated and subjected to TLC and HPLC for isolation and analysis of metabolites.

4. Pretreatment with PB and 3-MC

Male rats were subcutaneously treated with an aqueous solution of phenobarbital (PB) at 70 mg/kg or a suspension of 3-methylcholanthrene (3-MC) at 30 mg/kg once daily for three days. Liver microsomes of induced rats were prepared in the same manner as described above.

5. TLC

Precoated silica gel 60F₂₅₄ plates (0.25 mm thickness, Merck) and solvent systems (A) ethyl acetate/hexane (1/1, v/v) and (B) benzene/methanol (9/1, v/v) were used for separation of metabolites.

6. HPLC

Cosmosil 5-C₁₈ columns (4.6 mm i.d. × 150 mm and 10 mm i.d. × 250 mm, Nakarai Chemicals)

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were used for both analysis and separation of metabolites. The apparatus used were a Waters 6000 A pump, a Waters 440 UV absorbance detector at 254 nm and a Shimadzu Chromatopac C-R4A integrator at a flow rate of 1.0 or 2.0 ml/min with methanol/water (85/15, v/v).

7. Spectroscopy

Electron impact mass (MS) spectra were obtained with a Nippon Denshi (JMS-D300) mass spectrometer using a direct insertion probe, and ^1H NMR spectra with a Nippon Denshi (JNM-GX 270) FT NMR spectrometer in CDCl_3 .

RESULTS AND DISCUSSION

1. Structure of Metabolites

A predominant metabolite and several minor metabolites were detected besides the parent compound in ethyl acetate/hexane extracts after incubation of M.A_3 or M.A_4 with rat liver microsomes. Main metabolites of M.A_3 and M.A_4 were isolated and purified by TLC and HPLC. Table 1 shows R_f values on TLC and R_t (min) on HPLC of the main metabolites. Minor metabolites were in limited amounts when the incubation was terminated in the examined time.

The diagnostics ion structures of MS spectra and the ^1H NMR spectra of milbemycins have been reported^{3,5)} and were available for characterization of metabolites. The MS spectrum of the main metabolite of M.A_4 gave an ion at m/z 558, as shown in Fig. 1, indicating the metabolite was a mono-oxygenated compound because of the increase of 16 mass unit compared with M.A_4 (m/z 542). Fragment ions at m/z 279 and 195 provided certain information that the

metabolite of M.A_4 was hydroxylated at 13-position because the structure of these ions could be proposed as illustrated in Fig. 1.

The ^1H NMR spectrum of the metabolite of M.A_4 showed a characteristic doublet signal at 3.72 ppm (1H, $J=9.8$ Hz) which was assigned to be a proton at 13-position. The doublet signal was due to the coupling with a proton at 12-position.

These spectral data indicated that the main metabolite of M.A_4 was 13(β)-hydroxylated M.A_4 (13-OH- A_4), as illustrated in Fig. 2. The 13(β)-hydroxylated M.A_4 was also identified as one of the products by microbial conversion of M.A_4 . The ^1H NMR and MS spectra of 13(β)-hydroxylated M.A_4 , which have been described in detail,⁸⁾ were fully identical with those of the *in vitro* metabolite of M.A_4 . The main metabolite of M.A_3 was also identified as 13(β)-hydroxylated M.A_3 (13-OH- A_3).

Table 1 R_f values on TLC and R_t (min) on HPLC of main metabolites of M.A_3 and M.A_4 .

	Metabolite of M.A_3	Metabolite of M.A_4	M.A_3	M.A_4
R_f value ^{a)}	0.24	0.28	0.58	0.61
R_t (min) ^{b)}	3.2	3.8	10.8	14.0

^{a)} Solvent system: ethyl acetate/hexane (1/1, v/v).

^{b)} Column for HPLC: Cosmosil 5- C_{18} (4.6×150 mm), Mobile phase: methanol/water (85/15, v/v) at a flow rate of 1.0 ml/min.

Fig. 1 EI mass spectrum of the main metabolite of M.A_4 in rat liver microsomes and proposed structures of fragment ions at m/z 279 and 195.

Fig. 2 Metabolism of M.A₃ and M.A₄ in rat liver microsomes.

2. Kinetic Parameters

The hydroxylation rates at the 13-position of M.A₃ and M.A₄ were measured in rat liver microsomes. Apparent kinetic parameters of 13-hydroxylation were estimated from the Lineweaver-Burk plots. Figure 3 illustrates the plots and Table 2 the apparent K_m and V_{max} values.

The metabolic rates did not increase in proportion to the concentrations of substrates without BSA. It should be ascribed to the high hydrophobicity of M.A₃ and M.A₄. When incubation was done in BSA solution (in this case the concentration of BSA was 1%), the double-reciprocal plots became linear,^{9,10)} as illustrated in Fig. 3.

The V_{max} value of the hydroxylation of M.A₃ was 2.4-fold higher than that of M.A₄. The finding that M.A₃ was more readily metabolized than M.A₄ well corresponded to the *in vivo* observations using ³H preparations.⁶⁾ It is of interest that a substituent at 25-position distantly influenced 13-hydroxylation, although they had similar apparent K_m values. This similarity suggests that the substrate-binding step did not affect the metabolic rates. The

Table 2 Kinetic parameters of 13-hydroxylation of M.A₃ and M.A₄ by rat liver microsomes.

Substrate	Range of substrate (μM)	$V_{max}^a)$ (nmol/mg protein/min)	apparent $K_m^a)$ (μM)
M.A ₃	7.58–45.5	106	8.40
M.A ₄	6.13–36.8	43.6	10.9

^{a)} Data were estimated from the Lineweaver-Burk plots.

hydroxylation of M.A₃ and M.A₄ at 13-position was a cytochrome *P*-450 catalyzed reaction because it was enhanced when rats were pretreated with phenobarbital (PB), as shown later. Details of cytochrome *P*-450 catalyzed reaction have been described by White & Coon, in which rate-limiting steps of the reaction are proposed.¹¹⁾ Difference in the metabolic rates of M.A₃ and M.A₄ should originate from rate-limiting steps after substrate-binding step. For the difference in the extent of metabolism, the following explanations can be available: (1) steric factors at 25-position influence oxygenation, in other words, a bulky substituent at 25-position has an obstructive effect on 13-hydroxylation process, or (2) the rates of dissociation step are different between metabolites and enzyme involving the regeneration of cytochrome *P*-450.

3. Effect of PB and 3-MC

In order to characterize an enzyme which catalyzed the hydroxylation of M.A₃ and M.A₄ at 13-position, the influences of PB and 3-MC which could induce the activity of some kinds of cytochrome *P*-450(s) were investigated. Inducers were pretreated according to the method described elsewhere to produce sufficient effect.¹²⁾ Hydroxylation at 13-position of M.A₄ did not proceed without an NADPH-generating system. Hydroxylation of M.A₄ to 13-OH-A₄ was en-

Fig. 3 Lineweaver-Burk plots for 13-hydroxylation of M.A₃ and M.A₄.

Data: the mean value of three trials.

Table 3 Rate of 13-hydroxylation of M.A₄ by induced or uninduced rat liver microsomes.^{a)}

Substrate	Rate ^{b)} of 13-hydroxylation (%)		
	uninduced	PB ^{c)} induced	3-MC ^{d)} induced
M.A ₄	3.8	12.6	2.6

^{a)} All incubation mixtures had the same concentrations of M.A₄ and microsomal protein.

^{b)} Rate of 13-hydroxylation was calculated by the peak area of M.A₄ and 13-OH-A₄ on HPLC.

^{c)} Phenobarbital.

^{d)} 3-Methylcholanthrene.

hanced over three fold by pretreatment with PB but not with 3-MC, as shown in Table 3. This result indicates a phenobarbital-inducible form of cytochrome *P*-450 (s) is responsible for 13-hydroxylation of M.A₃ and M.A₄.

Some of the minor metabolites of M.A₄ in rat liver microsomes were assigned to an oxidation product at 5-position and a hydroxylation product at 26-position. Others were supposed to be dihydroxylated products of M.A₄ probably arising *via* 13-OH-A₄, which were detected in the excreta of rats. 13-OH-A₃ and 13-OH-A₄ were also detected as main metabolites in the tissues of rats orally administered with ³H-labeled M.A₃ and M.A₄,⁶⁾ indicating the *in vitro* metabolism well corresponded to the *in vivo* metabolism.

Avermectins are compounds related to milbemycins because both of them have a common macrolide structure. Avermectins, however, possess oleandrose disaccharide at 13(α)-position¹³⁾ and *iso*-propyl or *sec*-butyl groups at 25-position. *In vivo*¹⁴⁻¹⁶⁾ and *in vitro*^{17,18)} metabolites of avermectins in mammals have been reported to be a 24-hydroxymethyl metabolite, monosaccharide of a 24-hydroxymethyl metabolite, a 3''-*O*-desmethyl metabolite, and, so forth. It is noted that the predominant first step of the metabolism of M.A₃ and M.A₄ in rats is hydroxylation at 13-position, which probably accounts for the readiness of their degradabilities, while avermectins possessing a substituent at 13-position are fairly persistent in rats.

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要 約

ミルベマイシン A₃ と A₄ のラット肝ミクロゾームにおける比較代謝

貞包眞吾, 田中啓司, 佐藤一雄, 安藤 満
M.A₃ あるいは M.A₄ を NADPH 存在下でラット肝ミク

ロゾームと反応させたところ、主代謝物の生成が認められた。主代謝物を分離精製後、その MS, NMR スペクトルから、それらは M. A₃ および M. A₄ が 13(β) 位に水酸化を受けたものであった。13 位水酸化反応のキネティックパラメーターを調べたところ、M. A₃ の V_{max} 値は M. A₄ のそれより大きく M. A₃ の代謝が M. A₄ より速かった。このことは *in vivo* の実験結果

とよく一致した。一方、 K_m 値は両者でほぼ同じ値であった。M. A₃ と M. A₄ の代謝の受けやすさの差は本研究結果から、酸化酵素と基質の結合段階にあるのではなく、それ以降の過程にあるものと思われた。13 位水酸化反応はフェノバルビタールの処理により誘導される型のチトクローム P-450 が関与する酸化反応と考えられた。