COMPETITIVE INHIBITION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE BY ML-236A AND ML-236B FUNGAL METABOLITES, HAVING HYPOCHOLESTEROLEMIC ACTIVITY

Akira ENDO, Masao KURODA and Kazuhiko TANZAWA

Fermentation Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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1. Introduction

Fungal metabolites, ML-236A and ML-236B (fig.1), have been isolated from cultures of *Penicillium citrinum* as potent inhibitors of cholesterol synthesis in vitro in this laboratory [1].

These metabolites (LD_{50} for mice > 2 g, per os) cause a marked decrease in serum cholesterol levels in rats [1], and in hens and dogs (Kitano, Tsujita and Endo, in preparation). The experiments reported in this paper demonstrate that ML-236A and ML-236B inhibit specifically 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (EC 1.1.1.34), the rate-limiting enzyme in cholesterol synthetic pathway, without affecting the rest of the enzymes involved in this pathway, and that the inhibition is competitive with respect to the substrate HMG-CoA.

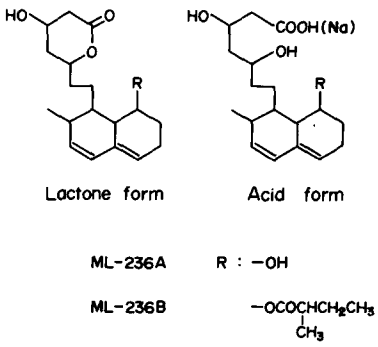

Fig.1. Structures of ML-236A and ML-236B.

2. Materials and methods

[1-^{14}C]Acetate (59.5 mCi/mmol) and D,L-[2-^{14}C] mevalonolactone (27.3 mCi/mmol) were obtained from Radiochemical Centre. [1-^{14}C]Acetyl-CoA (49.8 mCi/mmol) and D,L-[3-^{14}C]HMG-CoA (26.2 mCi/mmol) were purchased from New England Nuclear. Lactone forms of ML-236A and ML-236B were prepared as previously described [1]. Acid forms (sodium salts) (fig.1) of these compounds were prepared by saponification of their respective lactone forms in 0.1 N NaOH at 50°C for 2 h. Other chemicals were of the best grade commercially available.

2.1. *Incorporation experiments*

Rat liver microsomes and cytosolic enzyme fraction were isolated as described previously [2]. The reaction mixture (0.2 ml) contained: 1 mM ATP, 10 mM glucose-1-phosphate, 6 mM glutathione, 6 mM $MgCl_2$, 40 μM CoA, 0.25 mM NAD, 0.25 mM NADP, 100 mM potassium phosphate buffer (pH 7.4) 0.15 mg protein of microsomes, 1.5 mg protein of cytosolic enzyme fraction and 1 mM [1-^{14}C]acetate (1.5 mCi/mmol). Where indicated, [1-^{14}C]acetate was replaced by 0.15 mM [1-^{14}C]acetyl-CoA (1.0 mCi/mmol), 0.13 mM D,L-[3-^{14}C]HMG-CoA (7.3 mCi/mmol) or 0.52 mM D,L-[2-^{14}C]mevalonate (1.26 mCi/mmol). After incubation at 37°C for 60 min, the reaction was terminated by addition of 1 ml 15% alcoholic KOH. The synthesized nonsaponifiable lipids and fatty acid were measured as described previously [2]. Under these conditions, the incorporations of radiolabeled substrates were proportional to time up to 120 min.

2.2. *HMG-CoA reductase assay*

Rat liver microsomes were obtained as described previously [2], from which HMG-CoA reductase was solubilized by the method of Heller and Gould [3] and partially purified by fractionation with ammonium sulfate. The fraction precipitated by 35–50% saturation was used after dialysis for 3 h against 40 mM potassium phosphate buffer, pH 7.2, containing 100 mM sucrose, 50 mM KCl, 30 mM EDTA and 1 mM dithiothreitol. The reaction mixture (50 μl) contained: 100 mM potassium phosphate buffer (pH 7.4) 10 mM EDTA, 10 mM dithiothreitol, 5 mM NADPH, 0.11 mM D,L-[3-^{14}C]HMG-CoA (1.6 mCi/mmol) and 1–2 μg of enzyme protein. After incubation at 37°C for 20 min, the reaction was terminated by addition of 20 μl of 2 N HCl, and the mevalonolactone formed was isolated and counted as described previously [2]. The specific activity of the enzyme used was 10–17 nmol of mevalonate formed/min/mg protein under standard conditions.

Protein was determined by the method of Lowry et al. [4].

3. Results

3.1. *Incorporation of radiolabeled substrates into lipids*

As reported in a previous paper [1], ML-236B lactone is far more inhibitory in the [1-^{14}C]acetate incorporation into nonsaponifiable lipids than ML-236A lactone. The inhibitory potency was approximately doubled by the conversion of lactone forms to their respective acid forms (sodium salts). Of the four derivatives tested, ML-236B sodium salt was the most inhibitory. Concentrations required for 50% inhibition of nonsaponifiable lipid synthesis were: ML-236A 0.85 μM, ML-236A sodium salt 0.35 μM, ML-236B lactone 0.026 μM, ML-236B sodium salt 0.014 μM (0.006 μg/ml). None of these compounds had significant effects on the fatty acid synthesis from [^{14}C]acetate at concentrations which caused 50% or more reduction in the nonsaponifiable lipid synthesis.

Table 1 shows the effects of ML-236B sodium salt on the incorporation of various radiolabeled substrates into nonsaponifiable lipids. As indicated, conversions

Table 1
Inhibitory effects of ML-236B sodium salt on the incorporation of various radiolabeled substrates into nonsaponifiable lipids

Substrate	ML-236B sodium salt (nM)	Incorporation dpm/mg[a]	% of control
[1-^{14}C]Acetate	None	13 770	
	5.0	10 080	73.2
	50	4120	29.9
[1-^{14}C]Acetyl-CoA	None	8270	
	5.0	6020	72.8
	50	2410	29.2
D,L-[3-^{14}C]HMG-CoA	None	1050	
	5.0	570	53.7
	50	270	26.0
D,L-[2-^{14}C]Mevalonate	None	35 870	
	5.0	34 940	97.4
	50	34 180	95.3

[a] Counts incorporated/mg protein/60 min. The values for [1-^{14}C]acetate and [1-^{14}C]acetyl-CoA were calculated on the assumption that 33% of the radioactivity in these substrates were converted into CO_2 during their incorporation into nonsaponifiable lipids.

Experimental conditions are described in Materials and methods. The results are the average of duplicate incubations.

of [^{14}C]acetate, [^{14}C]acetyl-CoA and D,L-[^{14}C]HMG-CoA were inhibited to similar extent at two concentrations of the agent, 5 nM and 50 nM. On the other hand, D,L-[^{14}C]mevalonate conversion into non-saponifiable lipids was not affected by ML-236B sodium salt at concentrations up to 50 nM, indicating that this compound inhibited specifically the enzymatic step for the conversion of HMG-CoA to mevalonate catalyzed by HMG-CoA reductase.

3.2. *Inhibition of HMG-CoA reductase*

As shown in fig.2, all the four compounds were inhibitory to HMG-CoA reductase. The acid forms (sodium salts) of both ML-236A and ML-236B were more effective in inhibiting the reductase than their respective lactone forms, and the two forms of ML-236B were more potent inhibitors than those of ML-236A. Concentrations required for 50% inhibition were: ML-236A lactone 3.4 μM, ML-236A sodium salt 1.2 μM, ML-236B lactone 0.10 μM, ML 236B sodium salt 0.023 μM. The data correlated well with the results obtained for the inhibition of nonsaponifiable lipid synthesis from [^{14}C]acetate, although higher concentrations of the compounds were required for inhibition of the reductase.

The inhibition of HMG-CoA reductase by these compounds was competitive with respect to HMG-CoA and noncompetitive with respect to NADPH (fig.3). The K_i values were: ML-236A sodium salt 0.22 μM, ML-236B sodium salt 0.010 μM. Under these conditions, K_m values for the two substrates were: D,L-HMG-CoA 33 μM, NADPH 40 μM.

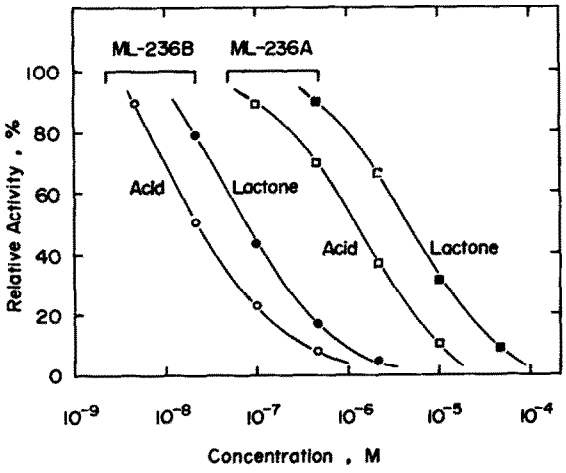

Fig.2. Inhibition of HMG-CoA reductase by ML-236A and ML-236B. Experiments were carried out as described in Materials and methods. The results are expressed as % of control (without inhibitor). The value for control was 16.5 nmol/min/mg protein.

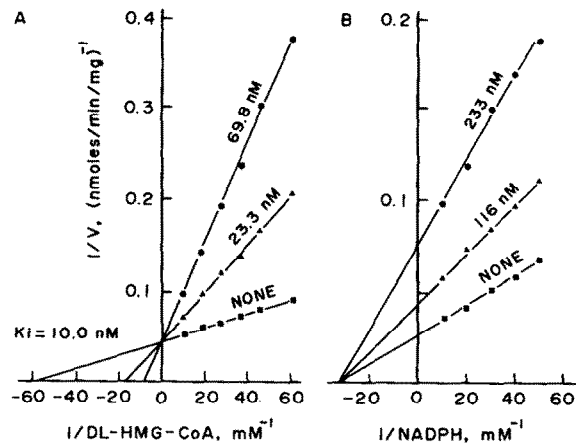

Fig.3. Double reciprocal plots of the inhibition of HMG-CoA reductase by ML-236B sodium salt. Experiments were carried out as described in Materials and methods, except that concentrations of HMG-CoA (A) and NADPH (B) were varied as indicated.

4. Discussion

The α-methylbutyrate residue of ML-236B (fig.1) appears to play a significant role in the inhibition of HMG-CoA reductase activity, since both lactone and acid forms of ML-236A, lacking such a residue in their structure, are far less inhibitory than ML-236B analogs. The acid forms of both ML-236A and ML-236B contain a portion having a chemical structure very similar to that of 3-hydroxy-3-methylglutarate (fig.1). This is compatible with the fact that these compounds are competitive inhibitors of HMG-CoA reductase (competitive against HMG-CoA) and further that the acid forms are more potent than the corresponding lactone forms. The present results, considered together with those given in the previous paper [1], provide good evidence that a specific inhibitor of HMG-CoA reductase is effective in reducing cholesterol synthesis in vivo, and thereby in lowering cholesterol levels in blood.

References

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