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Inhibitory Effect of Melinamide on Cholesterol Solubility in Mixed Micellar Solution of Sodium Taurocholate

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The inhibitory effect of melinamide, *N*-(α-methylbenzyl)-linoleamide, on cholesterol solubility in a mixed micellar solution of sodium taurocholate was investigated *in vitro* by the solubility method. At pH 7.4, melinamide decreased the amount of cholesterol by 30—40% without influencing the concentration of oleic acid in bile salt-rich micelles with a molar ratio of more than 10:10:5 of bile salt:oleic acid:monolein, while it had no effect on oleic acid–monolein-rich micelles. Further, the effect of melinamide was independent of the pH of the medium. Melinamide seems to exhibit its effect as a result of releasing cholesterol from the micellar phase. On the other hand, it is assumed that neomycin reduces the amount of cholesterol by destroying micellar structure, as it was shown to completely eliminate oleic acid as well as cholesterol from micellar solution.

Keywords——melinamide; mixed micelle; sodium taurocholate; oleic acid; monolein; cholesterol; solubility; inhibitor

Introduction

Melinamide, *N*-(α-methylbenzyl)linoleamide, an amide derivative of an unsaturated long-chain fatty acid (Chart 1), is a new cholesterol absorption inhibitor. It has become clear from many experiments using animals that melinamide remarkably reduces cholesterol absorption from the intestine,[1] and this effect has been confirmed clinically.[2] However, the mechanism has not yet been clarified in detail.

The transfer process of cholesterol from the intestinal lumen into the lymph system is thought to involve several stages.[3] One of the initial steps is the process of cholesterol solubilization by bile salts. In the intestinal lumen, cholesterol is solubilized by mixed micelles of bile salt–monoglyceride–free fatty acid, and transferred through an unstirred water layer to mucosal cells.[3] We therefore thought it important to establish the effect of melinamide on cholesterol solubilization by mixed micelles of bile salts in order to clarify its inhibitory effect on cholesterol absorption. Although there are many reports which deal with the properties of mixed micelles[4] or with the micellar solubilization of cholesterol,[5] no conclusive analyses of the effects of cholesterol inhibitors on cholesterol solubilization by mixed micelles of bile salts have yet been performed.[6]

In this study, the effect of melinamide on cholesterol solubility in a mixed micellar solution of bile salt was investigated from a physicochemical point of view. To obtain an *in*

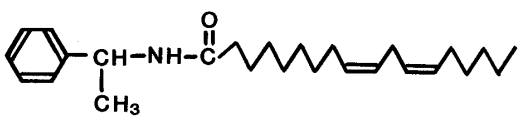

Chart 1. Structure of Melinamide

vitro model system of mixed micelles in the intestinal lumen, sodium taurocholate (STC) was selected as a bile salt, and a 2 : 1 (M/M) mixture of oleic acid and monolein as a fat, together with cholesterol. The mechanism was analyzed, as well as the effects of other agents.

Experimental

Materials——Melinamide used was the product of our own company. Methyl linoleate was prepared in the Synthesis Section of our Research Laboratory. Oleic acid (purity 99%) and neomycin sulfate were purchased from P-L Biochemicals, Inc. (Milwaukee). Monolein, the product of Kanto Chemical Co., Inc. (Tokyo, Japan), was of reagent grade. α-Methylbenzylamine (purity 99%) was obtained from Yamakawa Pharmaceuticals Co., Ltd. (Osaka, Japan). All other materials were of analytical grade. Sörensen buffer systems were employed as aqueous media.

Methods——Oleic acid, monolein, and cholesterol inhibitor in methanol or methanol–ethyl acetate were added to a given amount of STC and mixed, the organic solvents were removed *in vacuo*, and sufficient buffer (5 ml) was added to the residue. The contents were homogenized by sonication for 1 min, and transferred to a 10-ml tube. An excess amount of crystalline cholesterol was added, and the tube was purged with N_2 and shaken vigorously (400 rpm) for 4 h at room temperature (23 ± 1 °C). The mixture was ultracentrifuged (250000 *g*, 60 min) using a Hitachi automatic preparative ultracentrifuge, model 80P-7, then the transparent micellar phase was withdrawn with an injection-syringe and analyzed by gas chromatography (GC) and high performance liquid chromatography (HPLC). Each experiment was carried out in triplicate.

Analytical——1) Cholesterol and Melinamide: GC analysis was performed using a Hitachi model 163 instrument. The analytical conditions were as follows: column, 1 m × 3 mm i.d. packed with 3% OV-101 coated on Chromosorb W AW DMS (60—80 mesh); temperature, oven 240 °C and injector 290 °C; carrier, N_2 (flow rate, 50 ml/min); detector, flame ionization detector.

2) Oleic Acid (Except when α-Methylbenzylamine Was Used as the Cholesterol Inhibitor): HPLC analysis was performed using a Shimadzu model LC-3A apparatus. The analytical conditions were as follows: column, LiChrosorb RP-18, 30 cm × 4 mm i.d.; carrier, methanol/pH 7.4 (0.01 M) = 80/20; flow rate, 1 ml/min; detector, ultraviolet (UV) 210 nm.

3) Methyl Linoleate: The conditions were the same as those employed in the case of oleic acid except for the carrier, which was methanol only.

4) α-Methylbenzylamine and Oleic Acid: The conditions were the same as those employed in the case of oleic acid except for the carrier, which was methanol/pH 9.7 (0.01 M) = 8/2.

Results

(1) Dissolution Rate of Cholesterol in Mixed Micellar Solution

The dissolution rate of cholesterol in the mixed micellar solution (containing 10 mM STC, 4 mM oleic acid and 2 mM monolein) was determined at pH 7.4 in the presence and absence of melinamide. The concentration of cholesterol was constant between 2 and 8 h either in the presence or in the absence of melinamide. Hence, in later measurements the incubation time was set at 4 h.

(2) Solubilities of Cholesterol and Melinamide in Several Media

The solubitilies of cholesterol and melinamide were determined in a solution containing

TABLE I. Solubilities of Cholesterol and Melinamide in Several Media (Unit: mM)

	Aqueous media (pH 7.4)		
	None	10 mM STC solution	Mixed micellar solution[a]
Cholesterol[b]	0.001—0.002	0.005—0.006	0.4—0.5
Melinamide	0.001—0.002	0.004—0.007	0.4—0.5

a) Mixed micellar solution containing 10 mM STC, 4 mM oleic acid, and 2 mM monolein. *b*) States of compounds when added to media were as follows: cholesterol, crystalline; melinamide, oily. An excess amount of each compound was added in an appropriate medium of pH 7.4, and shaken (400 rpm) for 2 h at room temperature (23 ± 1 °C). The solution was then centrifuged (10000 *g*, 20 min) under cooling (20 ± 3 °C), and the transparent part was analyzed by GC or HPLC.

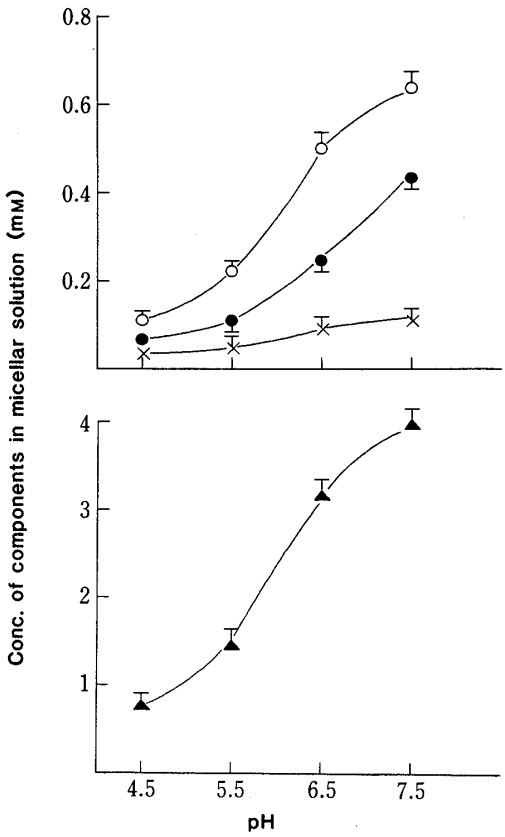

Fig. 1. Influence of pH on the Solubility of Cholesterol or Oleic Acid, and on the Cholesterol-Lowering Effect of Melinamide

Cholesterol (○) without melinamide, and (●) in the presence of 1 mM melinamide; melinamide (×); oleic acid (▲).

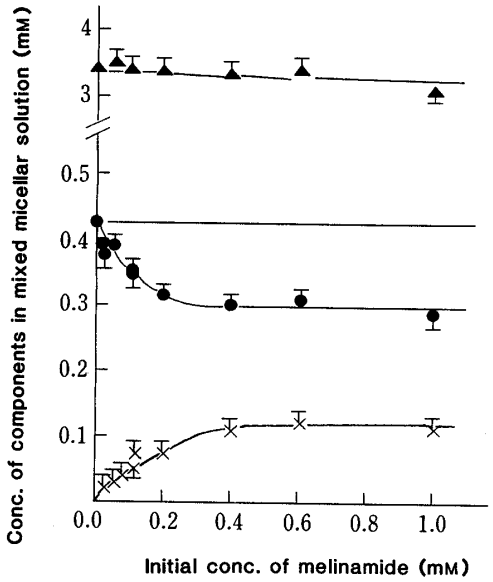

Fig. 2. Effect of Melinamide on Cholesterol Solubility in a Mixed Micellar Solution (Containing 10 mM STC, 4 mM Oleic Acid, and 2 mM Monolein) at pH 7.4

Cholesterol (●); melinamide (×); oleic acid (▲).

10 mM STC and a mixed micellar solution containing 10 mM STC, 4 mM oleic acid and 2 mM monolein at pH 7.4, and compared with that in phosphate buffer, pH 7.4 (Table I). The solubilities of α-methylbenzylamine and neomycin were more than 10 mM in each aqueous medium at pH 7.4.

(3) Influence of pH

We investigated the influence of pH change in the medium on the solubility of cholesterol or oleic acid, and on the cholesterol-lowering effect of melinamide. The results are shown in Fig. 1. It was found that the solubilities of both cholesterol and oleic acid decreased remarkably as the pH value was decreased. On the other hand, the effect of melinamide was almost constant, irrespective of pH.

(4) Influence of Added Amount of Melinamide

The concentrations of micellar components in an aqueous solution (pH 7.4) containing 10 mM STC, 4 mM oleic acid and 2 mM monolein were determined when the added quantity of melinamide was varied between 0 and 1 mM (Fig. 2). On addition of less than 0.4 mM melinamide, the concentration of cholesterol decreased and that of melinamide increased in the micellar solution. On addition of more than 0.4 mM melinamide, however, the concentrations of both components remained constant. The reduction in the amount of cholesterol was equivalent to the increase in the amount of melinamide in the micellar solution. At the leveling-off stage in Fig. 2, the percentage of cholesterol lowering reached 35% and the concentration of melinamide was only about one quarter of its solubility in the

absence of cholesterol. On the other hand, the concentration of oleic acid was almost independent of the amount of melinamide added.

$$\text{percentage of cholesterol lowering} = (C_0 - C_1)/C_0$$

C_0: solubility of cholesterol in the absence of melinamide.

C_1: solubility of cholesterol at the leveling-off state.

(5) Influence of Composition Ratio in Mixed Micellar Solution

When the concentration of STC was kept constant (10 mM) and the concentration ratio of the mixture solution of oleic acid: monolein was varied from 1 : 0.5 to 12 : 6 (mM: mM) at pH 7.4, the changes in the solubility of cholesterol and the effect of melinamide were as shown in Fig. 3. The effect of melinamide changed dramatically at a molar ratio of 10 : 10 : 5 of STC: oleic acid: monolein; melinamide had no effect at molar ratios of STC: oleic acid: monolein smaller than 10 : 10 : 5, but it lowered the cholesterol concentration by as much as 35—40% at molar ratios greater than 10 : 10 : 5. In addition, the solubility of cholesterol in the absence of melinamide rose rapidly below this molar ratio.

(6) Cholesterol-Lowering Effect of Other Agents

Using the same procedure as in the case of melinamide, described in section (4), the effects of other inhibitors or compounds were measured.

(i) α-Methylbenzylamine (Fig. 4A)——α-Methylbenzylamine, one of the constituent

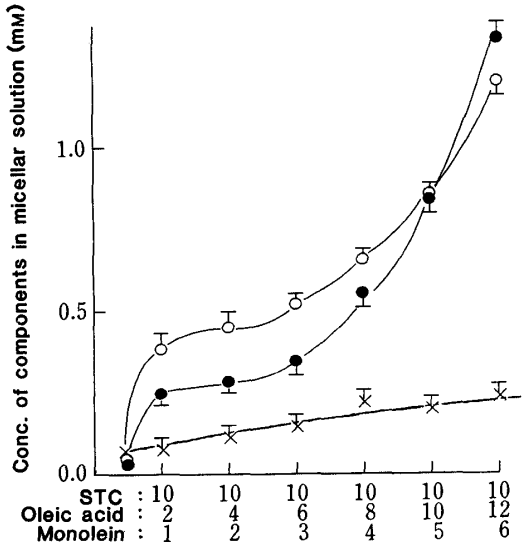

Fig. 3. Influence of Composition Ratio in STC–Oleic Acid–Monolein Micellar Solution at pH 7.4

Cholesterol (○) without melinamide, and (●) in the presence of 1 mM melinamide; melinamide (×).

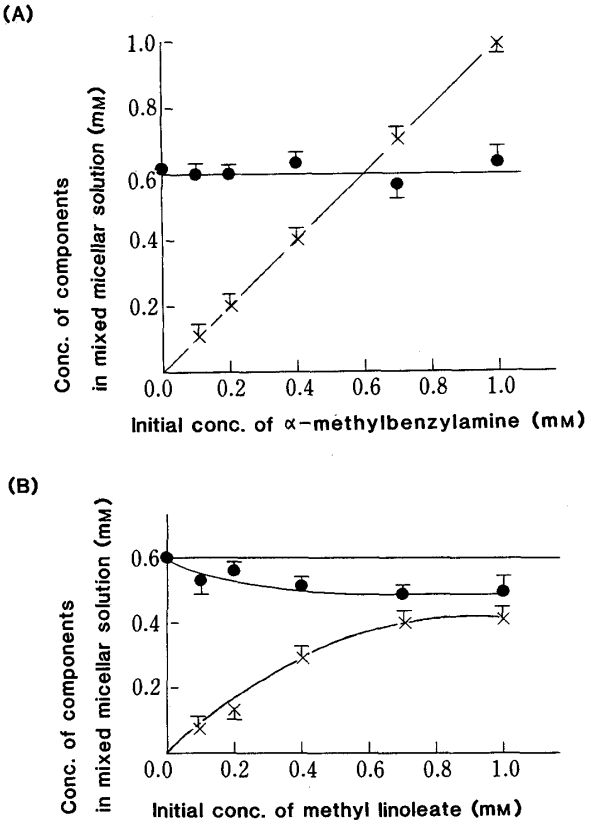

Fig. 4. Effect of α-Methylbenzylamine (A), and Methyl Linoleate (B) on Cholesterol Solubility in a Mixed Micellar Solution (Containing 10 mM STC, 4 mM Oleic Acid, and 2 mM Monolein) at pH 7.4

Cholesterol (●); agent (×).

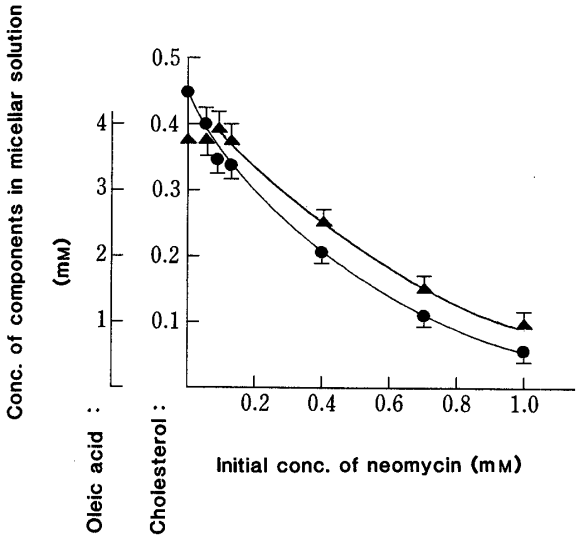

Fig. 5. Effect of Neomycin on Cholesterol Solubility in a Mixed Micellar Solution (Containing 10 mM STC, 4 mM Oleic Acid, and 2 mM Monolein) at pH 7.4

Cholesterol (●); oleic acid (▲).

species of the melinamide molecule, had no effect.

ii) Methyl Linoleate (Fig. 4B)——Methyl linoleate, one of the constituent parts of melinamide, showed some cholesterol-lowering effect, in contrast to α-methylbenzylamine. On addition of methyl linoleate in quantities smaller than 0.4 mM, the concentration of cholesterol decreased and that of methyl linoleate increased. However, the concentrations of both components levelled off on addition of more than 0.4 mM methyl linoleate. On the other hand, the concentration of oleic acid was not influenced by methyl linoleate.

Points which differed from the results obtained in the case of melinamide were that the molar ratio of the decreased cholesterol to the increased methyl linoleamide was about 1 : 4, while the percentage of cholesterol lowering at the leveling-off state was only 14%.

(iii) Neomycin Sulfate (Fig. 5)——In contrast to the case of melinamide, neomycin reduced the concentration of not only cholesterol but also oleic acid. Furthermore, these two components in the mixed micellar solution almost disappeared without reaching a leveling-off state on addition of more than 1 mM neomycin.

Discussion

(1) Dissolution Rate of Cholesterol in Mixed Micellar Solution

The incubation time (4 h) with vigorous shaking used in this experiment was different from that (from several days to several weeks) involving gentle shaking employed in many previous studies.[5] However, considering that the process of cholesterol solubilization terminates within several hours *in vivo*, it is thought that these experimental conditions are realistic, and that meaningful results can be obtained even if true equilibrium between cholesterol and mixed micelles is not reached.

(2) Solubilities of Cholesterol and Melinamide in Several Media

The solubilities of cholesterol and melinamide were negligible in phosphate buffer or the solution containing 10 mM STC, compared with that in the mixed micellar solution of STC–oleic acid–monolein at pH 7.4. Therefore, most of the compounds in the micellar solution are likely to exist in the micellar phase (within the mixed micelles or on their surfaces).

(3) Influence of pH

The decrease of the level of cholesterol in the mixed micellar solution with medium pH change from pH 7.4 to pH 4.5 was probably the result of a lowering of oleic acid solubility, owing to a decrease of the ionized form of oleic acid. On the other hand, the solubility of STC

does not change in this pH region because of its $pK_a < 2$.

(4) Influence of Added Amount of Melinamide

Oleic acid concentration was independent of the amount of melinamide added, and there was a 1 : 1 relationship between the decrease in the amount of cholesterol and the increase in the amount of melinamide in the micellar solution. Moreover, a leveling-off state appeared. These results suggest that the inhibitory mechanism of melinamide is as follows: the compound penetrates into mixed micelles without influencing the micellar structure (or size) and expels cholesterol from them.

(5) Influence of Composition Ratio in Mixed Micelles

The molar ratio of 10 : 10 : 5 of STC : oleic acid : monolein, at which the effect of melinamide changes, is consistent with that at which two different types of micellar structure are formed, as reported by Muller[7] and by Claffey and Holzbach.[8] On the assumption that this change of melinamide effectiveness is related to the transition between the two types of micellar structure, it can be envisaged that melinamide is effective for spherical-type bile salt-rich micelles, but ineffective for disk-type fatty acid-rich micelles.

(6) Cholesterol-Lowering Effects of Other Agents

(i) α-Methylbenzylamine——α-Methylbenzylamine is too hydrophilic to penetrate into the hydrophobic micellar phase, and therefore has no effect. Accordingly, most of the α-methylbenzylamine molecules found in micellar solution probably exist outside the micelles.

(ii) Methyl Linoleate——In contrast to α-methylbenzylamine, methyl linoleate, having a long hydrocarbon chain, one of the components of the melinamide molecule, shows a cholesterol-lowering effect, but this effect is evidently weaker than that of melinamide. From the results on melinamide, α-methylbenzylamine and methyl linoleate, it is clear that both the hydrocarbon chain and the benzylamine components of melinamide are necessary for a strong cholesterol-lowering effect.

(iii) Neomycin Sulfate——In the case of neomycin, the mechanism of the cholesterol-lowering effect is considered to be different from that of melinamide. That is to say, neomycin probably exerts its effect by destroying mixed micellar structure.[9]

Conclusion

(1) It is thought that melinamide displays a cholesterol-lowering effect as a result of expulsion of cholesterol in a molar ratio of 1 : 1 from the micellar phase.

(2) Melinamide exhibits its cholesterol-lowering effect even if the pH of the medium becomes acidic.

(3) Melinamide is effective for bile salt-rich micelles, but is ineffective for fatty acid-rich micelles.

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