Pharmacological Properties of YM17E, an Acyl-CoA:Cholesterol Acyltransferase Inhibitor, and Diarrheal Effect in Beagle Dogs

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ABSTRACT—YM17E (1,3-bis[[1-cycloheptyl-3-(p-dimethylaminophenyl)ureido]methyl]benzene dihydrochloride) was found to be a potent inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) in rabbit liver and intestine microsomes. Dixon plot analysis revealed that YM17E inhibited microsomal ACAT in a non-competitive manner. YM17E induced a marked decrease in serum cholesterol, especially in non-high-density lipoprotein (HDL) fractions, in cholesterol-fed rats and rats fed normal chow. Measurement of bile secretion after oral administration of YM17E in cholesterol-fed rats showed that the drug markedly accelerated the secretion of bile acids and neutral sterols. Furthermore, absorption of [3H]cholesterol from the gut of cholesterol-fed rats was significantly inhibited by YM17E. From these results, the hypocholesterolemic activity of YM17E in these animals resulted from both a decrease in cholesterol absorption from the gut and the stimulation of excretion of cholesterol from the liver into bile. However, YM17E caused secretory diarrhea in beagle dogs at near lipid lowering doses. When YM17E was administered at the same total dosage but divided into 5 daily administrations, the incidence of diarrhea was significantly reduced while its cholesterol lowering effect became stronger. These results suggest that the inhibition of intestinal and/or liver ACAT increases the risk of diarrhea development which, however, can be avoided by controlled drug administration in beagle dogs.

Keywords: YM17E, Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Cholesterol, Diarrhea

Acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is a microsomal enzyme that is found in various tissues including the intestine (1), liver (2), arterial wall (3) and adrenal gland (4). This enzyme catalyzes the cholesterol esterification reaction (5). The major physiologic role of ACAT is thought to be in the absorption of dietary cholesterol and intracellular storage of cholesterol as a non-toxic esterified form in various tissues. The enzyme is also involved in hepatic apolipoprotein B secretion (6, 7) and in macrophage or vascular smooth muscle cell-derived foam cell formation (8, 9). These findings support the involvement of ACAT in the development of atherosclerotic lesions. However, recent developmental studies of ACAT inhibitors have encountered obstacles such as adrenocortical toxicity (10). Furthermore, it is not clearly known whether ACAT is ubiquitous for maintaining the normal metabolic state in somatic cells.

A lot of reports on previous ACAT inhibitors have shown the data on hypocholesterolemic activities in animal models. Two mechanisms have been postulated for the blood cholesterol lowering effect of these compounds, namely that they inhibit cholesterol absorption from the gut (11, 12) and that they promote catabolic excretion of cholesterol from the liver into bile (12). Although malabsorption of lipids and bile acids are generally known to cause secretory diarrhea, little has been reported on whether ACAT inhibitors may cause diarrhea. In this study, we determined the mechanism of the hypocholesterolemic activity of a novel ACAT inhibitor, YM17E, in rat models. Furthermore, we also report the occurrence of diarrheal episodes in YM17E-treated beagle dogs and discuss the mechanism of this YM17E-induced diarrhea.
MATERIALS AND METHODS

Materials
YM17E (1,3-bis[1-cycloheptyl-3-(p-dimethylaminophenyl)ureido[methyl]benzene dihydrochloride) (Fig. 1) was synthesized at Yamanouchi Pharmaceutical Co., Ltd., Tokyo. Sodium pentobarbital was purchased from Muromachi Chemicals, Tokyo. KNa2-phosphate, dithiothreitol, bovine serum albumin, cholesterol, bovine brain extract and egg yolk phosphatidyl choline were purchased from Sigma (St. Louis, MO, USA). Disodium ethylenediaminetetraacetate (EDTA 2Na) and dimethyl sulfoxide (DMSO) were purchased from Dojin (Kumamoto). [14C]Oleoyl CoA, [14C]cholesterol and [3H]cholesterol were purchased from Dupont/NEN (Wilmington, DE, USA).

ACAT assays
The enzyme assay was performed with microsomes prepared from male Japanese white rabbit liver or intestine as described previously (13) with some modifications. Animals were killed by bleeding from the carotid artery under anesthesia with 30 mg/kg, i.v. of sodium pentobarbital. The liver and intestine were washed with ice-cold saline (0.9% NaCl), homogenized in 4 vol. of 0.25 M sucrose containing 1.5 mM EDTA (pH 7.4) and centrifuged at 1,000 x g for 10 min. The supernatants were recentrifuged several times at 12,000 x g until no pellets and lipids were observed. The supernatants were further centrifuged at 107,000 x g for 60 min to isolate microsomes. The microsomes were washed twice with 0.154 M KNa2-phosphate buffer (pH 7.4) containing 2 mM dithiothreitol. In each assay tube, 50 μl of diluted microsomes, 50 μl of 1.2% bovine serum albumin, 50 μl of liposomes containing 400 μg of cholesterol, 190 μg of bovine brain extract, 570 μg of egg yolk phosphatidyl choline, 5 μl of drug in DMSO and 70 μl of phosphate buffer were mixed and preincubated for 5 min at 37°C. Then 25 μl of 50 nCi/9.4 μmol [14C]Oleoyl CoA was added and the mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 2.5 ml of chloroform : methanol (2 : 1) and vortexing. Lipids were extracted by Folch's method (14), separated by thin layer chromatography and the bands corresponding to cholesteryl oleate were sectioned. The radioactivities in enzymatically synthesized cholesteryl oleate were determined by liquid scintillation counting (LSC) analysis (Model 2200CA; Packard, Meriden, CT, USA).

Serum lipid analysis
Serum total cholesterol, free cholesterol, triglycerides, phospholipids and non-esterized fatty acids were determined by a serum auto analyzer (Hitachi 736; Hitachi, Tokyo) with associated reagents (Monotest cholesterol, Triglycolor, Boehringer Mannheim, Mannheim, Germany; Clinimate NEFA, Clinimate F-CHO, Clinimate PL, Daiichi Pure Chemicals Co., Ltd., Tokyo). Serum high-density lipoprotein (HDL) was prepared by the heparin-Mn method (15). Briefly, 20 μl of 35.7 mg/ml heparin in saline and 25 μl of 1 M MnCl2 were added to 0.5 ml of serum, vortexed and incubated for 30 min on ice to precipitate non-HDL. After centrifugation at 4°C for 30 min, 0.4 ml of supernatants were removed and cholesterol content was analyzed directly with a serum auto analyzer (Hitachi 736, Hitachi). All measurements were performed within 24 hr after blood collection.

Animal studies
Male SD rats (SPF, 150~200 g) were purchased from Nippon SLC, Hamamatsu. They were kept under artificial lighting (7:30~20:30) and given free access to drinking water and normal rat chow (CE-2; Oriental Bioservice Kanto, Tokyo), an atherogenic diet (CE-2 supplemented with 1.5% cholesterol, 0.5% cholic acid, 10% coconut oil; Oriental Bioservice Kanto), or a cholesterol-enriched diet (CE-2 supplemented with 1% cholesterol, Oriental Bioservice Kanto). YM17E or vehicle (0.5% methylcellulose) were administered p.o. daily for 5 days. At 2 hr after the final administration, blood samples were collected from the inferior vena cava under anesthesia with diethyl ether. Liver and intestinal microsomes were prepared as described above for preparation of rabbit microsomes.

Male beagle dogs were purchased from Hamri Co., Ltd., Ibaraki. Animals were kept under artificial lighting (7:30~20:30) and give free access to drinking water and normal dog chow (DS, Oriental Bioservice Kanto) or the atherogenic diet according to the experimental procedures. All experiments were performed in accordance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd.
Study of bile excretion in cholesterol-fed rats

Rats were fed 1% cholesterol-containing CE-2 for 7 days before surgery. YM17E suspended in 0.5% methyl cellulose or vehicle was administered p.o. daily for 5 days before surgery. On the day of surgery, rats were anesthetized after the last administration of YM17E or vehicle with 60 mg/kg of sodium pentobarbital. The pancreas was exposed by abdominal incision and a polyethylene tube was inserted into the bile duct at the ventral side of the pancreas through a small incision. The abdominal incision was closed with surgical clips, and the animals were returned to cages for recovery from anesthesia. Bile samples were collected into glass tubes and measurement of secretory volume immediately after injection of 5 μCi/rat of [14C]cholesterol in saline containing 5% ethanol and 5% cremophor into the tail vein was started. Aliquots of bile samples collected every 2 hr were directly subjected to LSC to determine total counts secreted. Bile acid and neutral sterol separation were performed as follows: 1 ml of bile was added to 2 ml of 15% KOH in ethanol, the mixture was heated at 70°C for 2 hr, 1 ml of water and 2 ml of petroleum ether was added, and then the mixture was vortexed well. The petroleum ether layer (containing neutral sterols) was removed and subjected to LSC. HCl (0.5 ml) was added to the aqueous layer, and bile acids were extracted twice with 2 ml of diethyl ether and counted by LSC.

In vivo cholesterol absorption in cholesterol-fed rats

Rats fed a cholesterol-rich diet for 3 days were administered p.o. with 30 mg/kg YM17E (0.5% methyl cellulose suspension) or the same volume of vehicle, followed by p.o. administration of 10 μCi/animal of [3H]cholesterol. Blood samples were collected from the tail vein at 0, 4, 8, 24, 48 and 72 hr after the administration of [3H]cholesterol. Total serum cholesterol was determined enzymatically (Monotest cholesterol, Boehringer Mannheim). Radioactivities in plasma were determined by LSC. Rats had free access to a cholesterol-rich diet during the experiment.

Studies on diarrhea in cholesterol-fed beagle dogs

Fifteen male beagle dogs (average body weight 11 kg) were divided into 3 groups with similar serum cholesterol levels and fed an atherogenic diet [40 g chow (DS, Oriental Bioservice Kanto) containing 3% coconut oil and 12 g of dog meal (Oriental Bioservice Kanto) containing 4% cholesterol] at 8:00, 11:00, 14:00, 17:00 and 20:00. After 1 week of treatment with this diet, 5 animals were orally administered 30 mg/kg of YM17E filled in capsules after feeding at 8:00 and 6 mg/kg of lactose filled in capsules at 11:00, 14:00, 17:00 and 20:00. Another 5 animals were administered 6 mg/kg of YM17E after each feeding. Control animals (n = 5) received lactose capsules only 5 times daily. Drug treatment was continued for 2 weeks, followed by a 1-week observation period to confirm recovery. Blood samples for serum lipid analysis were collected from the cephalic vein of dogs fasted for 12 hr on days 0, 7 and 14. Blood samples for the analysis of blood drug concentration were also collected on day 14. Plasma concentrations of unchanged drug were determined as previously described (16) except that detection was done by UV absorbance at 265 nm.

RESULTS

Inhibition of microsomal ACAT

Investigation of the effect of YM17E on microsomal ACAT in vitro using rabbit liver and intestine showed that YM17E inhibited production of [14C]cholesteryl esterification. Control animals (n = 5) received lactose capsules only 5 times daily. Drug treatment was continued for 2 weeks, followed by a 1-week observation period to confirm recovery. Blood samples for serum lipid analysis were collected from the cephalic vein of dogs fasted for 12 hr on days 0, 7 and 14. Blood samples for the analysis of blood drug concentration were also collected on day 14. Plasma concentrations of unchanged drug were determined as previously described (16) except that detection was done by UV absorbance at 265 nm.

Fig. 2. Inhibition of microsomal ACAT by YM17E. A: microsomes were prepared from rabbit liver (○) or intestine (■) as described under Materials and Methods. Data indicate the mean of percent inhibition of cholesteryl oleate production. B: Dixon plot analysis of ACAT inhibition by YM17E. The rates of cholesterol esterification were estimated at substrate concentrations of 7.32 (○), 14.64 (■) and 36.56 (□) μM. V: nmol cholesteryl [14C]oleate /min/ml reaction mixture.
oleate from [14C]oleoyl CoA in a dose-dependent manner in both liver and intestinal microsomes used as enzyme sources (Fig. 2A). YM17E was as potent in inhibiting ACAT activity in the liver as in the intestine, with IC\textsubscript{50} values of 45 and 34 nM, respectively. Dixon plot analysis of ACAT inhibition by YM17E revealed non-competitive behavior with a \( K_i \) value of 86 nM when liver microsomes was used as the enzyme source (Fig. 2B).

**In vivo study in rats fed normal chow diet**

Effects of YM17E on body and liver weights and on serum lipid parameters were investigated in rats fed normal diets (Table 1). Rats given YM17E for 5 days showed no significant change in body weight gain or liver weight. A significant decrease was found in non-HDL cholesterol in animals treated with 10 mg/kg or more while a low dose (3 mg/kg) of YM17E increased non-HDL cholesterol. Non-esterized fatty acids were also decreased at the highest dose.

**In vivo study in rats fed an atherogenic diet**

Effects of YM17E on body and liver weights and on serum lipid parameters were investigated in rats fed atherogenic diets (Table 2). After 3 days of feeding the atherogenic diet, YM17E or vehicle only (0.5% methyl cellulose) was administered p.o. for 5 days. There was a significant weight loss in rats given the highest dose of YM17E. Liver weight was not affected by YM17E. After 8 days of treatment with the atherogenic diet, serum total cholesterol, triglyceride and phospholipids were elevated significantly (Control group in Table 1 vs Control group in Table 2). Total cholesterol was significantly decreased by YM17E in a dose dependent manner, with percentage decreases of 2.3%, 71% and 75% in groups dosed at 3, 10 and 30 mg/kg, respectively. The ED\textsubscript{50} value of YM17E for serum total cholesterol in this model was calculated to be 8 mg/kg. The decrease in total cholesterol came mainly from a marked reduction in non-HDL cholesterol, including both very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol. HDL cholesterol, however, was increased significantly by YM17E. No change in serum triglyceride level was observed. Serum phospholipid and non-esterized fatty acids were also decreased in YM17E-treated animals.

**Table 1. Effects of YM17E on body and liver weight and serum lipids in rats fed normal chow**

|                | Control | YM17E 3 mg/kg | YM17E 10 mg/kg | YM17E 30 mg/kg |
|----------------|---------|--------------|----------------|---------------|
| Number of animals | 10      | 6            | 6              | 6             |
| Body weight gain (g) | 5.4     | 5.0          | 4.8            | 5.5           |
| Liver weight (g)    | 1.4     | 0.9          | 0.6            | 0.9           |
| Total cholesterol (mg/dl) | 47.5    | 54.7         | 37.8           | 43.3          |
| HDL cholesterol (mg/dl) | 31.8    | 33.3         | 28.3           | 34.7          |
| Non-HDL cholesterol (mg/dl) | 15.7    | 21.3*        | 9.5*           | 8.7**         |
| Free cholesterol (mg/dl) | 7.1     | 8.7          | 6.2            | 6.8           |
| Cholesteryl ester (mg/dl) | 40.4    | 46.0         | 31.7           | 36.5          |
| Triglycerides (mg/dl)  | 47.4    | 51.7         | 31.0           | 38.3          |
| Phospholipids (mg/dl)   | 85.8    | 99.3         | 93.2           | 93.8          |
| Non-esterized fatty acids (mEq/l) | 1024.2  | 1026.3       | 964.0          | 618.5**       |

Data in the table represent means (upper) and standard errors of the means (lower). P values for differences in means of parameters between the control and YM17E-treated groups were determined by the Dunnett's test. *P<0.05, **P<0.01.

**Effect on cholesterol absorption from the gut**

To clarify the mechanism of the cholesterol lowering
Table 2. Effects of YM17E on body and liver weight and serum lipids in rats fed an atherogenic diet

|                      | Control | YM17E 3 mg/kg | YM17E 10 mg/kg | YM17E 30 mg/kg |
|----------------------|---------|---------------|----------------|---------------|
| Number of animals    | 5       | 10            | 6              | 6             |
| Body weight gain (g) | -0.7    | 2.3           | -2.5           | -10.0***      |
|                     | 1.2     | 0.6           | 1.8            | 1.2           |
| Liver weight (g)     | 6.1     | 6.1           | 5.6            | 5.5           |
|                     | 0.3     | 0.3           | 0.3            | 0.3           |
| Total cholesterol (mg/dl) | 363.2 | 354.8         | 104.8***       | 91.3***       |
|                     | 49.5    | 49.7          | 12.3           | 5.6           |
| HDL cholesterol (mg/dl) | 12.8   | 11.0          | 29.0***        | 43.3***       |
|                     | 1.0     | 1.1           | 1.5            | 4.3           |
| Non-HDL cholesterol (mg/dl) | 350.4 | 343.8         | 75.8***        | 48.0***       |
|                     | 49.8    | 49.4          | 12.8           | 4.4           |
| Free cholesterol (mg/dl) | 75.5   | 74.8          | 24.5***        | 24.2***       |
|                     | 10.9    | 11.1          | 3.3            | 2.1           |
| Cholesteryl ester (mg/dl) | 287.7  | 280.0         | 80.3***        | 67.2***       |
|                     | 38.7    | 38.6          | 9.1            | 3.7           |
| Triglycerides (mg/dl) | 34.4    | 41.7          | 35.2           | 27.3          |
|                     | 4.3     | 5.1           | 2.7            | 3.8           |
| Phospholipids (mg/dl) | 16.3    | 16.0          | 97.2***        | 99.8*         |
|                     | 15.4    | 17.4          | 6.5            | 6.1           |
| Non-esterized fatty acids (mEq/l) | 1403.6 | 1452.2        | 972.5**        | 959.5***      |
|                     | 23.5    | 137.1         | 79.0           | 75.3          |

Data in the table represent means (upper) and standard errors of the means (lower). P values for differences in means of parameters between the control and YM17E-treated groups were determined by the Dunnett’s test. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. ACAT activities in the liver (A) and the intestine (B) of rats administered 30 mg/kg/day of YM17E. The error bar shows the standard error of the mean of 10 (control group in panel A) or 6 (control group in panel B, YM17E group in both panels A and B) animals. Other experimental conditions were as described in Table 2. *P < 0.05, **P < 0.001 (Student’s t-test).
effect of YM17E, we first tested whether YM17E inhibited the absorption of cholesterol from the gut in cholesterol-fed rats. Serum total cholesterol decreased at 4 hr after a single administration of YM17E. The maximum decrease lasted another 4 hr, with recovery to the normal level surprisingly not occurring until 2 days later (Fig. 4B). When [3H]cholesterol was orally administered immediately after administrations of the drug or vehicle, radioactivity in the serum of control rats increased rapidly and remained at near-plateau level for 72 hr. This transient increase in serum radioactivity at 4–8 hr was strongly inhibited by 30 mg/kg of YM17E.

Studies on bile excretion in cholesterol-fed rats

Next, the effects of YM17E on cholesterol catabolism in bile excretion were studied using rats fed a cholesterol-enriched diet. Cumulative time-course changes in bile volume and radioactivity in neutral sterols and bile acids fractions are shown in Fig. 5. The volume of secreted bile was not affected by YM17E. In contrast, the secretion of neutral sterols derived from i.v. administered [14C]chole-

Fig. 4. Time course of changes in total radioactivity (A) and total cholesterol (B) in serum after oral administration of [3H]cholesterol (10 µCi/animal) to cholesterol-fed rats pretreated with 30 mg/kg of YM17E. The error bar shows the standard error of the mean of 4 animals. *P < 0.05, **P < 0.01 (Student’s t-test). ●: control, ○: YM17E.

Fig. 5. Effect of YM17E on bile excretion in cholesterol-fed rats. After oral administration of 0 (control ‐ ‐ ‐ ), 10 (■), 30 (□) and 100 (△) mg/kg of YM17E, bile samples were collected every 2 hr until 8 hr, and samples from 8 to 20 hr were pooled. Cumulated changes in bile volumes (left panel), counts in neutral sterol fractions (middle panel) and bile acids fractions (right panel) are shown. The error bar shows the standard error of the mean of 21 (control), 8 (10 mg/kg), 5 (30 mg/kg) and 16 (100 mg/kg) animals. *P < 0.05, **P < 0.01, ***P < 0.001 (Dunnett’s test).
ACAT Inhibitor and Diarrhea

Fig. 6. Effect of YM17E on diarrheal events (A) and serum total cholesterol (B) in cholesterol-fed beagle dogs. Animals received YM17E once per day (Single) or 5 times per day (Divided) as described under Materials and Methods. A: The sum of days during which diarrheal events occurred during the experimental period (2 weeks) is plotted. B: Percent decrease in serum total cholesterol vs pre-value after 2 weeks of administration. The error bar shows the standard error of the mean of 5 animals in each group. *P<0.05, **P<0.01, ***P<0.001 [Steel-Dwass test (A) or Tukey test (B)].

YM17E-induced diarrhea in beagle dogs

Finally, we tested the effects of YM17E in beagle dogs to study the diarrhea-inducing ability of the compound. In the preliminary study, the incidence of episodes of secretory diarrhea with soft or yellow mucous feces increased at dose of 15 mg/kg and higher in hyperlipidemic beagle dogs (frequency of diarrhea in dogs dosed at 0, 5, 15, 30 and 100 mg/kg was 2%, 2%, 33%, 38% and 60%, respectively). Diarrheal episodes also occurred at 50 mg/kg, s.c. in normal chow-fed animals (data not shown). These effects were transient and were reversed by the cessation of medication. Next, we compared the effects of administration at the same daily dosage but given in divided administrations. As shown in Fig. 6B, the potency of the effect of YM17E on serum total cholesterol was significantly stronger on divided administration than on single administration (60% vs 45% decrease compared to control, P<0.01). Furthermore, the incidence of diarrhea in animals receiving divided administration was also significantly lower (P<0.01) (Fig. 6A).

The plasma level of unchanged YM17E on the final day of this study is shown in Fig. 7. The area under the curve (AUC) of the divided administration group was somewhat higher than that of the single administration group (1708 ± 276 vs 1371 ± 163 ng/ml) while the maximum drug concentration (C_max) was lower (160 ± 26 vs 236 ± 30 ng/ml).

DISCUSSION

This study shows that the novel ACAT inhibitor YM17E strongly inhibits cholesterol esterification in microsomes of rabbit liver and intestine. In cholesterol-fed rats, YM17E strongly decreased serum total cholesterol, LDL cholesterol, esterified cholesterol, phospholipids and non-esterified fatty acid levels (Table 2). With regard to the mechanism of dietary cholesterol
induced hypercholesterolemia, Quarfordt et al. (17) showed that a triacylglycerol (TG)-rich particle such as the chylomicron stimulates the excretion of cholesterol-rich \(\beta\)-lipoprotein from hepatocytes. In these animals, dietary cholesterol is uptaken from the intestinal wall through the lymph and carried to the liver, then secreted as \(\beta\)-lipoprotein into the blood. To understand the mechanism of the hypocholesterolemic activity of YM17E, we first tested the effect of YM17E on biliary cholesterol-bile acid excretion in these animals. In this model, YM17E clearly increased the conversion of i.v. injected \([^{14}\text{C}]\)cholesterol into bile acid. Among the constituents of bile, cholesterol content tended to increase. Phospholipids also increased significantly (data not shown). Thomas and Hofmann’s lithogenic index (18) was unchanged, indicating that YM17E protects against gallstone formation by promoting phospholipid excretion into bile. In the same model, YM17E strongly inhibited the appearance of orally administered \([^{3}H]\)cholesterol in the blood, with this effect being parallel with the decrease in blood total cholesterol. In this experiment, YM17E appeared to inhibit the increase of radioactivity in plasma by two mechanisms. First, YM17E have the potency to block absorption of \([^{3}H]\)cholesterol into lymph flow at the intestine, and second, to block the assembly of \([^{3}H]\)-cholesterol into apoB lipoprotein at the liver. ACAT activity in the liver of these animals was inhibited more strongly than that in the intestine in the ex vivo assay (Fig. 3), while there was little difference between the in vitro potency in inhibiting liver and intestinal ACAT (Fig. 2A). We have confirmed in a previous report that the oral administration of radiolabeled YM17E in rats resulted in its specific distribution to the liver (16). However, because it is generally known that an inhibition of cholesterol absorption in the intestine is sufficient to reduce serum cholesterol in cholesterol-fed animals, it is unclear how significantly liver ACAT contributes to the reduction of serum cholesterol in cholesterol-fed animals. We then examined the effect of YM17E in normal chow-fed animals. Although, YM17E had a less potent effect on serum lipid profile in normal rats compared to the dramatic effect in hypercholesterolemic animals, it still significantly lowered non-HDL cholesterol and free fatty acids in these animals (Table 1). These observations were consistent with previous reports on other ACAT inhibitors that also showed hypocholesterolemic activity in normcholesterolemic animals (19). We have not examined the effect of YM17E on apoB protein production, but there is some evidence that ACAT inhibitor reduces hepatic apoB secretion. Carr et al. (7) showed ACAT inhibitors suppressed cholesteryl ester and apoB secretion in perfused liver. Decrease in plasma apoB lipoprotein level after ACAT inhibitor treatment was also reported in endogenous hypercholesterolemic animal models (20, 21). These results obtained in animal studies, however, were not reproduced successfully in HepG2 cells; i.e., Cianflone et al. (6) reported ACAT inhibitor reduced apoB secretion while Wu et al. (22) failed to modify apoB secretion by the same compound. Some alternate experimental procedures for cultured cells must be developed for further understanding of this issue.

YM17E raised HDL cholesterol in rats fed the atherogenic diet (Table 2). A possible explanation for this result is that the increase of free cholesterol on the cell surface by ACAT inhibition may promote the increase in HDL cholesterol with the involvement of the lecithin:cholesterol acyltransferase reaction. HDL cholesterol is also generally known to be related to the plasma triglyceride level. With respect to the ACAT inhibition-induced HDL cholesterol elevation, however, the influence of HDL generation after VLDL or chylomicron-triglyceride degradation by lipoprotein lipase has not been understood well. There was little change in the serum triglyceride level in cholesterol-fed rats by ACAT inhibition, suggesting that YM17E has no effect on lipase-induced HDL cholesterol generation in these animals. A similar result was reported in hamsters (19), but in cholesterol-fed rabbits, inhibition of ACAT decreased plasma TG significantly without affecting HDL cholesterol (23).

The LDL receptor pathway-depended cholesterol lowering effect is likely to be excluded since cholesterol feeding is generally known to down-regulate the LDL receptor mRNA. Furthermore, YM17E itself decreased LDL receptor mRNA in HepG2 cell (data not shown).

Finally, we concluded that the hypocholesterolemic activities of YM17E were due to the inhibition of dietary cholesterol absorption and the promotion of liver cholesterol secretion. Both these effects suggest the generally known risk of secretory diarrhea caused by malabsorption of dietary lipids and excess bile acids. Dominick et al. (24, 25) recently reported diarrheal episodes in ACAT inhibitor-treated beagle dogs, but did not refer to this in detail. We therefore performed several studies designed to clarify the relationship between the serum cholesterol lowering effect and the appearance of diarrhea after YM17E administration in beagle dogs. Results showed diarrhea in beagle dogs at doses that lowered serum cholesterol. The incidence of diarrhea, however, was decreased by reducing per-dose administration without any loss of the hypocholesterolemic activity of the drug (Fig. 6). The \(C_{\text{max}}\) of unchanged YM17E in the plasma of dogs receiving divided administrations was significantly lower, keeping the drug level very constant. In addition, the data shown in Fig. 3 suggests that partial inhibition of the intestinal ACAT is enough to reduce serum cholesterol. These results led us to hypothesize that unnes-
sarily raised YM17E level in plasma by bolus administration resulted in complete inhibition of ACAT in the intestine, disturbing the normal state of lipid absorption. Some explanations for YM17E-induced diarrhea can be postulated: a) disturbed micelle formation due to defective cholesterol absorption, b) extraordinary excretion of bile abnormally formed in the liver, c) functional defects in intestinal epithelium caused by the cytotoxic effect of YM17E-induced free cholesterol accumulation, and d) local stimulation of the intestine by the compound by a non-specific mechanism. The hypothesis b is supported by the previous observations that the common dihydroxy bile acids induce a concentration-dependent mucus secretion when infused into the colon (26) and by the enhanced bile excretion in YM17E-treated rats (Fig. 5). In this study, as shown in Fig. 5, the dose that accelerated bile acid synthesis was slightly higher (30–100 mg/kg) than the cholesterol lowering dose (10–30 mg/kg). This result is consistent with the more frequent occurrence of diarrhea with the bolus administration that resulted in a higher blood level of YM17E than the divided administration. To clarify the contribution of a and b, fecal contents of lipids and bile acids need to be analyzed. The possibility of d is likely to be low because diarrhea was also observed when YM17E was administered by a non-oral route, i.e., subcutaneously (data not shown). With regard to c, Warner et al. (27) recently reported that inhibition of ACAT in mouse peritoneal macrophages resulted in an increase in radiolabeled adenine release, a marker of cell toxicity. ACAT inhibitors including YM17E are generally known to raise cellular free cholesterol fraction. This phenomenon is suspected of causing functional defects with an unfavorable alternation of membrane dynamics by deposited free cholesterol. Only the liver can alleviate the increase in cellular free cholesterol by the mechanism of catabolic excretion into bile. Other tissues including the gut and the adrenal gland, however, do not have such a system. Moreover, these tissues highly depend on the ACAT reaction in order to perform their physiological function (i.e., chylomicron secretion and steroidogenesis) and may suffer more seriously from toxic effects than other tissues. The finding that repeated administration over a day reduced the frequency of diarrhea indicates that this unfavorable episode can be overcome by suitable drug delivery, such as a gradual drug release system, without loss of the hypocholesterolemic activities of this compound.

With regard to the effect of YM17E on the adrenal gland, daily oral administrations of 50 mg/kg for 13 weeks in rats did not cause degeneration nor necrosis of the adrenal gland but decreased intracellular lipid vacuoles in the zona fasciculata. Recent reports has revealed that adrenocortical toxicity is a common risk of ACAT inhibitors. Although we have no information of whether or not the adrenocortical toxicity is linked to diarrhea inducing, this critical toxicity can be avoided by choosing safer compounds by monitoring a marker such as the adrenocortical steroid level in the blood which would provide us with helpful information about the functional state of the adrenal gland. Diarrhea, however, is a nominal parameter and is difficult to quantitatively evaluate. Moreover, there are variations between species in the occurrence of diarrhea. We observed diarrhea in dogs and humans (M. Nakata, Clinical Development Department, Yamanouchi Pharmaceutical Co. Ltd.; unpublished data) but not in rats, hamster and rabbits at the dose of serum cholesterol lowering.

In summary, the novel ACAT inhibitor YM17E inhibited rabbit microsomal ACAT in a non-competitive manner and decreased serum lipids, especially non-HDL cholesterol, in cholesterol-fed rats. YM17E stimulated the excretion of cholesterol from the liver into bile and also inhibited the absorption of cholesterol from the gut in cholesterol-fed rats. Thus, the hypocholesterolemic activity of YM17E depends on both the promotion of cholesterol excretion into bile and the inhibition of extrinsic cholesterol absorption. In beagle dogs, YM17E caused serious diarrhea. The dose of lipid lowering seems to be slightly lower than that of diarrhea inducing, but they are very close, suggesting that these two effects of YM17E were closely related to each other.

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