Effect of the Hypocholesterolemic Agent YM-16638 on Cholesterol Biosynthesis Activity and Apolipoprotein B Secretion in HepG2 and Monkey Liver

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Received June 17, 1998  Accepted October 23, 1998

ABSTRACT—YM-16638 ([(5-[[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propyl]thio]-1,3,4-thiadiazol-2-yl]thio) acetic acid) showed a strong hypocholesterolemic effect in humans and monkeys. To clarify the mechanism of this hypocholesterolemic effect, the action of YM-16638 on cholesterol biosynthesis in the cultured human hepatoma cell line HepG2 and cynomolgus monkey liver was examined. Cholesterol biosynthesis activity derived from [14C]acetate, [3H/14C]mevalonic acid or [14C]isopentenyl pyrophosphate substrates was significantly decreased, but not that from [3H]farnesyl pyrophosphate or [3H]squalene substrates in HepG2 cells treated with YM-16638. Simultaneously, treatment of these cells with YM-16638 changed neither the rate of apolipoprotein B synthesis from [35S]methionine nor its secretion. In addition, the activities of hepatic cholesterol biosynthesis enzymes HMG-CoA reductase, mevalonate kinase (MK), isopentenyl pyrophosphate isomerase (IPPI), farnesyl pyrophosphate synthase (FPPS), squalene synthase and squalene epoxidase were measured in monkeys fed a diet supplemented with YM-16638. Among these enzymes, MK, IPPI and FPPS activities in the YM-16638-treated group significantly decreased by 38%, 56% and 30%, respectively, when compared to those from control animals receiving no drug treatment. These results indicate that YM-16638 has the characteristics of a cholesterol biosynthesis inhibitor.

Keywords: Cholesterol biosynthesis enzyme, Cynomolgus monkey, HepG2 cell, Hypocholesterolemic effect, YM-16638

Unexpectedly, during the course of clinical development as a leukotriene-receptor antagonistic drug, the compound YM-16638 ([5-[[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propyl]thio]-1,3,4-thiadiazol-2-yl]thio) acetic acid) showed a strong hypocholesterolemic effect without any serious side effects in normolipidemic human subjects. The leukotriene antagonist effects of this compound have been demonstrated in vitro and in vivo, and have been shown to arise by its similar acetonaphone structure to leukotriene D4, competing for the receptor binding site (1–3). It has also been previously reported that this hypocholesterolemic effect was species-specific and was only recognized in humans and old-world monkeys such as rhesus monkeys (4).

LY-171883 (Eli Lilly Co., Ltd., Indianapolis, IN, USA), which like YM-16638 has an acetonaphone structure, has also shown a potent anti-leukotriene effect in vitro and in vivo (5–7). LY-171883 is known to decrease blood triglyceride levels in rats and monkeys with a proliferatory action similar to that of clofibric acid (8) and to slightly decrease blood total cholesterol in rats by chronic treatment (9). However, little is known about either the mechanism of the hypolipidemic effect of this compound or the relevance of the hypocholesterolemic effect to the anti-leukotriene effect. Therefore, the mechanism of this hypocholesterolemic effect of YM-16638 in culture cells and monkeys was examined to partially elucidate the mechanism of its cholesterol-lowering effect. In this report, the inhibitory effects of YM-16638 on cholesterol biosynthesis enzymes in the human hepatoma cell line HepG2 and monkey liver are described.

MATERIALS AND METHODS

Materials

YM-16638 was synthesized at Yamanouchi Pharmaceut-
tical Co., Ltd. (Tokyo). Acetic acid, sodium salt, [1-14C]-
2.1 GBq/mmol; 3-hydroxy-3-methylglutaryl coenzyme A, dicarbonyl-[glutaryl-3-14C]-, 2.1 GBq/mmol; mevalonic acid DBED salt, dil-[mevalonic-2-14C]-, 2.2 GBq/mmol; meva-
lonic acid lactone, RS-[5-3H(N)], 1295.0 GBq/mmol;
farneisl pyrophosphate, trimmonium salt, [1-3H(N)],
995.3 GBq/mmol; and squalene, [4,8,12,13,17,21,23-H]-
477.3 GBq/mmol were purchased from New England Nuclear (Tokyo). Isopentenyl pyrophosphate, trimmoni-
unium salt, [1-14C]-, (1.85 GBq/mmol) and geranyl pyrophosphate, trimonium salt [1-2H]-, (370 GBq/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), and L-[35S]methionine (43.5 TBq/mmol) from Amersham (Tokyo). Human hepatoma cell line HepG2 cells (ATCC HB 8065) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Fetal calf serum (FCS) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA). Fetal calf lipoprotein-deficient serum (LPDS) was from Sigma (St. Louis, MO, USA). Anti-human apolipoprotein B (apoB) IgG (sheep IgG fraction) was purchased from The Binding Site Ltd. (Birmingham, UK). Human apoB standard serum was purchased from Daiichi Pure Chemicals (Tokyo). All other reagents were commercial high-purity materials obtained from standard sources.

Animals
Male cynomolgus monkeys (Macaca irus; 4.5-5.0 kg, n=9) were purchased from Hamri Co., Ltd. (Ibaraki) and maintained on a 12-hr light cycle (on 7:00-19:00). Monkeys were fed a banana (approx. 100 g per day) and a commercial solid diet (50 g, Primate diet 5048; PMI Feeds, Inc. (St. Louis, MO, USA), obtained from Oriental Yeast Co., Ltd. (Tokyo); containing 5% fat (including 4.3% cholesterol) supplemented with vitamin C (5 mg/kg/day).

Cholesterol biosynthesis activity in HepG2 cells
HepG2 cells were seeded into a 6-well plate at a density of 5 x 10^5 cells/well in Eagle’s minimal essential medium (MEM) supplemented with 10% FCS. After 48-hr incu-
bation, the medium was replaced with 10% FCS-MEM containing one of the following: [14C]acetic acid (2.1 GBq/mmol), [3H]mevalonic acid lactone (1295.0
GBq/mmol), [14C]isopentenyl pyrophosphate (1.85 GBq
/mmol), [3H]farneisl pyrophosphate (995.3 GBq/mmol) or
[3H]squalene (477.3 GBq/mmol). Media were then either supplemented with YM-16638 at the concentration of 10^-4 M or had no YM-16638 present. At the indicated times, the medium and cells were separated, and then the total lipids were extracted according to the method of Bligh and Dyer (10). Cholesterol in the total lipid fraction was separated by thin-layer chromatography (TLC) using a Silica Gel plate (Merck 60) with a petroleum ether / diethyl ether / acetic acid (80:30:1) solvent mixture (11). Radioactivity of the fraction corresponding to choles-
terol was measured by liquid scintillation counting (LSC) (Model 5003; Beckman, Palo Alto, CA, USA). Total cell protein content was measured by Lowry’s standard method (12).

ApoB synthesis and secretion in HepG2 cells
HepG2 cells were seeded into a 12-well plate (10^5 cells/well) and incubated in 10% FCS-MEM for 4 days. After incubation, the medium was exchanged with fresh 10% FCS-MEM, 1 µmol [14C]mevalonic acid DBED salt (2.1 MBq) and 22 µmol [35S]methionine (1 MBq) and either YM-16638 (10^-5 M-10^-4 M) or cycloheximide (CHX) (10^-6 M), and incubated for 12, 24 or 48 hr.

After treatment, cells were washed with phosphate buffered saline and harvested with buffer A (50 mM Tris /HCI, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulphonylfluoride, 1 µg/ml pepstatin, 1.5% soybean trypsin inhibitor). The cells were disrupted by sonication at 4°C. Each sample was mixed with 50 µg of anti-apoB IgG and the mixture incubated for 12 hr at 4°C. One half milliliter of immuno precipitant (100 mg/ml) was then added and incubation continued for 1 hr at room temperature with gentle stirring. The immunoprecipitin complex was precipitated by centrifugation at 2500 rpm for 20 min, washed twice with buffer A and resuspended in buffer B (5 mM Tris /HCl, pH 6.8, 20% glycerol, 10 mM dithiothreitol, 2% 2-mercaptoethanol, 5% sodium dodecylsulphate) (13, 14). After boiling for 5 min, the supernatant was analyzed by gradient SDS-PAGE (4-16%). The band corresponding to standard human apoB was calculated with an Auto Imaging Analyzer BAS2000 (Fuji Film, Tokyo). Labeled apoB secreted into the medium was measured with the same protocol. The lipid fraction containing [14C]choles-
terol in harvested cells and medium was extracted, and each lipid was separated by TLC. The radioactivity of the corresponding bands was measured by a liquid scintillation counter.

Preparation of enzyme source for measurement of cho-
sterol biosynthesis activity
Monkeys in both YM-16638 and control groups were sacrificed after overnight (12 hr) fasting and the liver was promptly excised. Both the liver homogenate and the microsome for measurement of cholesterol biosynthesis enzyme activities were prepared immediately at 4°C. A 1-
g portion of each fresh monkey liver was homogenized in 10 ml of 20 mM Tris HCI buffer with 5 strokes in a Teflon glass homogenizer. Homogenates for the measurement of mevalonate kinase (MK), squalene synthase (SOS) or
squalene epoxidase (SQE) activity were obtained by centrifugation at 10^4 g for 15 min. For measurement of HMG-CoA (Δ5-3-hydroxy-3-methylglutaryl-coenzyme A) reductase (HMGR) activity, the microsomal fraction was obtained by further ultracentrifugation between 3 × 10^5 g·min and 6 × 10^5 g·min.

Enzyme sources for the measurement of isopentenyl pyrophosphate isomerase (IPPI) and farnesyl pyrophosphate synthase (FPPS) were obtained by partial purification (15, 16). Briefly, after homogenization in 10 mM acetate buffer (pH 7.1) containing 10 mM 2-mercaptoethanol, the pH of the homogenate was adjusted to 5.2 with 3.5 N Na-acetate followed by ultracentrifugation at 1.35 × 10^5 g·min. The resulting supernatant was brought to 60% saturation with solid ammonium sulfate, stirred gently for 1 hr on ice and then centrifuged at 1.5 × 10^5 g·min. The precipitated material was dissolved in 10 ml of phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and dialyzed against 1 l of the same buffer for 16 hr at 4°C. Enzyme sources containing both IPPI and FPPS with a higher specific activity were obtained by using fractionation performance liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Tokyo) with a DEAE-Toyopearl 650M column (20 × 180 mm; Tosoh Co., Tokyo) under a linear gradient of NaCl from 0 to 1 M at 4°C. A fraction enriched with both IPPI and FPPS was eluted with 0.18 M NaCl.

Measurement of cholesterol biosynthesis enzyme activities in monkey liver

HMGR activity was determined by measurement of the conversion of [3H]HMG-CoA to [3H]mevalonic acid in an assay mixture that employed an NADPH-generating system (17). In this system, rat liver microsomes showed a linear increase in activity up to 100 µg protein and then reached a plateau. The radioactivity of [3H]mevalonate metabolite was 5,200 dpm using 500 µg protein and incubating for 20 min. In contrast, activity in both cynomolgus monkey liver homogenate and microsomes was extremely low at enzyme levels up to 2 mg protein (less than 100 dpm under the same assay condition).

MK activity was assayed according to the previously described method (18). The assay solution contained 50 mM Hepes (pH 7.4), 2 mM ATP, 4 mM MgCl2, 2 mM DTT, 10 mM KF, 1 mM EDTA, 10 mM iodoacetamide, [3H]mevalonic acid (10 kBq/assay, 2 GBq/mmol) and 20 µg of protein. Incubation was carried out for 15 min at 37°C and stopped by the addition of 1 N HCl. The radioactivity of the [3H]mevalonate 5-phosphate product separated by TLC with n-butanol / formic acid / water (77:10:13) as solvent was measured. The Rf value of mevalonate 5-phosphate was 0.25.

IPPI activity was measured as previously described (19). The reaction tubes contained, in a final volume of 1.0 ml, 20 mM Tris-HCl buffer, pH 7.7, 5 mM MgCl2, 1 mM DTT, 2.3 µM [3H]isopentenyl pyrophosphate (1.35 MBq/ml, 1.85 GBq/mmol) and 20 µg protein. Incubation was started by the addition of enzyme and incubated for 15 min at 37°C. The reaction was terminated by the addition of 1 N HCl, and then the mixture was incubated for 15 min to complete the hydrolysis of acid-labile allylic pyrophosphate. The mixture was then made alkaline by the addition of 0.5 ml of 1 N NaOH, and the free alcohol liberated was extracted with 4.0 ml of hexane. The hexane extract was washed with water, and the radioactivity in a 2-ml portion of the extract was counted by LSC.

FPPS activity was measured as in the IPPI assay except for the addition of 2.7 µM geranyl pyrophosphate to the solution (19, 20). One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [3H]isopentenyl pyrophosphate into allylic pyrophosphate per min. Specific activity is expressed in units of enzyme activity per mg of protein.

SQS activity was assayed by measuring the rate of formation of [3H]squalene from [3H]farnesyl pyrophosphate (21). The reaction tube contained, at a final volume of 200 µl, 50 mM Hapes (pH 7.5), 11 mM NaF, 5.5 mM MgCl2, 3 mM DTT, 1 mM NADPH, 10 µM NB-598 as an inhibitor for squalene epoxidase, [3H]farnesyl pyrophosphate (1,850 Bq/assay, 2.5 µM) and 50 µg of protein. The reaction was started by the addition of enzyme and incubated for 20 min at 30°C following pre-incubation for 5 min at 30°C. At the end of incubation, 100 µl of 40% KOH-EtOH was added and the incubation was continued for 30 min at 65°C. After saponification, the squalene product was extracted with 3 ml of petroleum ether and the radioactivity of a 1-ml aliquot was counted by LSC.

SQE activity was assayed with [3H]squalene as described previously (22). The assay buffer contained 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM NADPH, 0.1% Triton X-100, 0.1 mM FAD, 0.1 mM AMO1618, an inhibitor of 2,3-oxidosqualene lanosterol cyclase, [3H]squalene (833 Bq/assay, 10 µM) dispersed in 0.075% Tween 80 and 100 µg of protein. Incubation was carried out for 90 min at 37°C. After saponification by the addition of 300 µl of 15% KOH-EtOH, the reaction product was extracted with petroleum ether and separated by TLC with benzene / ethyl acetate (99.5:0.5) as a solvent. The band corresponding to authentic 2,3-oxidosqualene (Rf = 0.5) was scraped into a vial and the radioactivity was measured by LSC.

Statistical analyses
Statistical significance within each group was calculated by the paired Student's t-test. Differences between groups were analyzed by one-way analysis of variance (ANOVA).
RESULTS

Cholesterol synthesis inhibition in HepG2 cells

To study the inhibitory effect of YM-16638 on cholesterol biosynthesis, we measured the time course change of cholesterol biosynthesis activity in the human hepatoma cell line HepG2 using \(^{3}H\)mevalonic acid lactone as a substrate. As shown in Fig. 1, biosynthesis activity of \(^{3}H\)cholesterol from \(^{3}H\)mevalonic acid lactone was significantly decreased by 48 hr in the YM-16638-treated group in both medium and cells (51% and 40% versus control, respectively). This result suggests that YM-16638 decreased cholesterol biosynthesis activity in HepG2 cells by at least inhibiting an enzyme activity downstream of mevalonate.

To identify the enzyme(s) in the cholesterol biosynthesis pathway inhibited by treatment with YM-16638, the \(^{14}C\)cholesterol biosynthesis activity in HepG2 cells was measured using the radiolabeled intermediates, \(^{14}C\)acetic acid, \(^{3}H\)mevalonic acid lactone, \(^{14}C\)isopentenyl pyrophosphate, \(^{3}H\)farnesyl pyrophosphate and \(^{3}H\)squalene. After cells grew to near confluency, the medium was replaced with fresh 10% FCS-α-MEM supplemented with each substrate. The cells were then treated with or without YM-16638 and incubated for 24 hr at 37°C. Cholesterol biosynthesis rate using these substrates was calculated as percentage change versus control activity. As shown in Fig. 2, the cholesterol biosynthesis activity from \(^{14}C\)acetic acid, \(^{3}H\)mevalonic acid lactone and \(^{14}C\)isopentenyl pyrophosphate was significantly decreased in the cells treated with YM-16638 (66%, 60% and 47%, respectively). In contrast, \(^{3}H\)cholesterol biosynthesis activity using either \(^{3}H\)squalene or \(^{3}H\)farnesyl pyrophosphate did not change significantly in YM-16638-treated cells. Similar results were also obtained when monkey liver slices were used in the radiolabeled incor-

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Fig. 1. Inhibitory effect of YM-16638 on the cholesterol biosynthesis activity in HepG2 cells. HepG2 cells were incubated with 10% FCS-α-MEM supplemented with (■) or without (□) YM-16638 (10⁻⁴ M) and \(^{3}H\)mevalonic acid lactone (3.7 x 10⁷ Bq/ml medium) for the indicated times (6, 12, 24 and 48 hr). Total lipids in the medium (a) or cells (b) were extracted with chloroform/methanol (2:1), fractions were separated by TLC, and the radioactivity of cholesterol was measured. Each value represents the mean and range of values from three separate experiments. Statistical significance of the difference between the control and YM-16638 groups is shown as *P<0.05, **P<0.01.

Fig. 2. Inhibitory effect of YM-16638 on \(^{3}H/^{14}C\)cholesterol synthesis using labeled intermediates in HepG2 cells. HepG2 cells were incubated for 48 hr at 37°C in 10% LPDS-α-MEM containing only one labeled intermediate each with YM-16638 (10⁻⁴ M). After incubation, the cells were harvested and the cholesterol was extracted, separated and the radioactivity measured. These experiments were repeated a total of two to three times with essentially the same percentage changes. Statistical significance of the difference between the control and YM-16638 groups is shown as *P<0.05, **P<0.01. AcOH, \(^{14}C\)acetic acid; MVAL, \(^{3}H\)mevalonic acid lactone; IPP, \(^{14}C\)isopentenyl pyrophosphate; FPP, \(^{3}H\)farnesyl pyrophosphate; SQ, \(^{3}H\)squalene.
poration assay (data not shown). These results indicate that YM-16638 inhibits cholesterol biosynthesis located between farnesyl pyrophosphate and mevalonate or acetic acid.

**ApoB synthesis and secretion**

The incorporation rate of $[^{35}]$methionine into apoB in HepG2 cells and its secretion into the medium was measured (Fig. 3: a and b). The incorporation rate in controls showed an increase in a time-dependent manner. The translational inhibitor CHX strongly inhibited apoB protein synthesis in HepG2 cells. In contrast, YM-16638 treatment did not affect the intracellular level of $[^{35}]$methionine-labeled apoB content when compared to control treatment. At the concentration of $10^{-4}$ M with incubation for 48 hr, the radiolabeled apoB excretion rate into the medium was slightly decreased (89% of control, but no statistical significance). At the same time, newly synthesized $[^{14}]$cholesterol from $[^{14}]$mevalonate increased in a time-dependent manner in both cells and medium (Fig. 3: c and d), and the cholesterol level in cells treated with $10^{-3}$ M YM-16638 showed a significant decrease after 48 hr, and in those treated with $10^{-4}$ M, after 24- and 48-hr incubation.

**Cholesterol biosynthesis inhibition in monkeys**

The inhibitory effect of YM-16638 on cholesterol biosynthesis enzyme activities in monkey liver treated with YM-16638 was verified. Cynomolgus monkeys were fed a diet supplemented with or without YM-16638 at a daily dose of 60 mg/kg/day for 4 weeks. After treatment, the YM-16638 group showed a marked decrease in total blood cholesterol levels (baseline: 184±12 and 182±17 mg/dl and at 4 weeks: 181±13 and 136±11 mg/dl, in the control and YM-16638 group, respectively); these results are closely similar to the data obtained previously (4, 23).

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**Fig. 3.** Effect of YM-16638 on $[^{35}]$apoprotein B synthesis and secretion in HepG2 cells. HepG2 cells were incubated for 12, 24 and 48 hr at 37°C in 10% FCS-α-MEM containing $[^{35}]$mevalonic acid and 1 MBq of $[^{35}]$methionine with cycloheximide (CHX, $10^{-6}$ M), YM-16638 ($10^{-3}$ M, $10^{-4}$ M) or vehicle (Control). After incubation, $[^{35}]$labeled-apoB in cells (a) and medium (b) was recovered by immunoprecipitation with an antibody to apoB followed by SDS-PAGE (gradient gel 4–16%). The radioactivity of each corresponding band was measured and calculated with an Auto-Imaging Analyzer. $[^{14}]$C/Cholesterol in cells (c) and medium (d) was extracted. The corresponding band was localized by TLC, cut out, and the radioactivity was counted in a liquid scintillation counter. Statistical significance of the difference between the control and YM-16638 groups is shown as *P<0.05, **P<0.01.
Additionally, we found that three enzyme activities, MK, IPPI and FPPS, showed significant decreases. In contrast, the activities of enzymes located downstream of farnesyl pyrophosphate, SQS and SQE, did not change (Fig. 4a). HMGR activity was undetectable in both groups under the conditions we used. In contrast, YM-16638 did not inhibit any of these enzyme activities in vitro even when added at a concentration of 10^{-4} M (Fig. 4b). The most interesting result is that this compound suppressed the three enzyme activities, MK, IPPI and FPPS, only ex vivo.

**DISCUSSION**

Human hepatoma cell line HepG2 cells are known to have cholesterol homeostasis similar to that of human liver cells in terms of cholesterol biosynthesis, bile acid synthesis and secretion of a number of liver-specific proteins such as lipoproteins (24–26). Therefore, this study employed these cells for the easy investigation of the mechanism of the hypocholesterolemic effect of YM-16638 observed in humans and monkeys.

Treatment of HepG2 cells with YM-16638 caused a significant decrease in cholesterol biosynthetic activity when [1C]acetate, [3H/14C]mevalonate, or [3C]isopentenyl pyrophosphate. However, such inhibitory effects were not observed when [3H]farnesyl pyrophosphate and [3H]squalene was used as substrate (Figs. 1, 2, 3c and 3d). The cholesterol biosynthesis rate from each substrate was calculated as a percentage versus control treatment since the uptake rate of each radiolabeled substrate into cells may be different.

We have focused on the direct influence of this compound on synthesis and secretion of apoB, which is known as a major component of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) particles. Sato et al. demonstrated that neither inhibition of cholesterol synthesis is associated with any changes in the synthesis and secretion of apoB (27). The purpose of this experiment was to investigate whether the inhibitory effect on cholesterol biosynthesis of YM-16638 are due to modulation of apoB synthesis rate. However, our results indicated that YM-16638 caused little change in the amount of newly synthesized and secreted apoB protein in HepG2 cells (Fig. 3). In addition, no major change was detected in the activity and the mRNA level of microsomal triglyceride transfer protein (MTP), which is related to the assembly of apoB and lipid components into VLDL particle and their secretion (28). Although additional experiments are required to elucidate whether this compound influences the apolipoprotein metabolism, such as pulse chase studies to look at apoB degradation rate in the cells and studies on other factors necessary for synthesis and secretion of the apolipoprotein, it is inferred that the hypocholesterolemic effect of YM-16638 is primarily due to the inhibition of the cholesterol biosynthesis pathway and/or the enhancement of LDL-receptor activity (23).

Interestingly, YM-16638 inhibited in vivo activities of three enzymes, MK, IPPI and FPPS (Fig. 4a). However, none of these enzyme activities was inhibited directly when the compound was added to a reaction solution (Fig. 4b). These results suggest that an active metabo-
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Amo(s) may be produced in the cells during incubation and inhibit cholesterol biosynthesis activity. A major metabolite obtained from blood and urine was thiaazol (M1) produced by reduction of YM-16638. However, M1 itself had neither an anti LTD₄ effect nor a cholesterol inhibitory effect in vitro (data not shown).

Regarding HMG-CoA reductase activity in monkey liver, our results indicated an undetectable level. Judging from the human data indicating that diurnal variation of cholesterol formation exists and the highest rate of cholesterol synthesis occurs at midnight (29), the HMG-CoA reductase activity in monkey liver may be low at 10 a.m. Our results do not exclude the possibility of the inhibition of upstream enzymes such as HMG-CoA synthase and HMG-CoA reductase (Fig. 2). We assume that the hypocholesterolemic effect of this compound was due in part to an inhibitory effect on the cholesterol biosynthetic pathway between mevalonate and farnesylpyrophosphate. Further study on the target molecule(s) that suppresses the activities of the three enzymes, MK, IPPI and FPPS, is currently ongoing (Fig. 5).

In summary, these results indicate that YM-16638 decreases blood total cholesterol level partly due to the inhibition of de novo cholesterol biosynthesis. The inhibition of three enzyme activities (MK, IPPI and FPPS) in cynomolgus monkeys and in HepG2 cells may be caused by minor metabolite(s). This novel hypocholesterolemic compound proved effective in humans and monkeys and

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**Fig. 5.** An outline of the cholesterol biosynthetic pathway and a proposed model for the inhibitory effect of YM-16638.
should attract great attention, not only as a tool for the clarification of mechanisms of cholesterol regulation but also as a clinical agent as well.

Acknowledgments
We are grateful to Professors Kyozo Ojima (Tohoku University), Motohito Takeya (Kumamoto University) and Tsunehiko Kodama (Tokyo University) for their helpful discussions. We thank Drs. Yuichi Iizumi and Toichio Takenaka for helpful discussions. We thank Tohru Ugawa and Hiroshi Mori for their excellent technical assistance and thank Nobuyoshi Fukuda for his assistance in the preparation of this manuscript.

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