

Effect of ER-27856, a novel squalene synthase inhibitor, on plasma cholesterol in rhesus monkeys: comparison with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors

Hironobu Hiyoshi,¹ Mamoru Yanagimachi, Masashi Ito, Issei Ohtsuka, Ichiro Yoshida, Takao Saeki, and Hiroshi Tanaka

Eisai Company, Ltd., Tsukuba Research Laboratories, Tsukuba, Ibaraki 300-2635, Japan

Abstract Squalene synthase (SQS; EC 2.5.1.21) plays an important role in the cholesterol biosynthetic pathway. We discovered ER-28448, 5-*N*-[2-butenyl-3-(2-methoxyphenyl)]-*N*-methylamino-1,1-penthylidenebis(phosphonic acid) trisodium salt, as a potent and selective inhibitor of SQS. ER-28448 inhibited SQS in rat liver microsome with an IC₅₀ value of 3.6 nM. We also prepared ER-27856, the tripivaloyloxymethyl ester prodrug of ER-28448. Although less active than ER-28448 in a liver microsome assay, ER-27856 more potently inhibited cholesterol biosynthesis in rat hepatocytes; and ER-27856 orally inhibited de novo cholesterol biosynthesis in Sprague-Dawley rats, with an ED₅₀ value of 1.6 mg/kg. In HepG2 cells, ER-27856 upregulated low density lipoprotein receptor activity to 2.1 times that of control. A time-course study indicated that the inhibitory effect of ER-27856 on cholesterol biosynthesis in rats continued for up to 8 h. In a study of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMGRIs), atorvastatin actively suppressed cholesterol biosynthesis for 8 h, whereas the effect of pravastatin and simvastatin diminished at 4 and 8 h, respectively. In rhesus monkeys, 4 days of oral administration of ER-27856 lowered plasma total cholesterol (TCHO) more potently than did these HMGRIs. Whereas atorvastatin significantly elevated plasma alanine aminotransferase, a marker of hepatotoxicity, to 3.7 times at 100 mg/kg, ER-27856 increased the level only 1.4 times at 10 mg/kg, at which doses the hypocholesterolemic effect was equivalent. During 28 days of administration, ER-27856 reduced TCHO and non-high density lipoprotein (non-HDL) cholesterol by 72 and 95%, respectively. These results demonstrate that ER-27856 had more potent hypocholesterolemic activity and less hepatotoxic effect than HMGRIs. ER-27856 may contribute to the treatment of hypercholesterolemic patients.—Hiyoshi, H., M. Yanagimachi, M. Ito, I. Ohtsuka, I. Yoshida, T. Saeki, and H. Tanaka. **Effect of ER-27856, a novel squalene synthase inhibitor, on plasma cholesterol in rhesus monkeys: comparison with HMG-CoA reductase inhibitors.** *J. Lipid Res.* 2000. 41: 1136–1144.

Supplementary key words ER-27856 • squalene synthase • HMG-CoA reductase • enzyme inhibition • cholesterol biosynthesis • cholesterol-lowering • rhesus monkeys

Elevated plasma cholesterol, especially low density lipoprotein (LDL) cholesterol, is one of the major risk factors for atherosclerosis and coronary heart disease. A number of studies have shown that a decrease in plasma cholesterol leads to a reduction in the incidence of death by coronary heart diseases. The Lipid Research Clinics Coronary Primary Prevention Trial has concluded that every 1% reduction in plasma total cholesterol (TCHO) leads to a 2% decrease in risk of coronary heart disease (1). Reduction of plasma cholesterol can be achieved by inhibiting cholesterol biosynthesis. 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR; EC 1.1.1.34) is a key rate-limiting enzyme involved in the cholesterol biosynthetic pathway (2). Several inhibitors of the enzyme, now clinically available (3, 4), effectively lower plasma cholesterol levels; these inhibitors include pravastatin, simvastatin, and atorvastatin. Long-term treatment with HMG-CoAR inhibitors (HMGRIs) decreased the incidence of coronary events and improved survival rates in primary prevention (5) and secondary prevention trials (6).

HMGRIs occasionally induce adverse effects in liver and muscle. HMGRIs suppress the production of mevalonate, which is also used for the synthesis of nonsterol products such as isopentenyl tRNA, dolichol, coenzyme Q10, and isoprenylated proteins, and the depletion of these metabolites occasionally causes elevation of hepatic transaminase levels (7, 8). In rabbits, lovastatin-induced elevation of serum alanine aminotransferase (ALT) activity was completely prevented by coadministration of mevalonate but not by cholesterol supplementation (9). In dogs, sim-

Abbreviations: ALT, alanine aminotransferase; DiI, 3,3'-dioctadecylindocarbocyanine; FPP, farnesyl pyrophosphate; HDL, high density lipoprotein; HMG-CoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGRIs, HMG-CoA reductase inhibitors; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; LPDS, lipoprotein depleted fetal bovine serum; SCAP, SREBP cleavage-activating protein; SQS, squalene synthase; SREBP, sterol regulatory element binding protein; TCHO, total cholesterol; tri-POM, tripivaloyloxymethyl.

¹ To whom correspondence should be addressed.

vastatin decreased the myocardial level of coenzyme Q10 (10) and enhanced the contractile dysfunction of the myocardium in association with ATP reduction after reperfusion subsequent to ischemia (11). These data suggest that lipophilic HMGRI deteriorate the myocardial mitochondrial respiration during ischemia, and this side effect may restrict its beneficial application in the prevention of coronary heart disease.

To develop a new hypolipidemic agent and to avoid the adverse effect of mevalonate depletion, inhibition of cholesterol biosynthesis beyond the step of mevalonate production has recently attracted much attention. Squalene epoxidase (EC 1.14.99.7), oxidosqualene cyclase (EC 5.4.99.7), and lanosterol demethylase (CYP51, EC1.14.14) have been considered as targets to suppress cholesterol biosynthesis, but the inhibitors of these enzymes accumulated toxic lipophilic substrates in the cell (12, 13). Therefore, the most attention has been paid to inhibitors of squalene synthase (SQS; see refs. 14–24). Because SQS catalyzes the first step committed to cholesterol and forms squalene by the reductive dimerization of two molecules of farnesyl pyrophosphate (FPP; see ref. 25), an inhibitor of SQS would therefore be an ideal cholesterol-lowering agent.

We discovered ER-28448 and its prodrug ER-27856 as potent and selective inhibitors of SQS. In this article we report that ER-27856 exhibits the more potent activity in decreasing plasma cholesterol in rhesus monkeys, with less hepatotoxic effect than HMGRI.

MATERIALS AND METHODS

Reagents and animals

5-*N*-[2-Butenyl-3-(2-methoxyphenyl)]-*N*-methylamino]-1,1-penthyldienebis(phosphonic acid) trisodium salt, or ER-28448, and ER-27856 (the tripivaloyloxymethyl [tri-POM] ester prodrug of ER-28448) were synthesized in our laboratories (Fig. 1). Atorvastatin calcium was synthesized according to a method reported previously (26, 27). Pravastatin sodium and simvastatin were purchased from Sankyo (Tokyo, Japan) and Banyu Pharmaceuticals (Tokyo, Japan), respectively. Insulin and dexamethazone were purchased from Sigma (St. Louis, MO). Penicillin and streptomycin were from Life Technologies (Rockville, MD). Sprague-

Dawley rats were from Japan SLC (Shizuoka, Japan) and rhesus monkeys were from Charles River Laboratories (Key Lois, FL).

Preparation of rat liver microsomes

Lysosome-free and phosphatase-inactivated microsomes were prepared by modifying methods previously described (28, 29). Rat liver was removed; weighed; placed into 9 volumes of cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 10 mM EDTA, 10 mM dithiothreitol, and 50 mM sodium fluoride; minced with scissors; and homogenized with a loose-fitting Teflon homogenizer. The homogenate was centrifuged at 16,000 *g* for 15 min in a Beckman (Fullerton, CA) 50.2 Ti rotor at 4°C. The supernatant was centrifuged again at 16,000 *g* for 15 min. The clear supernatant was carefully collected and centrifuged at 105,000 *g* for 60 min. Precipitated microsomes were resuspended in 0.25 M sucrose at 1 mL/*g* of liver (approximately 20 mg of protein/mL) and stored at –80°C until use.

SQS activities in rat liver microsomes

SQS activities were determined by monitoring the conversion of [^3H]FPP into squalene as previously described (30) with some modifications. Reaction mixtures (500 μl) included 50 mM Tris-HCl (pH 7.4), 2 mM KF, 1 mM MgCl_2 , 1 mM NADPH, microsomes (0.1 mg of protein/mL), 10 μM [^3H]FPP (370 MBq/mmol, 3.7 kBq/mL; New England Nuclear, Boston, MA), and compounds of various concentrations. Reactions were started by adding [^3H]FPP. After a 10-min incubation at 37°C, reactions were terminated by adding 1 mL of ethanol. After 1 mL of H_2O was added, the mixtures were vigorously shaken with 3 mL of petroleum ether for 30 min. Extracted lipids were evaporated and resuspended in 25 μl of chloroform. Samples were applied to plastic-backed sheets (Silica gel 60, F254; Merck, Rahway, NJ) for thin-layer chromatography (TLC), and developed in heptane for 15 min. Radioactivities included in the squalene fraction were measured by liquid scintillation counting.

Preparation of rat primary hepatocytes

Primary hepatocytes were prepared from male Sprague-Dawley rats (150–200 g) as previously described (31). Briefly, rat liver was perfused with liver perfusion medium (GIBCO-BRL, Gaithersburg, MD) at 37°C for 15 min at 20 mL/min. The liver was then perfused with liver digest medium (GIBCO-BRL) for another 15 min. Hepatocytes were dispersed in Williams' E medium (pH 7.4) supplemented with 10% fetal bovine serum, 1 μM insulin, 1 μM dexamethazone, penicillin (100 units/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) by dissection and gentle shaking. After filtration through a 70- μm pore size nylon mesh filter, hepatocytes were isolated by centrifugation at 50 *g* for 2 min, repeated three times. Hepatocytes with >90% viability were cultured in type I collagen-coated 24-well plates (Iwaki, Chiba, Japan) at 2×10^5 cells/well. After 2 h of incubation in a 37°C, 5% CO_2 incubator, nonattached cells were removed by washing. Cells were incubated in culture medium at 37°C for 24 h before use.

Measurement of cholesterol biosynthesis activities in cells

Inhibition of cholesterol biosynthesis was determined by measuring the conversion of [^{14}C]acetate into cholesterol. The cells were incubated with compounds in the presence of [^{14}C]acetate (370 kBq/mL, 11.67–2.22 GBq/mmol; New England Nuclear) for 2 h. The media were removed and the cells were harvested in 1 mL of 4 N KOH. After adding an equal volume of ethanol, cell extracts were saponified at 65°C for 2 h. After addition of 1 mL of H_2O , nonsaponifiable lipids were extracted with 3 mL of petroleum ether. Evaporated extracts were resuspended in 25 μl of chloroform, applied onto plastic-backed TLC sheets, and developed with toluene–isopropyl ether (1:1). Cholesterol frac-

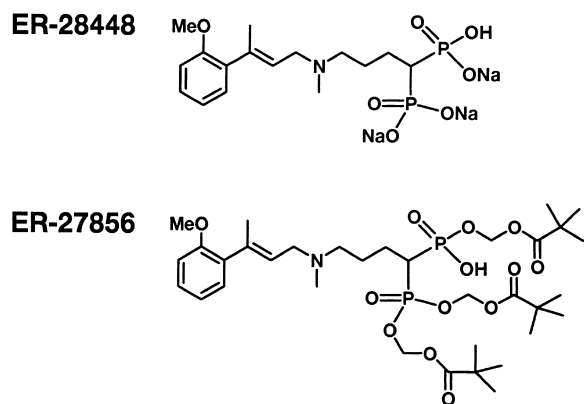


Fig. 1. The structures of ER-28448 and ER-27856.

tions were cut, and the radioactivities were measured by liquid scintillation counting.

Measurement of cholesterol biosynthesis activity in rats

The inhibition of de novo cholesterol biosynthesis in rats was determined by quantitating the conversion of intraperitoneally administered [^{14}C]acetate into plasma cholesterol as previously described (32) with some modifications. Compounds were intravenously or orally administered to male Sprague-Dawley rats (130–200 g). At the indicated times after administration, [^{14}C]acetate (555 kBq, 2,000 MBq/mmol) was intraperitoneally injected. One hour later, blood samples were taken from the abdominal aorta and 2-mL aliquots of heparinized plasma were prepared by centrifugation (1800 g, 10 min). After saponification in the presence of 1.2 N KOH and 25% ethanol at 75°C for 30 min, samples were extracted with 3 mL of petroleum ether. Evaporated nonsaponifiable lipids were separated by TLC with a developing solvent of toluene–isopropyl ether (1:1), and the radioactivities in cholesterol fractions were determined by liquid scintillation counting.

HepG2 cells

HepG2 cells, a human hepatoma cell line, were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). Subconfluent HepG2 cells grown in type I collagen-coated 24-well plates were preincubated with DMEM supplemented with 10% lipoprotein-depleted fetal bovine serum (LPDS) for 24 h before the experiments.

Isolation of DiI-labeled LDL

Human LDL was labeled with 3,3'-dioctadecylindocarbocyanine (DiI) by modifying the methods previously reported by Stephan and Yurachek (33). Human normolipidemic serum was incubated with DiI at 37°C for 20 h. DiI-LDL of 1.006–1.063 g/mL was isolated after sucrose gradient ultracentrifugation (100,000 g, 4 h). The collected DiI-LDL fraction was dialyzed against saline and sterilized by filtration. The protein composition of DiI-LDL was confirmed to be equivalent with normal LDL by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined as described by Lowry and coworkers (34).

Quantification of LDL-R activity in HepG2 cells

The cellular low density lipoprotein receptor (LDL-R) activity was determined by detecting the incorporation of DiI-labeled LDL. HepG2 cells were incubated in DMEM supplemented with 10% LPDS with or without compounds for 24 h. The cells were then incubated with DiI-LDL (23 $\mu\text{g}/\text{mL}$) at 37°C for 3 h in the absence or presence of an excess amount of unlabeled LDL. Media were removed, and the cells were washed three times with phosphate-buffered saline. After addition of 1 mL of 2-propanol, the cells were shaken for 15 min at room temperature. Aliquots were centrifuged at 3,500 rpm for 5 min to remove cell debris. The amount of DiI incorporated into the cells was determined by fluorescence intensity, with excitation and emission wavelengths of 520 and 566 nm, respectively. The LDL-R activities were calculated by subtracting nonspecific fluorescence intensity detected in the presence of excess unlabeled LDL.

Biochemical analyses of blood in rhesus monkeys

Male rhesus monkeys were allocated to each group and orally treated with compounds by intragastric intubation for the indicated periods of time (4–28 days). Blood samples were taken from radial veins of overnight-fasted monkeys 24 h after the previous administration or before feeding if during the recovery periods. Plasma samples were prepared by centrifugation (3,000

rpm, 10 min). Plasma ALT and TCHO levels were determined with an Olympus (Norwood, MA) AU550. Plasma high density lipoprotein (HDL) cholesterol levels were determined with an HDL cholesterol test (Wako Pure Chemical Industries, Kyoto, Japan). Plasma non-HDL cholesterol was calculated by subtracting HDL cholesterol from TCHO.

Statistical analysis

Statistical analysis was conducted with the software package SAS 6.12 (SAS Institute Japan, Tokyo, Japan). The IC_{50} and ED_{50} values were calculated by nonlinear regression analysis. Statistical evaluation was basically performed by means of a one-way analysis of variance (ANOVA), followed by the Dunnett *t*-test for comparisons with controls. The time-course study in rhesus monkeys was analyzed by repeated measures analysis of covariance (ANOCOVA) with pre-value as the covariate, followed by ANOCOVA at each point and the post hoc Dunnett *t*-test for comparisons with controls.

RESULTS

Inhibitory effect of ER-28448 and ER-27856 on SQS and HMG-CoAR activities in rat liver microsomes

We discovered ER-28448, 5-[*N*-(2-butenyl-3-(2-methoxyphenyl)]-*N*-methylamino)-1, 1-pentylidenebis (phosphonic acid) trisodium salt, as a novel, potent, and selective inhibitor of SQS (Fig. 1). ER-28448 dose dependently inhibited SQS with an IC_{50} value of 3.6 nM in a rat liver microsome assay (Fig. 2). We also prepared ER-27856, the tri-POM derivative of ER-28448. The IC_{50} value of ER-27856 was 39 μM . ER-28448 inhibited SQS activity in microsomes isolated from beagle dogs and rhesus monkeys to the same extent as in rats, and neither compound inhibited HMG-CoAR activity at concentrations up to 100 μM (data not shown).

Inhibition of cholesterol biosynthesis in rat primary cultured hepatocytes

We next determined the inhibitory effect of ER-28448 and ER-27856 on cholesterol biosynthesis in rat primary cultured hepatocytes. ER-28448 and ER-27856 dose de-

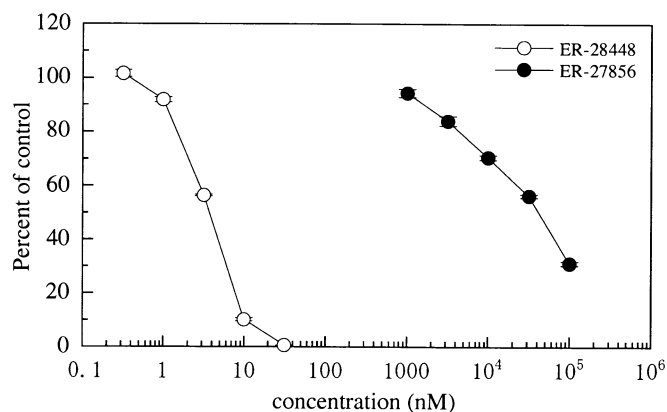


Fig. 2. Effect of ER-28448 and ER-27856 on rat liver microsomal SQS activity. The inhibition of rat liver microsomal SQS activity was determined by monitoring the conversion of [^{3}H]FPP into squalene. Reaction mixtures were incubated for 10 min at 37°C with the indicated concentrations of ER-28448 (open circles) or ER-27856 (closed circles). Each value represents the mean \pm SEM, as a percentage of control ($n = 3$).

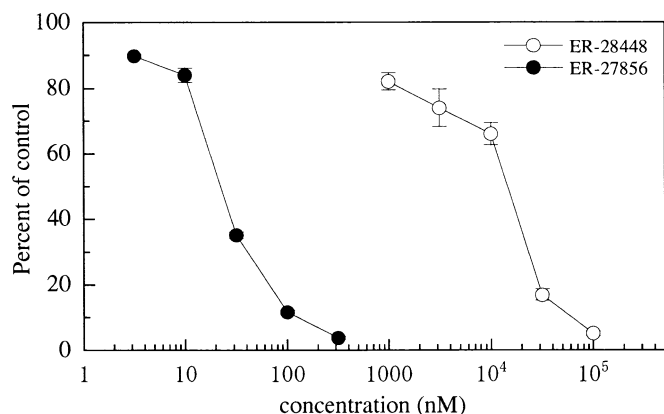


Fig. 3. Effect of ER-28448 and ER-27856 on cholesterol biosynthesis in rat primary cultured hepatocytes. The inhibitory activity on cholesterol biosynthesis was determined by quantifying the conversion of [$1\text{-}^{14}\text{C}$]acetate into cholesterol. Rat hepatocytes were incubated with various concentrations of ER-28448 (open circles) or ER-27856 (closed circles) in the presence of [$1\text{-}^{14}\text{C}$]acetate (185 kBq/well) for 2 h. Each value represents the mean \pm SEM, as a percentage of control ($n = 3$).

pendently inhibited cholesterol biosynthesis with IC_{50} values of 11 μM and 23 nM, respectively (**Fig. 3**). In contrast to the liver microsome assay, ER-27856 was 480 times more potent than ER-28448 in rat hepatocytes. These data indicate that ER-27856 was a potent prodrug of ER-28448 and was active in the cells.

Effect of ER-27856 on cholesterol biosynthesis in rats

We determined the dose-dependent effect of ER-28448 and ER-27856 on cholesterol biosynthesis in rats. The animals were treated by intravenous or oral adminis-

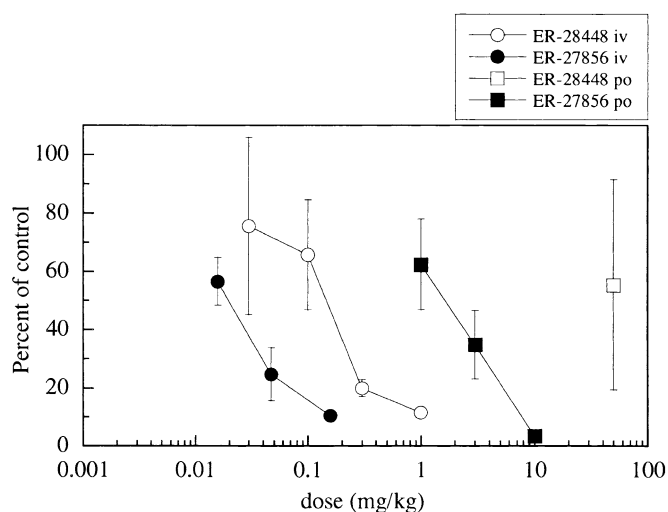


Fig. 4. Effect of ER-28448 and ER-27856 on cholesterol biosynthesis in rats. The inhibitory activity on in vivo cholesterol biosynthesis was determined by quantifying the conversion of intraperitoneally administered [$1\text{-}^{14}\text{C}$]acetate into plasma cholesterol in rats. Compounds were intravenously (circles) or orally (squares) administered to male Sprague-Dawley rats at 1 h (i.v.) or 2 h (p.o.) before the intraperitoneal injection of [$1\text{-}^{14}\text{C}$]acetate. Each value represents the mean \pm SEM, as a percentage of control ($n = 3$).

tration. Intravenously administered ER-27856 inhibited cholesterol biosynthesis in a dose-dependent manner, with an inhibition of 43% at 0.016 mg/kg, 65% at 0.047 mg/kg, and 90% at 0.16 mg/kg (**Fig. 4**). The ED_{50} value was estimated to be 0.022 mg/kg. ER-28448 also dose dependently inhibited cholesterol biosynthesis, with an ED_{50} value of 0.12 mg/kg when administered intravenously. ER-27856 was six times more potent than ER-28448. The net difference reached 10 times greater potency when ER-27856 was presented as molar equivalents. Orally administered ER-27856 inhibited cholesterol biosynthesis by 38% at 1 mg/kg, 65% at 3 mg/kg, and 96% at 10 mg/kg. The ED_{50} value was 1.6 mg/kg at 2 h after oral administration. In contrast, ER-28448 partially reduced cholesterol biosynthesis even at 50 mg/kg, when orally administered. Finding ER-27856 to be a potent inhibitor of cholesterol biosynthesis in rats, we decided to use ER-27856 for further in vivo studies.

Effects of ER-27856 on LDL-R up-regulation in HepG2 cells

To study the mechanism by which ER-27856 reduces plasma cholesterol levels, we determined its effect on LDL-R activity in HepG2 cells. Cells were treated with compounds for 24 h before the addition of DiI-LDL. ER-27856 significantly up-regulated LDL-R activity to 1.4 times at 1 μM and 2.1 times at 10 μM (**Fig. 5**). The effect on LDL-R activity was well correlated with the extent of cholesterol biosynthesis inhibition, and the LDL-R activity at the IC_{50} of cholesterol biosynthesis was approximately 2,000 ng of LDL protein/mg of cell protein. In addition, the maximum activity to induce LDL-R was equivalent with HMGRI (data not shown). These data suggest that ER-27856 decreased plasma cholesterol by enhancing LDL-R activity.

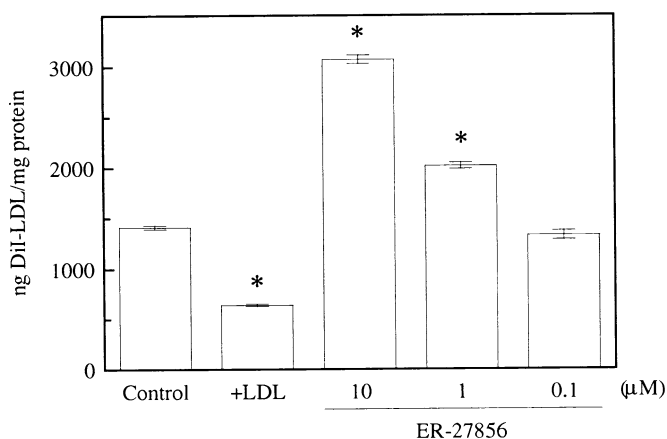


Fig. 5. Effect of ER-27856 on LDL-R activity in HepG2 cells. The LDL-R activity was determined by detecting the incorporation of DiI-labeled LDL. HepG2 cells were incubated in 10% LPDS-containing DMEM alone (Control), in the presence of LDL at 50 $\mu\text{g}/\text{mL}$ (+LDL), or with the indicated concentration of ER-27856 for 24 h. Each column represents the nanograms of DiI-LDL protein incorporated per milligram of cell protein (mean \pm SEM; $n = 3$). Asterisks represent a significant difference from control, with $P < 0.05$ by the Dunnett t -test.

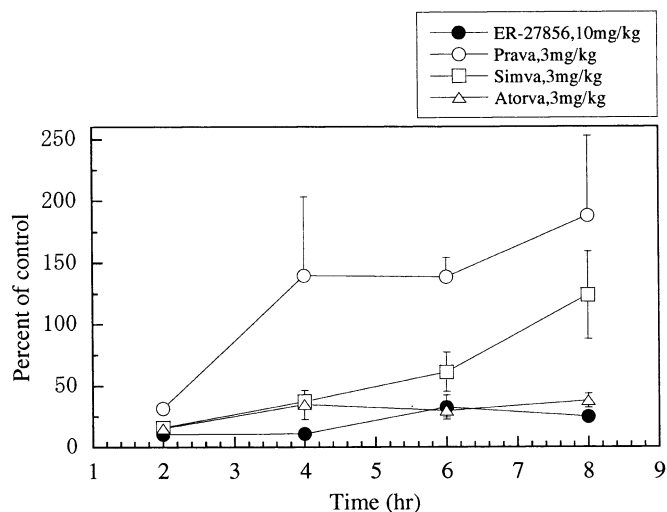


Fig. 6. Time course for cholesterol biosynthesis inhibition by ER-27856 and HMGRIs in rats. The inhibition of *in vivo* cholesterol biosynthesis was determined by quantifying the conversion of intraperitoneally administered [^{14}C]acetate into plasma cholesterol. ER-27856 (10 mg/kg; closed circles), pravastatin (3 mg/kg; open circles), simvastatin (3 mg/kg; open squares), and atorvastatin (3 mg/kg; open triangles) were orally administered to male Sprague-Dawley rats at the indicated times before the intraperitoneal injection of [^{14}C]acetate. Each value represents the mean \pm SEM, as a percentage of control ($n = 3$).

Effects of ER-27856 in rats and rhesus monkeys in comparison with HMGRIs

The period of time over which cholesterol biosynthesis is effectively suppressed (the effective period) may affect the potency of the hypolipidemic effect. We determined the effect of ER-27856 and compared it with HMGRIs at doses three to six times higher than the ED_{50} values in rats. The inhibitory effect of ER-27856 at 10 mg/kg continued up to 8 h after its administration, and the effective duration was equivalent to that of atorvastatin (Fig. 6). The inhibitory activity of simvastatin was observed at 2 to 6 h but had attenuated by 8 h after administration. Pravastatin was active only at 2 h. ER-27856 proved to be a potent and long-acting inhibitor of cholesterol biosynthesis in rats.

To compare the cholesterol-lowering activities of ER-27856 and HMGRIs, we administered these compounds to rhesus monkeys, whose plasma lipid composition is similar to that of humans. After 4 days of administration, ER-27856 lowered TCHO by 21% at 3 mg/kg, 26% at 10 mg/kg, and 38% at 30 mg/kg (Fig. 7). Pravastatin decreased plasma cholesterol only 5% at 30 mg/kg, simvastatin 19% at 30 mg/kg, and atorvastatin 20% at 30 mg/kg. The cholesterol-lowering activity of ER-27856 was significantly higher than those of HMGRIs.

Effects of ER-27856 and HMGRIs on hepatic toxicity in rhesus monkeys

HMGRIs occasionally caused hepatic damage and SQS inhibitors have been expected to avoid this side effect. To determine the hepatic toxicity of ER-27856 and HMGRIs, we measured ALT activity in rhesus monkeys. Atorvastatin,

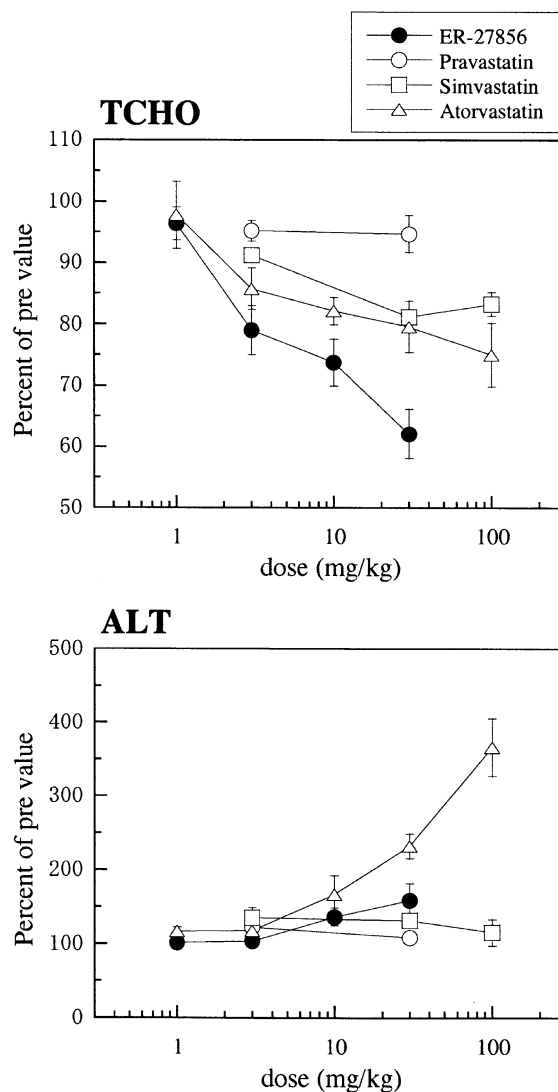


Fig. 7. Dose-dependent effects of ER-27856 and HMGRIs on TCHO and ALT levels in rhesus monkeys. Hypolipidemic activity of ER-27856 was compared with those of HMGRIs in rhesus monkeys. ER-27856 (closed circles), pravastatin (open circles), simvastatin (open squares), and atorvastatin (open triangles) were orally administered to male rhesus monkeys by intragastric intubation for 4 days. Each point represents the mean \pm SEM on day 4, as a percentage of the pretreatment value ($n = 4$). This figure was prepared by integrating 10 separate experiments, each of which used 8–16 monkeys.

the most potent HMGRIs in clinical studies, elevated plasma ALT levels in a dose-dependent manner (Fig. 7). The ALT activity reached 232 and 366% of prevalues at 30 and 100 mg/kg, respectively. In contrast, the ALT activity in ER-27856-treated monkeys was only 136 and 159% at 10 and 30 mg/kg, respectively.

The correlation between the elevation of plasma ALT levels and TCHO reduction is plotted in Fig. 8. The plasma ALT levels rose drastically in atorvastatin-treated animals as TCHO levels fell. The significant increase in ALT was 2.3-fold at 20% of TCHO reduction and 3.7-fold at 25% reduction. Although plasma ALT increased in ER-27856-treated monkeys, the increase was more moderate than that in atorvastatin-treated monkeys. Even at 38%

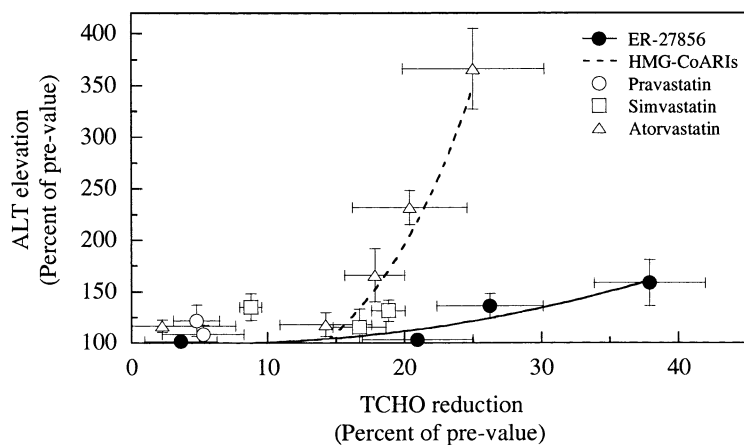


Fig. 8. Correlation of plasma ALT elevation and TCHO reduction after a 4-day treatment of rhesus monkeys. Results shown in Fig. 7 are expressed as the percent change in ALT elevation, and are plotted relative to TCHO reduction. Each point represents the mean \pm SEM of a single-dose group treated with ER-27856 (closed circles), pravastatin (open circles), simvastatin (open squares), or atorvastatin (open triangles).

TCHO reduction, the ALT elevation was only 1.6-fold. These data indicate that ER-27856 is a potent hypocholesterolemic agent and less hepatotoxic than HMGRIs.

Effects of long-term ER-27856 treatment on plasma TCHO and non-HDL cholesterol in rhesus monkeys

To determine the hypocholesterolemic effect of long-term treatment, we administered ER-27856 at 1 and 10 mg/kg to rhesus monkeys for 28 days. ER-27856 significantly reduced TCHO by 24% at 1 mg/kg and by 72% at 10 mg/kg over 28 days of administration (Fig. 9). The cholesterol-lowering effect reached a plateau on day 14, and the effect continued during administration. Plasma cholesterol rapidly recovered after the last administration.

Because non-HDL cholesterol enhances atherogenic disease in humans and HDL prevents it, we measured non-HDL cholesterol in rhesus monkeys treated with ER-27856. ER-27856 at 10 mg/kg significantly reduced non-HDL cholesterol on day 4 to day 31, and the decrease was 94% on day 14, 100% on day 21, and 95% on day 28. These data indicate that ER-27856 lowered plasma cholesterol mainly because of the complete reduction of non-HDL cholesterol.

DISCUSSION

In this article, we have reported that an SQS inhibitor, ER-27856, was more potent in suppressing cholesterol synthesis in rats and decreasing plasma cholesterol in rhesus monkeys than pravastatin, simvastatin, and atorvastatin. We also report that ER-27856 was less hepatotoxic in rhesus monkeys.

So far, several classes of SQS inhibitors have been discovered. SQS substrate analogs suppressed SQS activity (14–17), and RPR-107393, an SQS inhibitor with quinuclidine, reduced TCHO in marmosets (18). Zaragozic acids or squalostatins were potent inhibitors of SQS in mice, rats, and marmosets (19–24). We discovered ER-28448 as a novel 1,1-bisphosphonate inhibitor of SQS. Consistent with previous reports, ER-28448 was shown to be a potent SQS inhibitor, with an IC_{50} value of 3.6 nM (Fig. 2).

Although ER-28448 was active in a liver microsome assay,

ER-28448 showed poor activity in hepatocytes and poor oral availability in rats, probably because of its highly charged physicochemical properties (Figs. 3 and 4). Because the double esters have been utilized as prodrug moieties to mask the acidic charge of parent compounds (35), we prepared ER-27856, the tri-POM ester of a bisphosphonate derivative of ER-28448, as the prodrug. Whereas ER-27856 weakly inhibited SQS in rat liver microsomes, its activity in inhibiting cholesterol biosynthesis exceeded that of ER-28448 in rat hepatocytes, indicating that ER-27856 acted as a prodrug and was hydrolyzed to form pharmacologically active ER-28448 after being transported into the cells (Figs. 2 and 3). In vivo, intravenously or orally administered ER-27856 potently inhibited cholesterol biosynthesis in rats. Notably, ER-27856 was 10 times more potent than ER-28448 when intravenously administered, when compared on the basis of molar equivalents (Fig. 4). These results demonstrated that the high membrane permeability of ER-27856 resulted in its better distribution to liver. The tri-POM esters in ER-27856 also seemed to be resistant to esterases in the intestinal lumen and mucosa, and the stability may improve oral absorption. Therefore, the improvement of oral activity by tri-POM esterification should be attributed to both improved absorption and higher distribution to liver.

ER-27856 was more potent in cholesterol-lowering activity than HMGRIs in rhesus monkeys, whose plasma lipoprotein composition and metabolism resemble those in human (Fig. 7; see ref. 36). Several SQS inhibitors have been reported to reduce plasma (or serum) cholesterol in marmosets by either single intravenous dosing (20–22) or multiple oral dosing (18, 23, 24). RPR-107393 exhibited a greater reduction in plasma cholesterol than two HMGRIs, pravastatin and lovastatin (18). The results of those studies were consistent with our findings that ER-27856 reduced plasma cholesterol to an extent that no HMGRIs could reach even at higher doses. Moreover, ER-27856 reduced non-HDL cholesterol by 95% over 28 days of administration (Fig. 9). Even at the highest tolerance dose (30 mg/kg), atorvastatin did not eliminate non-HDL cholesterol after 21 days of oral administration (data not shown). ER-27856 is the first compound to eliminate non-HDL cholesterol from the plasma of rhesus monkeys. These results

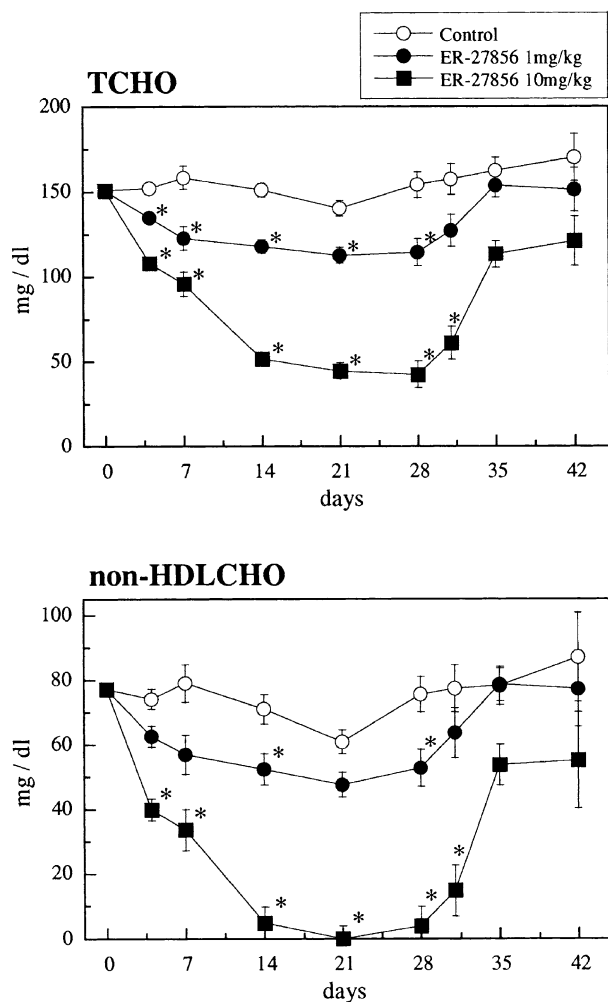


Fig. 9. Effect of ER-27856 on TCHO and non-HDL cholesterol in rhesus monkeys. ER-27856 (1, 10 mg/kg per day) was orally administered to male rhesus monkeys by intragastric intubation for 28 days. Blood samples were taken 24 h and 4, 7, 14, 21, and 28 days after administration, and on days 31, 35, and 42 for recovery. Each point represents a prevalue-adjusted mean \pm SEM ($n = 4$, except for days 35 and 42, where $n = 2$). Asterisks represent a significant difference from control, with $P < 0.05$ by the Dunnett t -test.

suggest that an SQS inhibitor may become a novel agent in the treatment of severe hypercholesterolemia.

The main mechanism by which HMGRIs reduce human plasma cholesterol is believed to involve the up-regulation of the hepatic LDL-R. Hepatic LDL-R activity is regulated at the transcriptional level by *cis*-acting basic helix-loop-helix leucine zipper proteins, designated sterol regulatory element-binding proteins (SREBPs; see ref. 37). Cellular sterols inhibit the activation of SREBPs by controlling the activity of SREBP cleavage-activating protein (SCAP). When cellular sterols decrease, the LDL-R gene is up-regulated after the sequential activation of SCAP and SREBPs, and its binding to SRE-1 in the promoter of the LDL-R gene. SREBPs are activated from membrane-bound precursors by a two-step proteolytic cascade: *i*) SCAP-controlled separation of NH₂- and COOH-terminal segments by site 1 protease, and *ii*) release of SREBPs from the


membrane by site 2 protease. In this study, we demonstrated that ER-27856 up-regulated LDL-R activity in correlation with the inhibition of cholesterol biosynthesis (Fig. 5), suggesting that ER-27856 decreased cholesterol by enhancing LDL-R activity in rhesus monkeys. The data on LDL-R increase in HepG2 cells, however, did not explain the larger reduction of plasma cholesterol by ER-27856 in rhesus monkeys, because the maximum activity required to induce LDL-R was equivalent to that of HMGRIs. Two other possibilities should be considered in determining how ER-27856 decreases plasma cholesterol more potently than HMGRIs.

First, the potent hypocholesterolemic effect of ER-27856 in rhesus monkeys could be attributed to a lasting inhibition of cholesterol biosynthesis. The duration of cholesterol biosynthesis inhibition seems to affect the cholesterol-lowering effect in primates, including humans. In rats, no significant difference between the three HMGRIs we used was observed in terms of inhibition of cholesterol biosynthesis, and a clear difference was seen in the duration of the activity (Fig. 6). Our hypolipidemic data on HMGRIs in rhesus monkeys and the therapeutic effects of those in humans (38) were well correlated with the duration of inhibition of cholesterol biosynthesis but not with the acute inhibitory potency of cholesterol biosynthesis in rats.

Second, the potent cholesterol-lowering effect of ER-27856 might be attributed to the repression of HMG-CoAR induction. HMG-CoAR induction was seen in the treatment with HMGRIs and might restrain their hypolipidemic effect. The nonsterol products of mevalonate regulate HMG-CoAR degradation (39–41). Several studies have shown that the degradation was induced by an increase in a nonsterol regulator, identifying FPP derivatives as candidates (42–44). Several studies, in which zaragozic acid suppressed the non-sterol-induced rapid degradation of HMG-CoAR, have evoked some controversy (45–47). More recent studies, however, have demonstrated that zaragozic acid might directly interfere with the production or action of the nonsterol regulator (48), and that FPP was the source of the positive signal for HMG-CoAR degradation in mammals and yeast (49). These results indicate that FPP derivatives, but not presqualene pyrophosphate or derivatives of squalene, are the nonsterol regulators of HMG-CoAR degradation. Therefore, the inhibition of SQS by ER-27856 might suppress the induction of HMG-CoAR by increasing the degradation, resulting in the superior hypocholesterolemic effect in rhesus monkeys. This idea is supported by a report that RPR-107393, another SQS inhibitor, achieved a greater reduction of plasma cholesterol than two HMGRIs, pravastatin and lovastatin (18).

Although the HMGRIs have been clinically well tolerated, they occasionally cause an elevation of hepatic transaminase levels (7, 8). There is an increased prevalence of myopathies when HMGRIs are coadministered with other drugs such as cyclosporin, niacin, and fibrates. The increase in adverse events makes the combination of the latter two medicines with HMGRIs difficult in clinical trials. In rabbits, all histopathological and serum biochemical changes

induced by lovastatin were completely prevented by co-administration of mevalonate (9). In contrast, supplementation of lovastatin-treated rabbits with cholesterol did not improve but instead worsened liver and kidney function. Consistent with these reports, a dose-dependent elevation of plasma ALT levels was detected in rhesus monkeys treated with atorvastatin (Fig. 7). Moreover, the elevation was prevented by mevalonate supplementation, indicating that the hepatotoxicity of atorvastatin was not due to the lack of cholesterol but resulted from depletion of non-sterol mevalonate derivatives (data not shown). Unlike atorvastatin, the plasma ALT increase by ER-27856 was moderate (Fig. 8). These results reveal the advantageous effect of SQS inhibitors over HMGRIs in terms of hepatotoxicity.

In conclusion, ER-27856, an SQS inhibitor prodrug, potently reduced TCHO and almost eliminated non-HDL cholesterol with few adverse effects in rhesus monkeys. ER-27856 may contribute to the treatment of severe hypercholesterolemic diseases and decrease coronary events and mortality better than HMGRIs. 

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