Ro 48-8071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys, and minipigs: comparison to simvastatin

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Abstract 2,3-Oxidosqualene:lanosterol cyclase (OSC, E.C. 5.4.99.7) represents a unique target for a cholesterol lowering drug. Partial inhibition of OSC should reduce synthesis of lanosterol and subsequent sterols, and also stimulate the production of epoxysterols that repress HMG-CoA reductase expression, generating a synergistic, self-limited negative regulatory loop. Hence, the pharmacological properties of Ro 48-8071, a new OSC inhibitor, were compared to that of an HMG-CoA reductase inhibitor, simvastatin. Ro 48-8071 blocked human liver OSC and cholesterol synthesis in HepG2 cells in the nanomolar range; in cells it triggered the production of monooxidosqualene, dioxidosqualene, and epoxycholesterol. It was safe in hamsters, squirrel monkeys and Göttingen minipigs at pharmacologically active doses, lowering LDL ~60% in hamsters, and at least 30% in the two other species, being at least as efficacious as safe doses of simvastatin. The latter was hepatotoxic in hamsters at doses >30 μmol/kg/day limiting its window of efficacy. Hepatic monooxidosqualene increased dose-dependently after treatment with Ro 48-8071, up to ~20 μg/g wet liver or less than 1% of hepatic cholesterol, and it was inversely correlated with LDL levels. Ro 48-8071 did not reduce coenzyme Q10 levels in liver and heart of hamsters, and importantly did not trigger an overexpression of hepatic HMG-CoA reductase, squalene synthase, and OSC itself. In strong contrast, simvastatin stimulated these enzymes dramatically, and reduced coenzyme Q10 levels in liver and heart. Altogether these findings clearly differentiate the OSC inhibitor Ro 48-8071 from simvastatin, and support the view that OSC is a distinct key component in the regulation of the cholesterol synthesis pathway.—**Morand, O. H., J. D. Aebi, H. Dehmlow, Y-H. Ji, N. Gains, H. Lengsfeld, and J. Himber.** Ro 48-8071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys, and minipigs: comparison to simvastatin. *J. Lipid Res.* 1997. **38**: 373–390.

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The relationship between coronary heart disease and elevated plasma LDL cholesterol is well established, and cholesterol lowering proved very beneficial for prevention of this disease (1–3). Medical guidelines emphasize that most survivors of a myocardial infarction and patients with angina pectoris or other atherosclerotic disease qualify for aggressive cholesterol-lowering therapy (4). Statins, inhibitors of HMG-CoA reductase, have been developed as efficacious cholesterol-lowering drugs, e.g., lovastatin and simvastatin which reduce LDL cholesterol by ~40% at recommended doses (5–7). Atorvastatin, a new HMG-CoA reductase inhibitor, exhibits high efficacy in man, reducing LDL cholesterol by ~60% (8); its long-term safety and therapeutical window have yet to be established.

Although inhibitors of HMG-CoA reductase are well tolerated at the recommended clinical dosage, adverse effects are observed at high doses of statins (5–8). Presumably, these drawbacks stem from the mode of action of statins because they inhibit not only the production of cholesterol, but also the synthesis of non-sterol isoprenoids. The latter are essential for functions that depend either on protein prenylation or on production of dolichol and coenzyme Q (CoQ), all being affected by statins (9–12). In rabbits (13) and hamsters (14) stat-

Abbreviations: apo, apolipoprotein; CoQ, coenzyme Q (ubiquinone); DOS, dioxidosqualene; HDL-C, high density lipoprotein cholesterol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; LDL-C, low density lipoprotein cholesterol; MOS, monooxidosqualene; OSC, 2,3-oxidosqualene:lanosterol cyclase; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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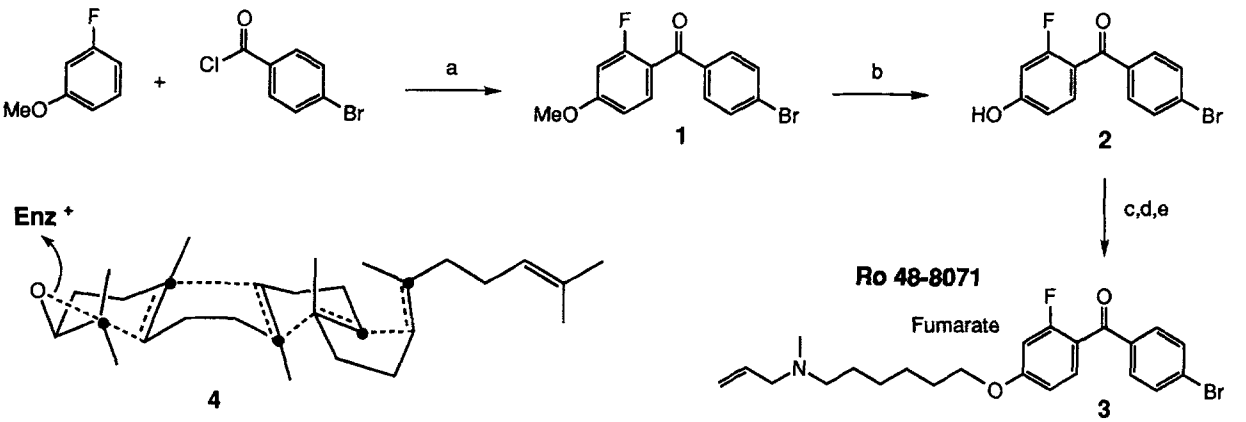

Fig. 1. Chemical synthesis of Ro 48-8071 [3] and high energy intermediate of cyclization [4]. (a) Nitrobenzene/$AlCl_3$ (41.6%), (b) HBr/CH_3COOH (96.9%), (c) 1,6-dibromohexane/K_2CO_3 in acetone, (d) N-allylmethylamine in N,N-dimethylacetamide, and (e) fumaric acid in ethanol (c,d,e: 53.4%). In [4], the five black dots denote the cationic centers of the folded, opened, high energy intermediate of MOS cyclization.

ins induce toxic effects that are neutralized by coadministration of mevalonate, the product of HMG-CoA reductase, suggesting that depletion of non-sterol isoprenoids is responsible for these effects. Not least, statins trigger overexpression of HMG-CoA reductase itself, a counterproductive, indirect effect (15–17) which may limit their efficacy or even abolish the cholesterol-lowering effect as in rats (18).

Among enzymes of the cholesterol synthesis pathway, 2,3-oxidosqualene:lanosterol cyclase (OSC, E.C. 5.4.99.7), a microsomal enzyme that catalyzes the cyclization of monooxidosqualene to lanosterol (19), is an attractive target for a safe, efficacious hypocholesterolemic drug. OSC is located downstream of farnesylpyrophosphate, and inhibitors of this enzyme should not block production of non-sterol isoprenoids nor decrease CoQ production and protein prenylation. Also, inhibition of OSC is unlikely to trigger an up-regulation of HMG-CoA reductase because of an indirect, synergistic, negative feedback mechanism regulating HMG-CoA reductase (20–27).

Hence, potent inhibitors of 2,3-oxidosqualene:lanosterol cyclase (28–31) were synthesized, and in this study, we examined the pharmacological properties of a new OSC inhibitor Ro 48-8071 (32), in hamsters, squirrel monkeys, and Göttingen minipigs, and compared them to that of simvastatin. Ro 48-8071 was apparently safe in all three species tested, and at least as efficacious as simvastatin at safe doses in lowering LDL cholesterol. For the first time, an OSC inhibitor has been clearly differentiated from a statin in vivo in hamsters for it showed no hepatotoxicity, did not reduce liver and heart coenzyme Q10 levels, and did not trigger an increase of ex vivo activity of hepatic HMG-CoA reductase, squalene synthase, and oxidosqualene cyclase.

MATERIALS AND METHODS

Chemicals

Simvastatin (MW = 418.57) was purchased as 20 mg Zocor® tablets from a retail pharmacy. The following were from commercial sources: [2-^{14}C]sodium acetate, 2.15 GBq/mmol (Amersham); [1,5,9,14,20,24-^{14}C]squalene, 7.4 GBq/mmol (American Radiolabeled Chemicals Inc., St. Louis, MO); [^{14}C]HMG-CoA, 2.1 GBq/mmol (DuPont-NEN); [^{3}H]farnesyl-pyrophosphate, 603 GBq/mmol (Amersham); [Na^{125}I] (Medipro AG, Teufen, Switzerland); Triton WR-1339 (Serva). CoQ9 and CoQ10 were from Fluka, and CoQ11 was a generous gift from Nisshin Flour Milling Co., Japan. 2,3-Monooxidosqualene (MOS) and 2,3-22,23-dioxidosqualene (DOS) were synthesized as reported (33), as well as 24,25-epoxycholesterol and 24,25-epoxylanosterol (refs. 34 and 35, respectively). [1,25-^{14}C]-(3S,R)-2,3-MOS (0.47 GBq/mmol) was synthesized as described (36, 37). Silica gel TLC plates were from E. Merck (Darmstadt). All solvents were from Fluka, and other chemicals from Sigma.

Synthesis of Ro 48-8071

Ro 48-8071 (fumarate, MW = 564.45) was synthesized as shown in **Fig. 1** (30, 31). All intermediates and Ro 48-8071 were characterized by 250 MHz ^{1}H-NMR, IR, MS, and microanalyses. Melting points (uncorrected)

were determined using a Büchi 510 apparatus. Proton NMR spectra were recorded on a Bruker AC250 spectrometer, and δ values are given in ppm relative to tetramethylsilane. IR spectra of KBr pellets were recorded using a Nicolet 7199-FT IR spectrometer. Mass spectra (MS) were obtained using the pneumatically assisted electrospray technique (Perkin-Elmer Sciex, type API-III). Results of elemental analyses were within 0.3% of theoretical values.

(4-Bromo-phenyl)-(2′-fluoro-4′-methoxy-phenyl)-methanone [1]

Aluminum chloride (144 g, 1.08 mol) was added to 450 ml precooled nitrobenzene keeping the temperature <8°C. Then, a suspension of 219.5 g (1 mol) 4-bromobenzoyl chloride in 200 ml nitrobenzene was added over 20 min, followed 10 min later by 108.5 ml (0.95 mol) 3-fluoroanisole. The reaction mixture was warmed to room temperature overnight, mixed into iced water (1.5l), and extracted with 3 × 1l dichloromethane. The three organic phases were washed sequentially with 2 × 1l water, pooled, and dried (Na_2SO_4). Evaporation (85°C, 1 Torr) provided a mixture of (4-bromo-phenyl)-(2-fluoro-4-methoxy-phenyl)-methanone and (4-bromo-phenyl)-(4′-fluoro-2′-methoxy-phenyl)-methanone which was immediately dissolved in 300 ml ethyl acetate, and crystallized at room temperature. The crystals were filtered off and washed with 100 ml ethyl acetate and 3 × 100 ml cyclohexane to give pure (4-bromo-phenyl)-(2′-fluoro-4′-methoxy-phenyl)-methanone [1] (122.4 g, 41.6%): mp 125–126°C; IR 1643 cm^{-1}; ^{1}H-NMR ($CDCl_3$) ∂ 3.87 (s,OCH_3), 6.66 (dd, J = 12.1, 2.4 Hz 1H, 3′-H), 6.80 (dd, J = 8.7, 2.4 Hz, 1H, 5′-H), 7.54–7.68 (m, 5H, arom H); EIMS *m/z* 308 (M^+, 1 Br). Calculated analysis for $C_{14}H_{10}BrFO_2$ C, 54.40; H, 3.26; F, 6.15; Br, 25.85. Found: C, 54.57; H, 3.35; F, 6.21; Br, 26.07.

(4-Bromo-phenyl)-(2′-fluoro-4′-hydroxy-phenyl)-methanone [2]

A suspension of 61.8 g (200 mmol) of [1] in 400 ml acetic acid was treated with 230 ml 62%-aqueous hydrobromic acid, and stirred at 125°C for 8 h prior to evaporation. The residue was dissolved in 500 ml ethyl acetate and washed with 300 ml saturated sodium bicarbonate and 300 ml 10%-sodium chloride solution. The aqueous phases were extracted with 2 × 500 ml ethyl acetate. The organic phase was dried (Na_2SO_4) and evaporated to give orange crystals of (4-bromo-phenyl)-(2′-fluoro-4′-hydroxy-phenyl)-methanone [2] (57.2 g, 96.9%): mp 62–63°C; IR 1652 cm^{-1}; ^{1}H-NMR (DMSO-d_6) ∂ 6.68 (dd, J = 12.6, 2.2 Hz, 1H, 3′-H), 6.77 (dd, J=8.5, 2.2 Hz, 1H, 5′-H), 7.49 (dd, J=8.5, 8.5 Hz, 1H, 6′-H), 7.64 and 7.75 (AA′BB′, 4H, 2,3,5,6-H), 10.85 (br s, 1H, OH); EIMS *m/z* 294 (M^+, 1Br). Calculated analysis for $C_{13}H_8BrFO_2$: C, 52.91; H, 2.73; F, 6.44; Br, 27.08. Found: C, 52.94; H, 2.74; F, 6.42; Br, 26.84.

[4′-(6-Allyl-methyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone fumarate [3]

A mixture of 35.4 g (120 mmol) [2], 54.9 ml (360 mmol) 1,6-dibromohexane and 49.8 g (360 mmol) potassium carbonate in 1100 ml acetone was vigorously stirred at 75°C for 5 h. After filtration and evaporation, the residue was dissolved in dichloromethane treated with sodium sulfate, filtered again, and evaporated. Crystallization with 400 ml cyclohexane–hexane 1:3 (v/v) first at 0°C and then at −78°C gave 53.2 g (116 mmol) crude [4′-(6-bromo-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone. This product was dissolved in 390 ml N,N-dimethylacetamide, cooled to 0°C, and 22.5 ml (232 mmol) N-allylmethylamine was added dropwise. After 22 h at room temperature the reaction was cooled to 0°C, and treated again with 22.5 ml (232 mmol) N-allylmethylamine. After 5 h the solution was evaporated (70°C, 1 Torr), neutralized with 300 ml saturated sodium bicarbonate, and extracted with 3 × 400 ml dichloromethane. The organic phase was dried (Na_2SO_4), evaporated to dryness, and purified by flash column chromatography (silica gel 0.04–0.063 mm, dichloromethane–methanol 95:5 (v/v), producing 37.7 g (84.1 mmol) of [4′-(6-allyl-methyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromo-phenyl)-methanone. The free amine and 8.8 g (75.7 mmol) of fumaric acid were dissolved in 200 ml ethanol, evaporated, and crystallized from acetone–ethylacetate–ether to give [4′-(6-allyl-methyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromo-phenyl)-methanone fumarate [3] (36.2 g, 53.4%): mp 86–88°C; IR 1653 cm^{-1}; ^{1}H-NMR (DMSO-d_6)∂ 1.25–1.60 (m, 6H, $OCH_2CH_2\mathit{CH_2CH_2CH_2}CH_2N$), 1.70–1.80 (m, 2H, $OCH_2\mathit{CH_2}CH_2CH_2CH_2CH_2N$), 2.24 (s, 3H, NCH_3), 2.40–2.50 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2\mathit{CH_2}N$), 3.11 (d, J = 6.5 Hz, 2H, $N\mathit{CH_2}CHCH_2$), 4.08 (t, J = 6.4 Hz, 2H, $O\mathit{CH_2}CH_2CH_2CH_2CH_2CH_2N$), 5.17–5.27 (m, 2H, $NCH_2CH\mathit{CH_2}$, 5.75–5.90 (m, 1H, $NCH_2\mathit{CH}CH_2$), 6.67 (s, 2H, fumarate), 6.91–7.00 (m, 2H, 3′,5′-H), 7.56 (dd, J = 8.6,8.6 Hz, 1H, 6′-H), 7.65 and 7.76 (AA′BB′, 4H, 2,3,5,6-H); EIMS *m/z* 448 (M^+, 1Br). Calculated analysis for $C_{23}H_{27}NBrFO_2 \cdot C_4H_4O_4$; C, 57.45; H, 5.54; N, 2.48; F, 3.37; Br, 14.16. Found: C, 57.39; H, 5.57; N, 2.50; F, 3.38; Br, 14.15.

2,3-Oxidosqualene cyclase assay

A section of liver from a 18-year-old healthy donor was obtained at the hepatic transplantation unit of Hôpital Bicêtre, Paris, and frozen at −80°C. Liver micro-

somes were prepared as described (38) and stored in sodium phosphate buffer at −80°C. OSC activity was measured in 100 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA, and 1 mM DTT in a detergent-free assay. [^{14}C]R,S-MOS was diluted in ethanol and mixed into buffer–1% BSA. A stock solution of Ro 48-8071 in DMSO was diluted at increasing concentrations in buffer–1% BSA. The assay was carried out in screw-cap glass tubes by mixing 40 µl of diluted microsomes and 20 µl of the drug dilution, and initiating the reaction with 20 µl of the [^{14}C]R,S-MOS dilution. Final conditions were: 0.4 mg microsomal protein/ml and 30 µM [^{14}C]R,S-MOS in buffer (pH 7.4) containing 0.5% albumin, <0.1% DMSO, and <0.5% ethanol, in a volume of 80 µl for 1 h. The reaction was stopped with 0.6 ml 10% KOH–methanol, 0.1 ml n-hexane–ether 1:1 (v/v) containing an excess of non-labeled MOS and lanosterol as carriers, and 0.6 ml water. Then, each tube received 1 ml n-hexane–ether 1:1 (v/v), was vortexed and centrifuged. The upper organic phase was collected, the lower phase was extracted once more with 1 ml n-hexane–ether, and the two upper organic phases were pooled and evaporated to dryness. Radioactive MOS and lanosterol in each lipid extract were separated by TLC in n-hexane–ether 1:1 (v/v). Sensitive Phosphor screens (PhosphorImager, Molecular Dynamics) were then exposed to the TLC plates for recording radioactivity. Data were calculated as a percent conversion of S-MOS to lanosterol, and expressed in pmol [^{14}C]lanosterol formed, considering that only the enantiomer (3S)-MOS is cyclized to lanosterol (39).

Incorporation of [^{14}C]acetate into nonsaponifiable lipids of cultured HepG2 cells

Human hepatoma HepG2 cells (ATCC) were cultured routinely at 37°C, in a humidified 5% CO_2 95% air atmosphere, in Dulbecco's Modified Eagle medium (DMEM, Gibco) containing penicillin (100 IU/ml, Gibco) and streptomycin (100 IU/ml, Gibco), supplemented with 10% (v/v) FCS (Gibco). Lipoprotein-deficient serum (LPDS) was prepared from FCS by KBr density flotation, followed by repeated dialysis and sterile filtration.

HepG2 cells were seeded in DMEM–5% LPDS in sterile, collagen-coated screw-cap glass tubes at a density of 7×10^4 cells in 0.8 ml DMEM–5% LPDS per tube. After 24 h, a stock solution of Ro 48-8071 in DMSO was diluted in DMEM–5% LPDS at different concentrations. Then, each tube received *i*) 0.1 ml DMEM–5% LPDS containing [^{14}C]acetate and *ii*) 0.1 ml of intermediate dilutions of Ro 48-8071, to achieve a volume of 1 ml (0.1% DMSO, v/v). The final concentration of [^{14}C]-acetate was 74 kBq/ml. After 18 h at 37°C, the medium was aspirated, and cells were washed once with 2 ml PBS. Next, each tube received 0.6 ml methanol–KOH 10% and 0.7 ml water. After closing, the tubes were incubated for 1 h at 70°C for saponification. After cooling, nonsaponifiable lipids were extracted with 2 × 1 ml n-hexane–ether 1:1 (v/v) and the two extracts were pooled and dried. Lipids were separated by TLC in n-hexane–ether–acetic acid 60:40:1 (v/v) along with standards applied on separate lanes. Sensitive Phosphor screens were then exposed to the TLC plates for recording radioactivity. Arbitrary units from the Phosphorimager were converted to dpm using a linear calibration curve obtained with known amounts of a ^{14}C-labeled standard spotted on the side of the plate after the separation. Data for synthesis of cholesterol and other lipids were expressed as dpm of [^{14}C]acetate incorporated in each lipid per tube.

For further identification, nonsaponifiable lipids were also analyzed by reverse phase HPLC (40) using a C18 Versapack column (10 µm, 300 × 4.1 mm I.D., Alltech) under isocratic conditions with HPLC grade acetonitrile–water 93:7 (v/v) at 2 ml/min and monitoring at 205 nm. Elution times of authentic standards were as follows: 24,25-epoxycholesterol (7.0 min), 2,3-22,23-DOS (9.9 min), 24,25-epoxylanosterol (11.2 min), desmosterol (18.0 min), 2,3-MOS (22.6 min), cholesterol (28.4 min), lanosterol (30.4 min), and squalene (56.0 min).

Administration of Ro 48-8071 and simvastatin to hamsters

Six-week-old male golden Syrian hamsters (Fume SPF from BRL, Füllinsdorf, Switzerland), weighing 120–130 g were housed individually on nesting material in Makrolon® cages with 12-h alternating periods of light and darkness, and had free access to standard rodent chow (Kliba Mühlen, Kaiseraugst, Switzerland) and to water. During the week preceding treatment, hamsters received 9 g of a 40 cal.% fat diet (coconut kernel in standard chow, Kliba Mühlen) every morning. The daily serving of 9 g of fat diet was mixed with 9 ml of water to produce a paste (41). Hamsters consistently ate more than 90% of their daily serving, and exhibited a new steady state of plasma lipoproteins (41). For drug administration, the fumarate salt of Ro 48-8071 or simvastatin (ground Zocor® pills) was suspended in water prior to mixing into the diet. Each group consisted of five hamsters receiving 9 g of fat diet containing either Ro 48-8071 (25–300 µmol/kg per day) or simvastatin (10–200 µmol/kg per day) every morning for 10–20 days. Food consumption and body weight were monitored daily throughout the treatment period. Blood samples were collected on EDTA via the jugular vein under

light halothane anesthesia at different time points, and plasma was prepared by centrifugation and stored at −20°C. Liver and heart were excised under anesthesia.

Administration of Ro 48-8071 and simvastatin to squirrel monkeys

Twenty-year-old male squirrel monkeys (*Saimiri sciureus,* ~1 kg) were housed in pairs in metal cages under controlled temperature and hygrometry, with normal daylight and free access to water. They were maintained on a 40 cal.% fat diet (coconut kernel in standard chow, Kliba Mühlen #23-2551), 40 g once a day. The fat diet was mixed with water to produce a paste, extruded in small pellets and dried. For drug administration, the fumarate salt of Ro 48-8071 or simvastatin (ground Zocor® pills) was suspended in water prior to mixing into the diet, followed by extruding and drying. Each group consisted of four animals that received either Ro 48-8071 (30–60 μmol/kg per day) or simvastatin (110 μmol/kg per day) in 40 g of dry, pelleted fat diet, every morning for 14 days. Fasting blood samples were collected from the tail vein, without anesthesia, in the morning every 3–4 days before and during drug administration, and plasma was prepared by centrifugation and stored at −20°C. Body weight was also monitored.

Administration of Ro 48-8071 and simvastatin to Göttingen minipigs

Ten- to 14-month-old female Göttingen minipigs (17–21 kg) were housed by three in 20 m^2 cages under controlled temperature and hygrometry, with 12-h alternating periods of light and darkness and free access to water. They were maintained on a 40 cal.% fat diet (coconut kernel in standard chow, Kliba Mühlen #23-2551), 125 g twice a day. Each group consisted of three animals that were treated for 14 days with either the fumarate salt of Ro 48-8071 (30–40 μmol/kg per day) formulated in gelatin capsules or simvastatin (Zocor®), 3 or 9 pills 20 mg each (~10–30 μmol/kg per day), both administered in pieces of apple. Fasting blood samples were collected from an ear vein, without anesthesia, in the morning every 7 days before and during drug administration, and plasma was prepared by centrifugation and stored at −20°C. Food intake and body weight were also monitored.

Analytical procedures for plasma parameters

Alkaline phosphatase, transaminases (ALAT, ASAT, GGT), bilirubin, and urea were determined in plasma with automated kinetic or enzymatic UV assays, using Cobas Fara or Cobas Mira robots (F. Hoffmann-La Roche Ltd.); free fatty acid levels were measured using the test kit NEFAC (Wako), and testosterone levels using a commercial Testosterone[^{3}H] assay (TRK600, Amersham).

Analytical procedures for plasma cholesterol, triglycerides, lipoproteins, and apolipoproteins

Plasma total cholesterol and triglycerides were measured by colorimetric enzymatic methods using the <Roche> Cholesterol PAP and the <Roche> Triglycerides PAP kits (Roche Diagnostica). Plasma lipoproteins were separated and identified by size-exclusion Superose-6 gel chromatography (Smart™, Pharmacia), and total cholesterol in 50-μl fractions was quantified using a fluorometric enzymatic assay (41). Lipoprotein distribution was calculated assuming a Gaussian distribution for each peak, using a nonlinear, least-squares curve-fitting procedure to calculate the area under the curve. Hamster apoB-100 and apoA-I were determined in a sandwich-format ELISA, using rabbit polyclonal IgG antibodies as described (41).

Quantitative determination of monooxidosqualene, dioxidosqualene, and cholesterol in liver of hamsters

Each liver was homogenized with two volumes of bidistillated water (Polytron); 5 ml of homogenate was mixed with 20 ml of ethanol and 7.5 ml of 10 N NaOH and saponified at 60°C for 90 min with shaking, under nitrogen. Nonsaponifiable lipids were extracted with 3 × 50 ml of petroleum ether. The petroleum ether extract was washed twice with 15 ml and 7.5 ml of water, evaporated to dryness, resuspended into 2 × 0.75 ml of n-heptane, transferred into a screw-cap vial, and stored dry under nitrogen. Cholesterol and intermediates along with squal*a*ne as internal standard were quantified by GC using a 0.32 mm × 15 m, 0.32 μm methylsilicon, capillary column (DB-1) with helium at 4.9 ml/min. GC was performed with two consecutive temperature gradients of 20°C/min (50–200°C) and 3°C/min (200–280°C). The injector was maintained at a temperature 5°C higher than column temperature, and a flame ionization detector was used at 300°C.

Intravenous administration of [^{14}C]squalene to hamsters and incorporation into liver nonsaponifiable lipids

First, 480 kBq of [1,5,9,14,20,24-^{14}C]squalene and 0.65 mg of non-radioactive squalene were dried under nitrogen, mixed into 0.1 ml of ethanol and added to 12 ml of sterile homologous hamster plasma. For acute experiments, Ro 48-8071 was administered to fat-fed hamsters by gavage with 3 ml of a 5% (w/v) milk-powder suspension. After 2 h, 500 μl of [^{14}C]squalene formulated in plasma was injected in the jugular vein under light anesthesia. After another 12 h, hamsters were

killed under anesthesia, livers were excised and homogenized in bidistilled water, and lipids were extracted and saponified as above. Nonsaponifiable lipids were separated quantitatively by TLC in n-hexane–ether–acetic acid 60:40:1 (v/v/v) along with lipid standards. Sensitive Phosphor screens were then exposed to the TLC plates for recording radioactivity. For chronic experiments, fat-fed hamsters received Ro 48-8071 in food-admix for 10 days. Radioactive squalene was injected i.v. on the last day of treatment; hamsters were killed 12 h after injection, and hepatic nonsaponifiable lipids were prepared and analyzed as above. Radioactivity in each lipid was recalculated as a percent of the total radioactivity injected.

Analytical procedure for coenzymes Q9 and Q10

Hamster hearts and livers were homogenized in bidistilled water, and coenzymes Q were extracted in ethanol–n-hexane 2:5 (v/v) containing the internal standard CoQ11 (42). Each organic phase was collected, dried under nitrogen, and chromatographed on silica gel F_{254}-TLC plates in diethyl ether–n-hexane 4:6 (v/v). CoQ9, CoQ10, and CoQ11 which comigrated in a single band were detected by fluorescence. The CoQ band was scraped off and extracted with dichloromethane. The three CoQ subspecies were separated and quantified by reverse phase HPLC using an ODS Spheri column (5 µm, 220 × 4.6 mm I.D., Brownlee) under isocratic conditions with HPLC grade ethanol–water 99:1 (v/v) and monitoring at 275 nm.

Ex vivo assays for hepatic HMG-CoA reductase, squalene synthase, and oxidosqualene cyclase

Hamster livers were homogenized, and cell-free extracts and microsomes were prepared as described (38). HMG-CoA reductase activity was measured (43) in the presence of 345 µM [^{14}C]HMG-CoA, 90 mM glucose-6-phosphate, 9 mM NADP, 0.66 units/ml of glucose-6-phosphate dehydrogenase and 8 mg protein/ml for 20 min at 37°C in 50 mM potassium phosphate, 250 mM NaCl, 1 mM EDTA and 5 mM DTT (pH 7.4). Squalene synthase activity was measured in cell-free extracts (44), in the presence of 80 µM [^{3}H]farnesyl-pyrophosphate, 0.5 mM NADPH, 50 mM ascorbate, 20 units/ml of ascorbate oxidase and 2 mg protein/ml for 30 min at room temperature in 50 mM MOPS, 10 mM KF and 10 mM $MgCl_2$ (pH 7.5). Oxidosqualene cyclase activity was measured in cell-free extracts as described above, in the presence of 30 µM [^{14}C]R,S-MOS and 4 mg protein/ml for 1 h at 37°C.

Determination of VLDL secretion rate in hamsters

Intravenous injection of Triton WR-1339 blocks the lipolytic degradation of newly secreted VLDL (45). As a consequence VLDL cannot be removed from the circulation or be converted to LDL, causing a rapid and linear elevation in plasma triglycerides matching a rapid accumulation of VLDL, reflecting the rate of VLDL secretion from the liver (41, 46). VLDL secretion was examined in fat-fed hamsters after 10 days of treatment with either Ro 48-8071 or simvastatin. Hamsters were fasted for 4 h prior to an intravenous injection of Triton WR-1339 (600 mg/kg b.w.). Then, blood was collected at different time points, and plasma triglycerides were measured as above.

Isolation of hamster lipoproteins and ^{125}I labeling

VLDL and LDL were isolated from pooled EDTA hamster plasma (41) prior to radioiodination (47). Free ^{125}iodine was removed by Sephadex G-25 gel filtration on PD10 columns (Pharmacia) and ^{125}I-labeled lipoproteins were dialyzed against 150 mM NaCl, 0.27 mM EDTA (pH 7.4) at 4°C with repeated changes of the buffer. The ^{125}I-labeled VLDL fraction was incubated for 1 h at 37°C in VLDL-free hamster plasma and isolated again by tube slicing after flotation at 440,000 *g* for 2 h at 4°C in a TLA-100.2 rotor (Beckman), in order to decrease radioactivity of apoE and apoC, and to increase the relative radioactivity of apoB-100 (41). The ^{125}I-labeled LDL fraction was used as such. VLDL and LDL with a specific radioactivity of 180–360 cpm/ng protein were stored under nitrogen at 4°C, and used within 1 week.

Liver VLDL uptake in hamsters

^{125}I-VLDL were injected in the left jugular vein of hamsters under light halothane anesthesia, and 100 µl of blood was collected from the right jugular vein after 15 s to determine the total plasma radioactivity. Hamsters were killed by decapitation 3 min after injection of ^{125}I-labeled VLDL when VLDL uptake reaches a maximum (48). Livers were immediately perfused in situ with saline through the portal vein for 3 min; the whole liver was excised and weighed. Three samples, 0.8–1.0 g each, were taken from the three main lobes, and radioactivity was measured directly in a gamma-counter (Cobra Auto-Gamma, Packard). VLDL uptake was expressed as a percent of radioactivity injected.

Plasma LDL turnover in hamsters

Hamsters kept under light halothane anesthesia received an intravenous bolus injection of hamster ^{125}I-labeled LDL, 15 µg protein per animal in 0.15 ml of 150 mM NaCl, via the left jugular vein. The first blood sample (150 µl) was collected 5 min after injection, and the amount of radioactivity circulating at this time point was set at 100%. Blood samples were collected subsequently from the right jugular vein at different time points. The TCA precipitable ^{125}I radioactivity associated with LDL was counted. Plasma decay curves were analyzed by a nonlinear, least-squares curve-fitting pro-

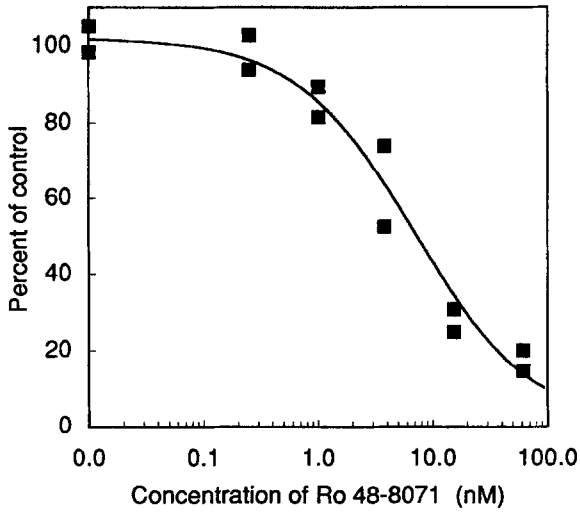

Fig. 2. Inhibition of human liver microsomal oxidosqualene cyclase by Ro 48-8071. Human liver microsomes (0.4 mg protein/ml) were incubated with increasing concentrations of Ro 48-8071 in the presence of 30 μM [^{14}C]R,S-MOS and 0.5% albumin in phosphate buffer, pH 7.4, for 1 h at 37°C. The reaction was stopped, and samples were processed as described.

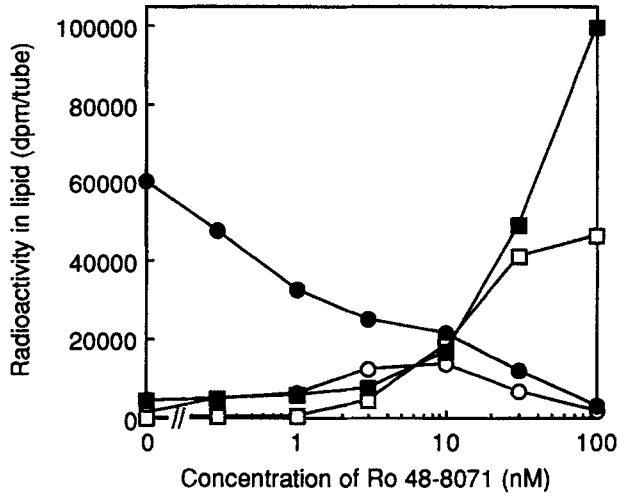

Fig. 3. Effects of increasing concentrations of Ro 48-8071 on the incorporation of [^{14}C]acetate into nonsaponifiable lipids in cultured HepG2 cells. HepG2 cells were preincubated for 24 h in DMEM–5% LPDS, and then incubated for 18 h with increasing concentrations of Ro 48-8071 in the presence of [^{14}C]acetate. After washing the cells with PBS, lipids were extracted, saponified, extracted, and separated by TLC, prior to identification and quantification by Phosphorlmager scanning. The symbols are: 24,25-epoxycholesterol, R_f = 0.16 (○); cholesterol, R_f = 0.20 (●), DOS, R_f = 0.53 (□); and MOS, R_f = 0.78 (■). Data are expressed as dpm of [^{14}C]acetate incorporated per lipid in each culture tube, and each point is the mean value of two determinations.

cedure, and the fractional catabolic rate (FCR) was calculated using a two-pool model (49).

Statistical methods

All statistical comparisons were performed using the unpaired, two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Inhibition of hepatic oxidosqualene cyclase by Ro 48-8071

The effect of increasing concentrations of Ro 48-8071 on the enzymatic conversion of MOS to lanosterol was measured in a detergent free buffer using a concentration of radiolabeled substrate of 30 μM, i.e., within the range of first-order enzymatic reaction of mammalian OSC (50, 51). Ro 48-8071 inhibited OSC activity with an IC_{50} of ~6.5 nM **(Fig 2).** Under similar conditions it was ~6-times less potent against hamster and Göttingen minipig liver OSC, and ~10-times more potent against squirrel monkey liver OSC (data not shown).

Incorporation of [^{14}C]acetate into nonsaponifiable lipids of HepG2 cells

In HepG2 cells, cholesterol synthesis assessed by [^{14}C]acetate incorporation was reduced dose-dependently by Ro 48-8071 with an IC_{50} of ~1.5 nM **(Fig. 3).** As assessed by TLC and HPLC, the inhibition of cholesterol synthesis was associated with the production of MOS and DOS at concentrations of Ro 48-8071 >3 nM. A polar lipid with an R_f of 0.16 on TLC increased with increasing concentrations of Ro 48-8071 up to 10 nM, and decreased further at higher concentrations. In HPLC this lipid co-eluted with an authentic standard of 24(R,S),25-epoxycholesterol (data not shown), in agreement with previous works using other OSC inhibitors (20, 27, 40). Epoxycholesterol radioactivity reached almost one-fourth of the radioactivity in cholesterol of control cells, although the mass of epoxycholesterol relative to cholesterol in treated cells must have been less because of preexisting cholesterol. The relative increase in radioactive epoxycholesterol was higher than that reported for BIBX79 (27); in the latter work, cells were exposed to the OSC inhibitor for 16 h prior to a coincubation of 2 h with [^{14}C]acetate, which might have limited the amount of epoxycholesterol produced. Importantly, Ro 48-8071 was specific for OSC causing no accumulation of desmosterol, lanosterol, squalene, or other nonsaponifiable lipids.

Safety parameters in hamsters treated with Ro 48-8071 and simvastatin

Food consumption in hamsters treated with Ro 48-8071 was normal up to a dose of 200 μmol/kg per day

TABLE 1. Food intake, body weight increase, liver weight, plasma bilirubin, urea, free fatty acids, and testosterone in hamsters treated with Ro 48-8071 and simvastatin

Dose	Food Intake (Daily average)	Body Weight Increase	Liver Weight	Plasma Bilirubin	Plasma Urea	Plasma FFA	Plasma Testosterone
μmol/kg/day	*g/day*	*g*	*g*	*μmol/l*	*mmol/l*	*mmol/l*	*ng/ml*
0	8.3 ± 0.6	19.6 ± 2.7	5.9 ± 0.2	1.3 ± 0.3	6.5 ± 0.3	1.2 ± 0.1	5.8 ± 0.8
Ro 48-8071							
25	7.5 ± 0.2	18.7 ± 2.2	5.6 ± 0.2	0.7 ± 0.1	7.1 ± 0.5	1.0 ± 0.1	n.d.
50	8.7 ± 0.4	24.1 ± 1.1	6.4 ± 0.3	0.9 ± 0.1	7.2 ± 0.2	0.9 ± 0.1	8.0 ± 1.3
100	8.1 ± 0.3	16.9 ± 3.2	6.0 ± 0.3	0.8 ± 0.1	8.2 ± 0.6[a]	1.1 ± 0.1	n.d.
150	7.7 ± 0.2	18.8 ± 2.2	6.4 ± 0.2	0.8 ± 0.1	6.7 ± 0.6	0.8 ± 0.1[a]	6.3 ± 0.6
200	7.5 ± 0.4	18.1 ± 2.3	6.3 ± 0.5	0.7 ± 0.1	6.5 ± 0.3	0.8 ± 0.1[a]	n.d.
300	7.2 ± 0.3[a]	12.1 ± 2.9	6.3 ± 0.3	0.7 ± 0.1	5.9 ± 0.2	0.9 ± 0.1	7.2 ± 1.4
Simvastatin							
10	8.3 ± 0.3	21.1 ± 2.3	5.9 ± 0.2	0.7 ± 0.1	6.2 ± 0.3	0.7 ± 0.1[c]	6.8 ± 0.4
20	7.6 ± 0.2	13.5 ± 2.2	5.7 ± 0.4	0.6 ± 0.1	6.6 ± 0.5	0.8 ± 0.2	n.d.
30	7.2 ± 0.2[a]	14.5 ± 1.7	5.8 ± 0.2	0.7 ± 0.1	6.5 ± 0.5	1.0 ± 0.2	6.4 ± 0.7
60	6.7 ± 0.2[b]	11.6 ± 2.4[a]	7.3 ± 0.5[b]	2.1 ± 0.4[a]	5.9 ± 0.4	0.3 ± 0.1[c]	6.3 ± 1.0
100	6.1 ± 0.2[b]	1.4 ± 3.3[b]	8.5 ± 0.4[b]	2.2 ± 0.6[a]	6.0 ± 0.3	0.3 ± 0.1[c]	3.2 ± 1.1[a]
200	4.6 ± 0.3[a]	−11.1 ± 5.2[b]	6.9 ± 0.3[b]	3.8 ± 1.9[a]	8.6 ± 0.8[a]	0.7 ± 0.1[b]	n.d.

Hamsters received increasing doses of either Ro 48-8071 or simvastatin in food-admix for 20 days. Food intake, body weight increase, liver weight, plasma bilirubin, urea, free fatty acids (FFA), and testosterone were determined as described in Materials and Methods. Mean value ±SEM (n = 10 for control group; n = 5 animals for treated groups), significantly different from control group at [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.005$; n.d., not determined.

(Table 1). It was slightly reduced at 300 μmol/kg per day already on the first day of administration, most likely because of aversion to taste. Body weight increase was normal, and there was no sign of liver toxicity, i.e., no increase in liver weight and no increase in plasma alkaline phosphatase, transaminases, bilirubin, and urea. In contrast, simvastatin at doses >30–60 μmol/kg per day caused a reduction in food consumption (Table 1) that amplified over time (data not shown), and a reduction in body weight (Table 1). Also, simvastatin at doses >30–60 μmol/kg per day caused a dose-dependent increase in liver weight, and in plasma bilirubin and urea, as well as a decrease in plasma free fatty acids, all indicative of liver dysfunction (Table 1). It had no effect on plasma alkaline phosphatase and transaminases (data not shown). Simvastatin at 100 μmol/kg per day reduced plasma testosterone level by ~50%, in contrast to Ro 48-8071 which had no effect.

Effects of Ro 48-8071 and simvastatin on plasma cholesterol, triglycerides, lipoprotein, and apolipoprotein levels in hamsters.

In fat-fed hamsters, plasma total cholesterol at baseline was 200–250 mg/dl consisting of ~8% VLDL, ~28% LDL, and ~64% HDL. After 10 days of treatment with Ro 48-8071 total cholesterol, LDL-C and apoB-100 levels were reduced dose-dependently **(Table 2).** Ro 48-8071 lowered LDL-C maximally ~60% at 150 μmol/kg per day, with no further reduction up to 300 μmol/kg per day, leaving HDL-C unchanged at all doses **(Fig. 4,** top-left). Consistently, Ro 48-8071 lowered apoB-100 levels maximally ~40%, whereas apoA-I levels were slightly elevated (Fig. 4, bottom-left).

Simvastatin lowered total cholesterol and LDL-C levels more than Ro 48-8071 (Table 2), but at the expense of adverse effects at doses >30 μmol/kg per day as discussed above in the section Safety parameters; it also markedly lowered HDL-C and apoA-I. In addition, simvastatin up to 100 μmol/kg per day caused an increase in triglyceride and apoB-100 levels, followed by a reduction at 200 μmol/kg per day, most likely because of low food consumption. Such an increase in triglyceride levels has been reported before in hamsters (52), and is associated with an increase in apoB100-containing VLDL. At the highest safe dose of 30 μmol/kg per day, simvastatin lowered LDL-C ~50%, leaving, HDL-C unchanged (Fig. 4, top-right); at this dose, apoB-100 and apoA-I levels were unaffected (Fig. 4, bottom-right). At higher but toxic doses, simvastatin reduced LDL-C and HDL-C dramatically (Fig. 4, top-right), reduced apoA-I levels, and increased apoB-100 levels (Fig. 4, bottom-right).

Monooxidosqualene, dioxidosqualene, and cholesterol in liver of hamsters treated with Ro 48-8071 and simvastatin

Lipids were extracted from hamster liver homogenates after treatment with either Ro 48-8071 or simvastatin in order to quantify cholesterol and nonsaponifiable intermediates. MOS was found in trace amounts in liver of control animals, and increased in response to treatment with Ro 48-8071 dose-dependently **(Fig. 5).**

TABLE 2. Plasma cholesterol, triglycerides, lipoproteins, and apolipoproteins in hamsters treated with Ro 48-8071 and simvastatin

Dose	Total Cholesterol	Triglycerides	VLDL-C	LDL-C	HDL-C	ApoB 100	ApoA-I
μmol/kg/day	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*	*mg/ml*	*mg/ml*
0	244 ± 14	417 ± 67	20 ± 6	74 ± 10	150 ± 16	0.97 ± 0.09	2.10 ± 0.12
Ro 48-8071							
25	215 ± 5	374 ± 42	5 ± 2[a]	64 ± 7	146 ± 10	0.93 ± 0.09	2.40 ± 0.22
50	233 ± 7	356 ± 23	9 ± 3	60 ± 7	163 ± 11	0.83 ± 0.06	2.60 ± 0.19[a]
100	236 ± 7	458 ± 72	14 ± 6	59 ± 5[a]	164 ± 15	0.80 ± 0.06[a]	2.40 ± 0.28
150	200 ± 7[a]	417 ± 33	13 ± 5	44 ± 5[b]	143 ± 7	0.77 ± 0.07[a]	2.20 ± 0.10
200	192 ± 10[a]	320 ± 51	5 ± 3[a]	49 ± 11[a]	137 ± 10	0.77 ± 0.17	1.90 ± 0.16
300	174 ± 6[b]	288 ± 72	20 ± 7	47 ± 4[b]	106 ± 11[a]	0.79 ± 0.11	1.70 ± 0.20[a]
Simvastatin							
10	241 ± 10	306 ± 62	5 ± 4[a]	63 ± 4	174 ± 12	0.84 ± 0.10	2.40 ± 0.16
20	196 ± 17	498 ± 102	18 ± 6	53 ± 7[a]	124 ± 17	1.20 ± 0.07[b]	1.70 ± 0.18[a]
30	192 ± 5	267 ± 29[a]	13 ± 1	49 ± 5[b]	130 ± 4	1.10 ± 0.11	2.10 ± 0.06
60	149 ± 8	483 ± 49	28 ± 5	25 ± 5[b]	96 ± 9[b]	1.10 ± 0.12	2.00 ± 0.20
100	123 ± 14	584 ± 36[a]	18 ± 7	22 ± 3[b]	83 ± 6[b]	1.30 ± 0.13[a]	1.80 ± 0.15
200	44 ± 15	199 ± 43[b]	4 ± 2[b]	5 ± 3[b]	36 ± 10[b]	0.50 ± 0.11[b]	0.76 ± 0.21[b]

Hamsters received increasing doses of either Ro 48-8071 or simvastatin in food-admix for 10 days. Blood was collected on days 0 and 10, and plasma was prepared by centrifugation. Plasma lipoproteins, cholesterol, triglycerides, and apolipoproteins were analyzed as described in Materials and Methods. Mean value ±SEM (n = 10 for control group; n = 5 animals for treated groups), significantly different from control group at [a]$P < 0.05$ and [b]$P < 0.01$.

The maximum concentration of MOS detected was ~20 μg/g wet liver or only less than 1% of the cholesterol content. The concentration of MOS in liver was inversely correlated with LDL-C **(Fig. 6),** in agreement with the mode of action of Ro 48-8071. No DOS, the epoxidation product of MOS, as well as no other non-saponifiable lipid could be found under our experimental conditions. The cholesterol concentration in liver (mg/g) remained unchanged at all doses of Ro 48-8071 (Fig. 5) as well as total cholesterol (mg/liver).

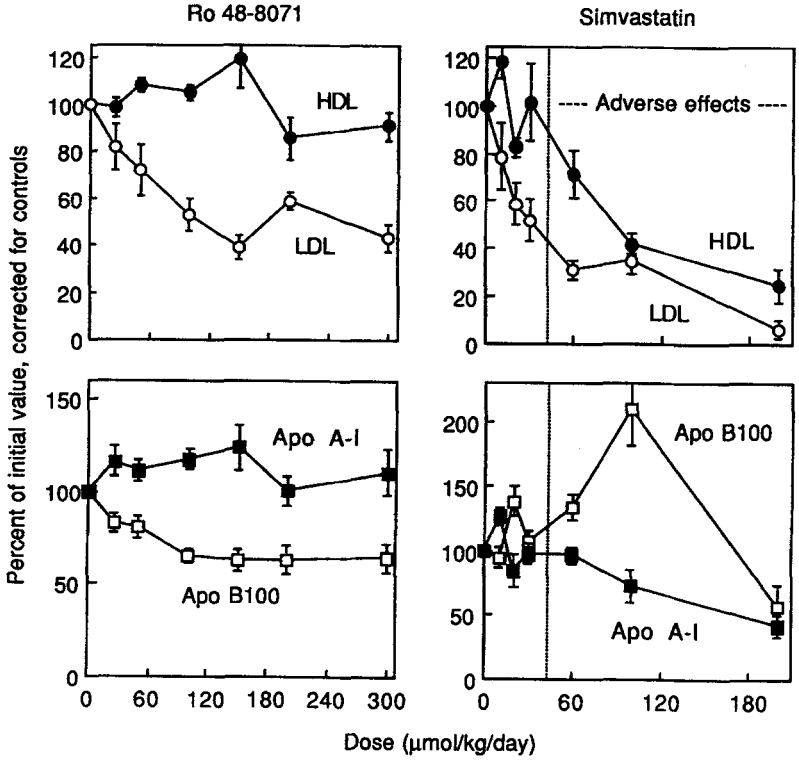

Fig. 4. Effects of Ro 48-8071 and simvastatin on lipoprotein cholesterol and apolipoprotein levels in hamsters. Hamsters were treated with either Ro 48-8071 or simvastatin, and plasma samples collected and processed as in Table 2. Data at day 10 are expressed as a percent of initial value (day 0) corrected with respect to control animals (no drug), and each point is a mean value ±SEM (n = 10 for control group; n = 5 for treated groups). The reduction in LDL-C and apoB-100 was statistically significant at $P < 0.01$ with Ro 48-8071 at doses ≥100 μmol/kg per day, and with simvastatin at doses ≥20 μmol/kg per day. Symbols are: LDL cholesterol (○); HDL cholesterol (●); apoB-100 (□); and apoA-I (■).

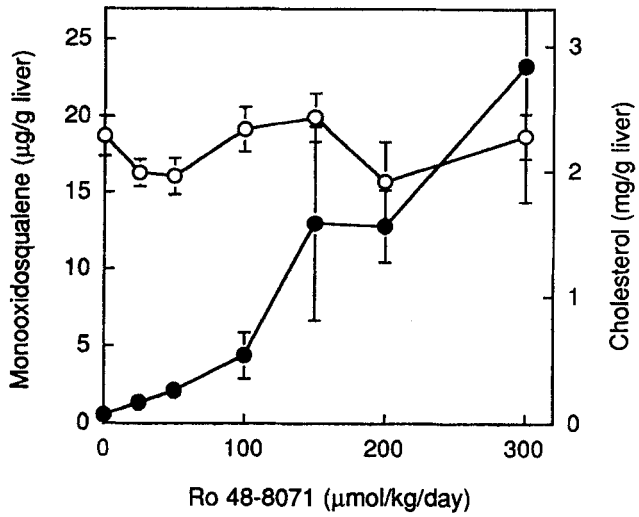

Fig. 5. Monooxidosqualene and cholesterol in liver of hamsters treated with Ro 48-8071. Hamsters were treated for 20 days with increasing doses of Ro 48-8071. Livers were collected and homogenized; lipids were extracted and saponified. MOS and cholesterol were identified and quantified by GC–MS. Data are expressed in µg MOS (●) and mg cholesterol (○) per gram wet liver, and each point is the mean value of five determinations ± SEM.

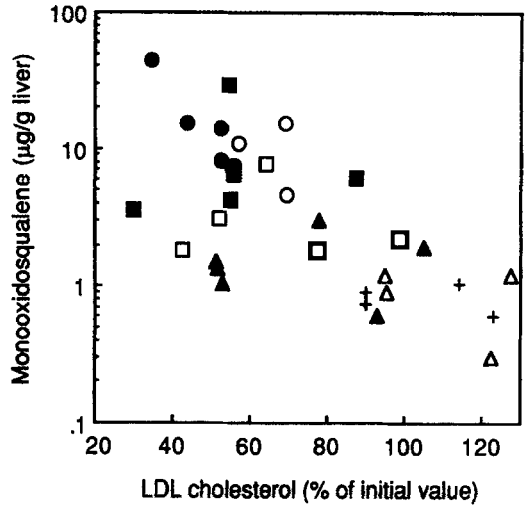

Fig. 6. Monooxidosqualene in liver versus LDL cholesterol lowering in hamsters treated with Ro 48-8071. Hamsters were treated with Ro 48-8071, and liver samples were analyzed as in Fig. 5. Plasma LDL cholesterol was determined as described. The MOS content of each single liver expressed in µg/g wet tissue was plotted against LDL-C of the corresponding animal expressed as a percent of initial value (day 0) corrected for controls (n = 5 for each dose). Symbols for doses are: 0 (+), 25 (△), 50 (▲), 100 (□), 150 (■), 200 (○) 300 (●) µmol/kg/per day (n = 35, $R = 0.618$, $P = 0.0002$).

As expected from its mode of action, simvastatin did not increase MOS and DOS (data not shown); it had no effect on cholesterol concentration in liver expressed in mg/g, although total hepatic cholesterol was elevated, matching the increase in liver weight.

Incorporation of i.v. injected [^{14}C]squalene into nonsaponifiable lipids of liver of hamsters treated with Ro 48-8071

When [^{14}C]squalene was injected to control animals, >16% of the radioactivity injected was recovered in hepatic cholesterol after 12 h, 2–3% in lanosterol, 2–4% as unchanged squalene, and <0.2% in MOS. After acute or chronic treatment with the OSC inhibitor, radioactivity increased dose-dependently in MOS up to ~2% of the radioactivity injected, and radioactivity in lanosterol decreased **(Fig. 7).** Cholesterol synthesis measured by this method was reduced ~25% at a dose of 200 µmol/kg per day. Upon treatment with Ro 48-8071 no radioactivity was found in DOS and in other nonsaponifiable lipids. Together with the results shown in Fig. 5 and 6, these data demonstrate that inhibition of OSC took place in vivo, and strongly suggest that it is responsible for the cholesterol-lowering effect of Ro 48-8071; it also confirms the specificity of Ro 48-8071 for OSC.

Effects of Ro 48-8071 and simvastatin on heart and liver coenzyme Q levels in hamsters

Because mevalonate, the product of HMG-CoA reductase, is a precursor of nonsterol isoprenoids and subsequently of CoQ10 (53), inhibition of HMG-CoA reductase by statins can reduce tissue CoQ10 levels in vivo (54–56). In order to determine whether inhibition of OSC and/or HMG-CoA reductase affects this pathway in vivo, CoQs were quantified in liver and heart of hamsters treated with either Ro 48-8071 or simvastatin. CoQ9 was found in relatively large amounts in heart and liver of hamsters, in contrast to human tissues which contain mostly CoQ10. In liver, CoQ9 levels were somewhat increased after treatment with Ro 48-8071 at 50–150 µmol/kg per day, and noticeably more after treatment with simvastatin **(Table 3).** At doses of 100–200 µmol/kg per day of simvastatin, CoQ9 levels in liver almost tripled as compared to untreated animals. The significance of this increase is unclear, and it might well reflect the involvement of HMG-CoA reductase in determining the side-chain length of CoQs (57). In heart, CoQ9 levels were unchanged in both groups of treated animals. In liver, CoQ10 levels were increased 30–80% after treatment with Ro 48-8071 (Table 3); in contrast, liver CoQ10 levels were reduced 30–50% after treatment with simvastatin at doses >30 µmol/kg per day. In heart, CoQ10 levels were unchanged after treat-

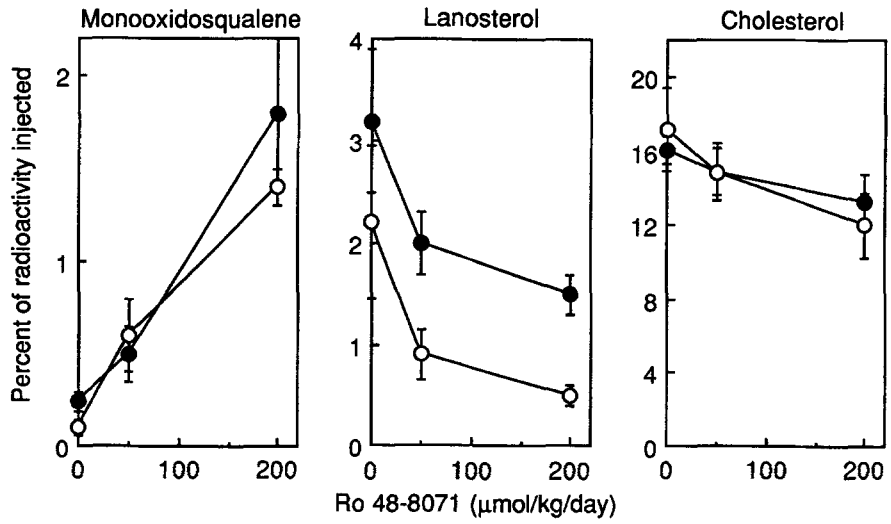

Fig. 7. Effects of Ro 48-8071 on the incorporation of [^{14}C]squalene injected i.v. into non-saponifiable lipids of liver in hamsters. In the acute experiment (●), Ro 48-8071 was administered to hamsters by gavage at 50 and 200 μmol/kg per day, and 2 h later [^{14}C]squalene formulated in plasma was injected intravenously. After 12 h, hamsters were killed, livers were collected and homogenized, and lipids were extracted, saponified and separated by TLC for quantification of radioactivity. In the chronic experiment (○), hamsters received Ro 48-8071 in food-admix at 50 and 200 μmol/kg per day for 10 days. [^{14}C]squalene was injected intravenously on the last day of treatment, and hamsters were killed 12 h after injection, and lipids were prepared and analyzed as above. Data are expressed as a percent of the radioactivity injected, and each point is the mean value of four determinations ± SEM. Radioactivity recovered as intact squalene precursor was less than 0.2% (not shown).

ment with Ro 48-8071; in contrast, CoQ10 levels in heart were reduced 10–25% after treatment with simvastatin, in agreement with previous studies (58). In hamster plasma, CoQ10 and CoQ9 were below detection limit.

Enzymes of cholesterol synthesis in liver of hamsters treated with Ro 48-8071 and simvastatin

In humans and animals, inhibition of HMG-CoA reductase with statins triggers an overexpression of HMG-

TABLE 3. Coenzyme Q levels in liver and heart of hamsters treated with Ro 48-8071 and simvastatin

	Coenzyme Q9		Coenzyme Q10	
Dose	Liver	Heart	Liver	Heart
μmol/kg/day	*μg/g of wet tissue*			
0	14.4 ± 0.3	115.1 ± 5.9	20.8 ± 1.2	70.8 ± 2.7
Ro 48-8071				
25	14.7 ± 1.7	118.4 ± 5.0	32.0 ± 3.1	72.6 ± 2.1
50	22.0 ± 1.8[a]	130.5 ± 6.6	34.0 ± 1.8[a]	75.4 ± 3.1
100	17.1 ± 1.3[a]	106.5 ± 12.7	29.8 ± 2.8[a]	64.3 ± 7.0
150	17.0 ± 0.7[b]	128.3 ± 2.5	28.7 ± 1.4[a]	75.4 ± 8.3
200	16.7 ± 1.1	106.6 ± 5.8	37.8 ± 2.1[a]	64.3 ± 3.8
300	13.8 ± 0.9	115.6 ± 10.5	27.8 ± 1.5[a]	72.3 ± 3.4
Simvastatin				
10	17.9 ± 0.9[a]	115.9 ± 8.6	18.4 ± 1.6[a]	60.8 ± 4.1[b]
20	21.9 ± 2.1[a]	123.6 ± 3.4	17.7 ± 2.0[a]	62.5 ± 2.0[b]
30	25.5 ± 2.4[a]	124.5 ± 13.3	17.6 ± 1.3[a]	72.0 ± 5.0
60	29.6 ± 3.3[a]	116.6 ± 6.5	13.9 ± 0.8[a]	59.6 ± 3.1[a]
100	45.3 ± 1.4[a]	136.3 ± 4.1	11.4 ± 0.7[a]	63.0 ± 2.2[a]
200	43.3 ± 1.8[a]	106.1 ± 5.4[b]	10.2 ± 1.2[a]	53.8 ± 3.2[a]

CoQ9 and CoQ10 were extracted from liver homogenates of hamsters treated with increasing doses of either Ro 48-8071 or simvastatin in food-admix for 20 days. Samples were pre-purified by TLC; CoQ9 and CoQ10 were quantified by HPLC using CoQ11 as an internal standard. Mean value ±SEM (n = 10 for control group; n = 5 animals for treated groups), significantly different from control group at [a]$P < 0.01$ and [b]$P < 0.005$.

TABLE 4. Ex vivo activity of enzymes of cholesterol synthesis in liver of hamsters treated with Ro 48-8071 and simvastatin

Dose	HMG-CoA Reductase	Squalene Synthase	Oxidosqualene Cyclase
μmol/kg/day		*pmol/mg/min*	
0	10.2 ± 1.2	2.4 ± 1.1	1.6 ± 0.1
Ro 48-8071			
25	n.d.	4.5 ± 2.0	1.1 ± 0.1
50	n.d.	3.6 ± 1.5	1.0 ± 0.1[a]
100	n.d.	4.1 ± 1.3	0.9 ± 0.1[a]
150	11.9 ± 2.6	3.2 ± 1.7	0.8 ± 0.2
200	8.8 ± 5.3	3.8 ± 0.8	1.1 ± 0.1
300	n.d.	4.4 ± 0.9	1.3 ± 0.2
Simvastatin			
10	20.1 ± 7.4	n.d.	n.d.
30	170 ± 111[a]	n.d.	n.d.
60	512 ± 37[a]	88 ± 45[a]	10.9 ± 38[a]

Enzyme activities were measured in liver cell-free extracts (squalene synthase and oxidoqualene cyclase) or liver microsomes (HMG-CoA reductase) prepared from hamsters treated with increasing doses of either Ro 48-8071 or simvastatin in food-admix for 20 days. Mean value ±SEM (n = 2), significantly different from control group at [a]$P < 0.05$; n.d., not determined.

CoA reductase (15–17), as a result of a positive feed-back regulatory mechanism (25). Previous reports suggest that epoxysterols produced upon inhibition of OSC could maintain HMG-CoA reductase and other enzymes of the pathway at normal or subnormal levels via a negative feed-back regulatory mechanism (20–24, 27). In order to discriminate the indirect effects triggered upon inhibition of either OSC or HMG-CoA reductase, the activities of HMG-CoA reductase, squalene synthase, and oxidosqualene cyclase were determined ex vivo in liver of hamsters treated with increasing doses of either Ro 48-8071 or simvastatin.

HMG-CoA reductase. Treatment of hamsters with Ro 48-8071 did not affect HMG-CoA reductase activity **(Table 4).** In contrast, treating hamsters with simvastatin at doses of 10, 30, and 60 μmol/kg per day resulted in 2-, 17-, and 50-fold increases in ex vivo hepatic HMG-CoA reductase activity, respectively.

Squalene synthase. Previous studies suggested that squalene synthase is coordinately regulated with HMG-CoA reductase, thereby maintaining levels of key intermediates (59). Accordingly, a 37-fold increase of ex vivo hepatic squalene synthase activity was found in hamsters treated with simvastatin at 60 μmol/kg per day (Table 4). In contrast, squalene synthase activity remained unchanged in animals treated with Ro 48-8071.

Oxidosqualene cyclase. Treating hamsters with Ro 48-8071 triggered a 20–50% decrease in ex vivo hepatic oxidosqualene cyclase activity (Table 4). It is unlikely that this decrease is due to endogenous Ro 48-8071 as liver cell-free extracts are very diluted in the assay. In contrast, treating hamsters with simvastatin at a dose of 60 μmol/kg per day resulted in a 7-fold increase in ex vivo hepatic oxidosqualene cyclase activity, suggesting that oxidosqualene cyclase might also be coordinately regulated with HMG-CoA reductase.

VLDL and LDL metabolism in hamsters treated with Ro 48-8071 and simvastatin

Hamsters were treated for 10 days with either Ro 48-8071 or simvastatin. VLDL secretion was determined indirectly by measuring plasma triglycerides 2 h after injection of Triton WR-1339. VLDL secretion was markedly and significantly reduced by 44% after treatment with 300 μmol/kg per day of Ro 48-8071, and by 25% after treatment with 30 μmol/kg per day of simvastatin **(Table 5).** At a dose of 150 μmol/kg per day simvastatin reduced VLDL production further, but at the expense of adverse effects as discussed above in the section Safety parameters. ^{125}I-labeled VLDL liver uptake was reduced by 10–20% (not significant) after treatment with Ro 48-8071. In contrast, ^{125}I-labeled VLDL liver uptake was reduced almost 60% after treatment with simvastatin. ^{125}I-labeled LDL fractional catabolic rate remained unaffected after treatment with either Ro 48-8071 or simvastatin (Table 5). These data suggest that Ro 48-8071 and simvastatin modify lipoprotein metabolism in distinct ways, although both cause a reduction in LDL-C of similar amplitude.

Effects of Ro 48-8071 and simvastatin on lipoprotein levels in squirrel monkeys

In fat-fed squirrel monkeys, Ro 48-8071 was more potent than simvastatin in lowering plasma cholesterol, and as efficacious. Ro 48-8071 at 30 μmol/kg per day lowered LDL-C 27% leaving HDL-C unchanged **(Table**

TABLE 5. VLDL and LDL metabolism in hamsters treated with Ro 48-8071 and simvastatin

Dose	Plasma Triglycerides (Post-Triton WR1339)	^{125}I-Labeled VLDL Liver Uptake	^{125}I-Labeled LDL Fractional Catabolic Rate
μmol/kg/day	*mg/dl*	%	*pools per day*
Control			
0	520 ± 45 (6)	17.3 ± 1.5 (4)	2.18 ± 0.15 (6)
Ro 48-8071			
30	504 ± 53 (4)	15.8 ± 1.3 (4)	2.11 ± 0.23 (5)
300	290 ± 52[b] (4)	13.8 ± 1.3 (4)	2.05 ± 0.22 (5)
Simvastatin			
30	390 ± 37[a] (4)	7.3 ± 1.3[a] (3)	2.09 ± 0.18 (3)
150	196 ± 36[b] (4)	10.3 ± 1.2[a] (3)	3.24 ± 0.73[a] (3)

VLDL and LDL metabolism was examined in hamsters after 10 days of oral treatment with either Ro 48-8071 or simvastatin. Plasma triglyceride levels were determined 2 h after i.v. administration of Triton WR1339. Uptake of ^{125}I-labeled VLDL by liver was measured 3 min after injection of the radiolabeled lipoprotein, and expressed as a percent of radioactivity injected. To determine LDL fractional catabolic rate, ^{125}I-labeled LDL were injected i.v.; plasma samples were collected during the next 30 h; and TCA-insoluble radioactivity of each sample was measured. Mean value ±SEM (n per group shown in parentheses), significantly different from control group at $^{a}P < 0.05$ and $^{b}P < 0.01$.

6); at 60 μmol/kg per day it lowered LDL-C 30% and HDL-C 17%. Simvastatin at 110 μmol/kg per day lowered LDL-C 26% and HDL-C 16% (Table 6); at 60 μmol/kg per day it had no effect on plasma cholesterol (data not shown). Both Ro 48-8071 and simvastatin at the highest doses reduced body weight 5–10% though not significantly (data not shown). There was no evidence of liver toxicity in monkeys treated with Ro 48-8071 or simvastatin, i.e., no increase in plasma alkaline phosphatase, transaminases, bilirubin, and urea (data not shown).

Effects of Ro 48-8071 and simvastatin on lipoprotein levels in Göttingen minipigs

In minipigs, Ro 48-8071 was 5- to 10-times less potent than simvastatin in lowering plasma cholesterol, and at least as efficacious. Ro 48-8071 at 30 μmol/kg per day lowered LDL-C 33%, leaving HDL-C unchanged (Table 6); at 40 μmol/kg per day it lowered LDL-C 34% and HDL-C 17%. Simvastatin at 10 μmol/kg per day lowered LDL-C 28%, leaving HDL-C unchanged (Table 6); at 30 μmol/kg per day it lowered both LDL-C and HDL-

TABLE 6 Effects of Ro 48-8071 and simvastatin on lipoprotein levels in squirrel monkeys and Göttingen minipigs

Squirrel Monkeys			Minipigs		
Dose	LDL	HDL	Dose	LDL	HDL
μmol/kg/day	%	%	*μmol/kg/day*	%	%
Control					
0	100 ± 10	100 ± 4	0	100 ± 11	100 ± 5
Ro 48-8071					
30	73 ± 9[a]	98 ± 3	30	67 ± 3	94 ± 6
60	71 ± 5[b]	83 ± 8[a]	40	66 ± 3[a]	83 ± 7
Simvastatin					
110	73 ± 5[b]	84 ± 9	10	72 ± 4	105 ± 5
			30	66 ± 6[a]	66 ± 5[b]

Squirrel monkeys and minipigs were treated with either Ro 48-8071 or simvastatin for 14 days, in food-admix and in gelatine capsules, respectively. Blood was collected at several time points, plasma was prepared, and lipoproteins were quantified. In squirrel monkeys total cholesterol at base-line ranged from 160 to 240 mg/dl, consisting of ~3% VLDL, ~32% LDL, and ~65% HDL; in minipigs it ranged from 100 to 210 mg/dl, consisting of ~54% LDL and ~46% HDL. LDL cholesterol and HDL cholesterol levels after 14 days of treatment were expressed as a percent of initial value corrected with respect to control animals (no drug). The initial value is the average of base-line values of pretreatment samples collected at day −10, day −7, day −3, and day 0 for squirrel monkeys, and at day −14, day −7, and day 0 for minipigs. Mean value ±SEM significantly different from control group at $^{a}P < 0.05$ and ^{b}P 0.01 (n = 4 per group for squirrel monkeys; n = 3 per group for minipigs).

C 34%. Both Ro 48-8071 and simvastatin at all doses had no significant effect on food intake nor body weight increase (data not shown). There was no evidence of liver toxicity in minipigs treated with Ro 48-8071 or simvastatin, i.e., no increase in plasma alkaline phosphatase, transaminases, bilirubin, and urea (data not shown).

DISCUSSION

Enzymatic cyclization of oxidosqualene remains a remarkable, but not fully understood, mechanism (60–62) although some structural information on the OSC protein and its catalytic site is available (51, 62–64). Our approach for the design of new OSC inhibitors consisted in superimposing a prototype inhibitor on the high energy intermediate of oxidosqualene (28–31) to optimize hydrophobic and ionic interactions with the transition state of the enzyme. The folded, high energy intermediate of oxidosqualene cyclization was modeled as an opened protosterol (Fig. 1), using the computerized modeling program MOLOC (65). Axial delivery of negative point-charges by the enzyme would establish ion pairs, and consequently stabilize cationic centers that are produced during cyclization of the substrate (66, 67). Hypothetically, the amine of our prototype inhibitor Ro 48-8071 (Fig. 1) would interact with the positive charge of the epoxide-opening region of the enzyme, whereas the carbonyl of the benzophenone system would interact with the negative point-charge stabilizing the last protosterol cation. Ro 48-8071 is one of the most potent inhibitors of its class, bearing noticeable structural similarities with BIBX79 (27) for they share a tertiary amine and a keto function, with a lipophilic halogen substitution on the aromatic moiety. The distance between the amine and the ketone is about the same in both agents, and these two functions are separated by a lipophilic system: a rigid trans-cyclohexylaryl system for BIBX79 and a more flexible aryloxyalkyl system for Ro 48-8071.

In cells, Ro 48-8071 at 1–3 nM was able to reduce cholesterol synthesis by as much as 50–60%, with little build-up of MOS and DOS. This would be the consequence of the feed-back regulatory loop triggered by partial inhibition of OSC resulting from *i*) formation of MOS followed by *ii*) secondary epoxidation of MOS to DOS, *iii*) preferential cyclization of DOS (versus MOS) to 24(S),25-epoxylanosterol (24, 27, 68), and *iv*) production of 24(S),25-epoxycholesterol (22, 23, 26). The latter has been found previously in cells incubated with other OSC inhibitors (27, 40); it would be acting as a potent down regulator of HMG-CoA reductase expression (21–24). HMG-CoA reductase itself is not directly inhibited by Ro 48-8071 (data not shown). Preferential cyclization of DOS versus MOS in the presence of Ro 48-8071 would be critical for initiating the negative feedback loop, allowing net blockade of cholesterol synthesis with little accumulation of intermediates.

Hamsters on a human-like fat diet were used for in vivo evaluation as hepatic cholesterol metabolism in this model is somewhat similar to that of humans (41, 69). Simvastatin caused marked dose-dependent adverse effects (Table 1) comparable to those reported previously (14). The adverse effects of statins, particularly in hamsters but also in guinea pigs and rabbits, are fully or partially antagonized by coadministration of mevalonate (13, 14, 70), suggesting that these are mechanism-based effects linked to an excessive blockade of the pathway and not to an intrinsic toxic property of the molecule. The reduction in CoQ10 levels in heart and liver (Table 3) is an indirect evidence for the blockade of the non-sterol isoprenoid pathway by simvastatin. Hence, the adverse effects of simvastatin define the limits of a window of safety in hamsters, in which maximum reduction of plasma LDL-C is ~50%. In contrast, the safety window of Ro 48-8071 in hamsters extended throughout the whole dose-range tested in our conditions; the OSC inhibitor lowered LDL-C by ~60% and correspondingly apoB-100 by ~40%, with little alteration of HDL-C and apoA-I levels.

The mode of action of Ro 48-8071 was confirmed in vivo as evidenced by the occurrence of MOS in liver, whereas the inverse correlation between the amount of MOS in liver and the decrease in LDL-C supports the view that the cholesterol-lowering effect of Ro 48-8071 is indeed due to inhibition of OSC. Importantly, MOS built up safely in the organ to only less than 1% of hepatic cholesterol or less than 0.002% of liver wet weight. The feed-back regulatory loop preventing overexpression of enzymes of the cholesterol synthesis cascade would reduce the flux of MOS in the pathway, whereas partial inhibition of OSC would not allow the formation of detectable amounts of DOS. Epoxycholesterol could not be found in liver of hamsters treated with Ro 48-8071, possibly because lipoprotein-derived cholesterol is able to lower the overall rate of the synthetic pathway keeping the amount of epoxycholesterol relatively low, and because of inadequate timing for sampling liver combined to rapid elimination of such epoxysterol (71).

The hypothesis that simvastatin stimulates enzymes of the cholesterol synthetic pathway in vivo was confirmed here with a dramatic increase in ex-vivo activity of liver HMG-CoA reductase and squalene synthase, in agreement with previous reports (15–18). Expression of HMG-CoA reductase is regulated at different levels

(59), and blockade of the pathway by a statin would be responsible for *i*) abolition of the feedback suppression of transcription from sterol responsive control elements of the reductase gene (72), *ii*) abolition of suppressive posttranscriptional control by nonsterol isoprenoids and oxysterols (73), and *iii*) abolition of sterol and nonsterol isoprenoid-accelerated degradation of the reductase (74, 75). Up-regulation of squalene synthase by simvastatin is consistent with previous reports (17, 76), whereas the overexpression of OSC induced by simvastatin is reported here for the first time. The latter would suggest that the capacity of the cholesterol synthesis pathway is coordinately regulated at the level of several intermediate enzymes, and not only at the level of HMG-CoA reductase. In contrast, Ro 48-8071 did not increase or decrease the enzymatic capacity of the cholesterol synthesis pathway. As discussed above, partial inhibition of OSC would still permit the cyclization of DOS and further conversion to 24(S),25-epoxycholesterol (20, 68) triggering a synergistic, self-limited regulatory loop preventing overexpression of HMG-CoA reductase (21–24, 27) and of squalene synthase and OSC itself (this paper). Overall, OSC would be partially inhibited in vivo as demonstrated by the occurrence of MOS and the absence of DOS in liver of hamsters treated with Ro 48-8071. Partial OSC inhibition avoids *i*) overproduction of MOS, *ii*) accumulation of DOS, and *iii*) overcapacity of the cholesterol synthesis pathway, with no effects on the synthesis of non-sterol isoprenoids used for protein farnesylation and CoQ production. Also, the reduction of VLDL production by Ro 48-8071 is consistent with the hypothesis that inhibition of OSC would result in a net reduction of cholesterol production, and subsequently of VLDL/LDL production to maintain liver cholesterol homeostasis (27).

Inhibiting OSC is unique as compared to inhibition of other target enzymes of this pathway, which would not allow for the production of oxysterols or trigger a synergistic, self-limited negative regulatory loop, causing an increase of the flux of non-sterol isoprenoids and subsequent accumulation of precursors. Consistently, the squalene synthase inhibitor zaragozic acid prevents the mevalonate-mediated suppression of HMG-CoA reductase (77). Zaragozic acid induces a marked increase in HMG-CoA reductase mRNA and protein synthesis, resulting in an accumulation of farnesol-derived dicarboxylic acid (78, 79); in rats, it exhibits a mechanism-based toxicity where the production of the dicarboxylic acid parallels the increase in HMG-CoA reductase (79).

Taken together, the findings presented in this paper clearly differentiate an OSC inhibitor from an HMG-CoA reductase inhibitor; they support the view that OSC is a distinct, key component in the regulation of the cholesterol synthesis pathway and a very attractive target to design an inhibitor as a cholesterol lowering drug with *i*) no compensatory increase in the activity of HMG-CoA reductase, squalene synthase, and oxidosqualene cyclase, avoiding massive accumulation of potentially harmful intermediates, *ii*) no reduction of CoQ levels in liver and in peripheral tissues, and *iii*) good safety records. The pharmacological activity of the OSC inhibitor was substantiated in two other species, squirrel monkeys and minipigs, where Ro 48-8071 was safe and at least as efficacious as simvastatin in reducing LDL-C. Whether these findings can be extrapolated to humans remains to be determined.

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