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AZALANSTAT (RS-21607), A LANOSTEROL 14α-DEMETHYLASE INHIBITOR WITH CHOLESTEROL-LOWERING ACTIVITY

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Abstract—Agents that inhibit hepatic cholesterol biosynthesis reduce circulating cholesterol levels in experimental animals and humans, and may be of pharmacological importance in the prevention of atherosclerosis. Azalanstat (RS-21607), a synthetic imidazole, has been shown to inhibit cholesterol synthesis in HepG2 cells, human fibroblasts, hamster hepatocytes and hamster liver, by inhibiting the cytochrome P450 enzyme lanosterol 14α-demethylase. When administered orally to hamsters fed regular chow, RS-21607 (50 mg/kg/day) lowered serum cholesterol in a dose-dependent manner (ED_{50} = 62 mg/kg) in a period of 1 week. It preferentially lowered low density lipoprotein (LDL) cholesterol and apo B relative to high density lipoprotein (HDL) cholesterol and apo A-1. It also lowered plasma cholesterol levels in hamsters fed a high saturated fat and cholesterol diet. RS-21607 inhibited hepatic microsomal hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity in hamsters in a dose-dependent manner (ED_{50} = 31 mg/kg), and this was highly correlated with serum cholesterol lowering (r = 0.97). Cholesterol lowering by azalanstat and cholestyramine was additive, and the increase in HMG-CoA reductase brought about by cholestyramine was attenuated significantly by azalanstat. *In vitro* studies with HepG2 cells indicated that this modulation of reductase activity was indirect, occurring at a post-transcriptional step, and it is proposed that a regulatory oxysterol derived from dihydrolanosterol (or lanosterol) may be responsible for this regulation. Azalanstat does not appear to lower circulating cholesterol in the hamster by up-regulation of the hepatic LDL receptor, suggesting that other mechanisms are involved. Orally administered azalanstat (50–75 mg/kg) stimulated hepatic microsomal cholesterol 7α-hydroxylase activity by 50–400% in hamsters, and it is postulated that this may result from modified cholesterol absorption and bile acid synthesis.

Key words: lanosterol 14α-demethylase; HMG-CoA reductase; cholesterol 7α-hydroxylase; LDL receptor; lanosterol; dihydrolanosterol; regulatory oxysterol

Elevated circulating levels of LDL† cholesterol have been correlated with coronary heart disease [1], and clinical trials designed to reduce serum cholesterol levels by diet or pharmacological means have been shown to lead to a reduction in the incidence of this disease [2, 3]. One of the most effective ways of reducing circulating cholesterol levels is to inhibit endogenous cholesterol synthesis in the liver, and it has been demonstrated that inhibitors of HMG-CoA reductase, the enzyme that catalyzes the rate-determining step of cholesterol biosynthesis [4], effectively lower LDL cholesterol levels in humans [5]. It would be advantageous, however, to block cholesterol biosynthesis at a step distal to HMG-CoA reductase, to preserve intermediate products and metabolites such as coenzyme Q, an important component of the respiratory chain in mammalian mitochondria and essential for cardiac function [6]. Coenzyme Q has been shown recently to be reduced substantially in the serum of both normal subjects and patients with cardiomyopathies, following administration of lovastatin [7, 8]. Although a number of late-stage inhibitors of cholesterol biosynthesis have been described [9–12], the only one to be used successfully as a cholesterol-lowering agent in humans is the antimycotic ketoconazole, an LDM inhibitor [13]. Ketoconazole, however, is a non-selective inhibitor of cytochrome P450 isoenzymes, including those involved in steroid synthesis and metabolism, and can lead to side-effects related to hormone imbalance [14]. Therefore, it would be desirable to develop an LDM inhibitor that is selective for LDM but lacks the hormonal side-effects resulting from inhibition of other P450

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† Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; HMG-CoA reductase, hydroxymethylglutaryl-CoA reductase; LDM, lanosterol 14α-demethylase; LPDS, lipoprotein-deficient fetal bovine serum; MEM, modified Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; PMSF, *p*-methylsulfonyl fluoride; BHT, butylated hydroxytoluene; DHL, dihydrolanosterol; LAN, lanosterol; and TC, total cholesterol.

enzymes. We describe here the effect of azalanstat (RS-21607), a substituted aryl alkyl imidazole [(2*S*,4*S*) - *cis* - 2 - [(4 - chlorophenyl)ethyl] - 2 - [(1*H* - imidazol - 1 - yl)methyl] - 4 - [(4 - aminophenylthio) - methyl]-1, 3-dioxolane] [15] and a selective and potent inhibitor of mammalian LDM *in vitro* [16], on cholesterol metabolism *in vitro* in hepatic and extrahepatic cells and *in vivo* in male Syrian hamsters.

MATERIALS AND METHODS

Materials

[2-^{14}C]Acetate (45–60 Ci/mol), DL-[2-^{14}C]-mevalonate (50 Ci/mol), [^{3}H]cholesterol (46–52 Ci/mol) and ^{125}I-protein A (9 μCi/μg) were obtained from DuPont New England Nuclear (Wilmington, DE). [^{14}C]HMG-CoA (55–57 Ci/mol) was obtained from either Dupont New England Nuclear or Amersham Radiochemical Center (Arlington Heights, IL). [^{3}H]7α-Hydroxycholesterol was prepared as described by Swinney *et al.* [16]. [32,32,32-$^{2}H_3$]Dihydrolanosterol was prepared from dihydrolanosterol by reduction with tritium gas in the presence of palladium. Human ^{125}I-LDL (sp. act. = 150 cpm/ng), LPDS and human LDL were obtained from the Organon Teknika Biotechnology Research Institute (Rockville, MD). DMEM, MEM, Williams E medium and FBS were obtained from Gibco (Grand Island, NY). HMG-CoA, DTT, alkaline phosphatase (Type III), glucose-6-phosphate dehydrogenase (Type XXIV), NADP, NADPH, glucose-6-phosphate, HEPES, collagenase, fatty acid-free bovine serum albumin, bovine insulin, dexamethasone, cholestyramine, cholesterol, lanosterol, squalene, 25-hydroxycholesterol, benzamidine, Triton X-100, Nonidet P-40, PMSF and BHT were obtained from the Sigma Chemical Co. (St. Louis, MO). Squalene-oxide was a gift from Dr. E. E. van Tamelen (Stanford University, CA). Silica gel G and H plates were purchased from Analtech Inc. (Newark, DE). Nitrocellulose paper and Nitran filters were purchased from Schleicher & Schuell (Keene, NH). Soybean trypsin inhibitor and protein A were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Acrylamide/BIS-acrylamide solution (37.5:1) was purchased from the Amersco Corp. (Solon, OH). Oligo dT-cellulose was obtained from Pharmacia LKB Biotech (Uppsala, Sweden). All other chemicals were of reagent grade quality.

RS-21607 was prepared as described previously [15] and used as the dihydrochloride salt. Ketoconazole was isolated from an aqueous suspension of Nizoral® tablets (Janssen Pharmaceuticals, Piscataway, NJ) by extraction with dichloromethane and crystallized from ethyl acetate. Lovastatin was isolated from Mevacor® tablets (Merck & Co., West Point, PA) by extraction of an aqueous suspension with dichloromethane, filtered and crystallized from ethyl acetate–hexane. 5β-Cholan-3β-ol was a gift from Dr. Norman Javitt (New York University Medical Center, NY).

Hamster LDL and VLDL

These lipoproteins were isolated from pooled normal hamster serum by density gradient ultracentrifugation. Densities were adjusted with KBr, and solutions were centrifuged at 100,000 *g* for 20 hr in a T45 rotor in a Beckman ultracentrifuge at 4°. VLDL was isolated at a density of $\leq$1.006 g/L and LDL in a range of 1.19 to 1.063 g/L. Lipoproteins were washed with density-adjusted KBr solution, centrifuged at 100,000 *g* and dialyzed exhaustively against 0.15 M NaCl–0.24 mM EDTA (pH 7.4). The dialyzed lipoproteins were concentrated under N_2 using an Amicon YM-10 membrane and stored at 4°. Lipoproteins were characterized by agarose gel electrophoresis at pH 8.6 after staining with Sudan Black and Coomassie Blue dyes and used within 2–3 weeks of preparation. ^{125}I-Labeled LDL and VLDL were prepared by the iodine-monochloride method as described by Bilheimer *et al.* [17]. Specific activities of ^{125}I-LDL and ^{125}I-VLDL were 400–700 and 300–500 cpm/ng, respectively. In each case, 98% of the radioactivity was precipitable with 10% trichloroacetic acid, and less than 3% was extractable with $CHCl_3$–CH_3OH (2:1).

cDNA probes

The human LDL receptor probe (pLDL R-3) and the human HMG-CoA reductase probe (PH RED-102) were obtained from the American Type Culture Collection (ATCC). The human transferrin receptor cDNA probe was obtained from Clontech (Palo Alto, CA).

Animals and diets

Male Golden Syrian hamsters (90–110 g) were obtained from Charles River Laboratories (Wilmington, VA). Purina Rodent Chow 5001 was obtained from Ralston Purina (Richmond, IN) and contained 0.027% cholesterol by weight. The high fat diet containing 0.1% cholesterol, 5.9% butter fat and 6% peanut oil was prepared by ICN Biomedicals Inc. (Costa Mesa, CA).

Cell culture

Human skin fibroblasts were obtained from ATCC and were pooled from 2 male and 2 female donors between 1 week and 1 month of age. Cells were grown to approximately 90% confluence in 60-mm culture dishes in 10% FBS in MEM in a humidified atmosphere containing 5% CO_2 and 95% air. HepG2 cells were obtained from ATCC after 75 passages and grown to approximately 80–90% confluence in 10% FBS–MEM. Hamster hepatocytes were prepared from the livers of male Golden Syrian hamsters (100 g) and harvested after sterile liver perfusion with 0.14 M NaCl, 6.7 mM KCl, 10 mM HEPES, 25 mM EDTA (pH 7.4) at 37°. Connective tissue was degraded by perfusion with 0.05% collagenase in 66.7 mM KCl, 100 mM HEPES, 4.8 μM $CaCl_2$ and 1% fatty acid-free bovine serum albumin (pH 7.6). The livers were rinsed with Williams E medium containing 10^{-5} M hydrocortisone, 100 U of bovine insulin, 23 mM HEPES, 15 mM dexamethasone, 1 U/mL of penicillin, 1 μg/mL of streptomycin and 10 mM non-essential amino acids in 20% FBS. The hepatocytes were teased out into 20 mL of Williams medium, passed through a 40 μm wire mesh filter, and washed twice with medium. Hepatocytes were plated onto 25 cm^2

Falcon Prismasid flasks at a density of 2.5×10^6 per 4 mL of medium and incubated in 10% CO_2/90% air in a humidified atmosphere at 37° for 3 days.

Cholesterol synthesis from [^{14}C]*-acetate* in vitro

Human fibroblasts, HepG2 cells or hamster hepatocytes were washed twice with serum-free medium (MEM) and incubated in 10% LPDS in MEM for 24 hr. Cells were washed twice and placed in MEM containing RS-21607 (0.1 nM to 10 μM) in triplicate for 1 hr at 37°. Cells were then pulsed with 10 μCi [^{14}C]sodium acetate for 2 hr at 37°. The reaction was terminated by placing the cells on ice and washing three times with ice-cold PBS. Cells were harvested with a rubber policeman and suspended in 5 mL PBS containing 250 μg cholesterol and 250 μg lanosterol, as carrier sterols. Approximately 30,000 dpm of [^{3}H]cholesterol was added to each dish as an internal standard, and the lipids were saponified by incubation with 7.5% KOH in 90% ethanol at 80° for 1 hr. After cooling, lipids were extracted four times with 2 vol. of petroleum ether, concentrated under N_2 to 200 μL, and fractionated by TLC on 250 μm silica gel H plates in a solvent containing ethyl acetate:hexane (25:75) with authentic reference samples of cholesterol, lanosterol, squalene-oxide and squalene. Sterols were visualized by exposure to I_2 vapor, scraped from the plate, and counted in 10 mL of Aquasol scintillation liquid. After correction for recovery of the internal standard, cholesterol, methyl-sterols, squalene-oxide and squalene synthesis were expressed as disintegrations per minute of [^{14}C]acetate incorporated per milligram of cell protein per hour.

Measurement of HMG-CoA reductase and LDL receptor mRNA in HepG2 cells

HepG2 cells were grown to 90% confluence in 10% FBS in DMEM and exposed to 10% LPDS in DMEM for 24 hr prior to treatment. The cells were exposed to compound at the concentrations indicated in Results for 18 hr and washed three times with ice-cold PBS. A 1.8 kb *Escherichia coli*/Xho fragment of the human LDL receptor gene (pLDLR3) and a 2.5 kb Bgl II fragment of the human HMG-CoA reductase gene (PHRED-102) were labeled with ^{32}P by the random prime method [18]. Total HepG2 cell RNA isolation and northern blots were performed as described by Heller *et al.* [19]. Blots were stripped by emersion in boiling water and re-probed with a human transferrin receptor ^{32}P-labeled cDNA probe. Signals were quantitated by densitometric scanning of the autoradiographs after correction for the loading control.

Cholesterol synthesis from [2-^{14}C]*mevalonate* in vivo

Hamsters (8 per group) were administered RS-21607 (50 mg/kg/day) orally by gavage in 0.5 mL propylene glycol or 0.5 mL vehicle once daily for 15 days. Animals were fasted overnight on day 14 and received the last dose of RS-21607 at 9:00 a.m. on day 15. After 1 hr, each animal was administered 10 μCi [^{14}C]mevalonate intraperitoneally in saline. After 1.5 hr, animals were bled by cardiac puncture under nembutal anesthesia, and livers, spleen, kidneys, adrenals, testes, gall bladder and ileum were excised, rinsed in ice-cold saline, blotted and weighed. Adrenals, spleen, gall bladders and testes from treatment and control groups were pooled from two groups of four animals. Livers, kidneys and small intestines were processed separately. Tissues were chopped with scissors and homogenized in 7.5% KOH in 90% ethanol in an ice-bath using a Polytron tissue homogenizer. [^{3}H]Cholesterol (30,000 dpm) was added to each homogenate as an internal standard, and tissues were saponified at 80° for 2 hr. Non-saponifiable sterols were extracted with 3 vol. of petroleum ether and concentrated to 0.5 mL by rotary evaporation and under N_2. The extracts were fractionated by thin-layer chromatography on 500 μm preadsorbant silica gel G plates in a solvent containing ethyl ether–heptane (55:45). Isotopically labeled sterols were identified by autoradiography and compared with authentic standards of cholesterol, lanosterol, squalene-oxide and squalene, after visualization with I_2 vapor. The sterols were then extracted with 40 vol. of anhydrous ethyl ether, concentrated to 1 mL under N_2, and stored in 10% methanol–90% ethyl ether, containing 4.5 mg BHT in the dark at −20°. Aliquots were counted in 10 mL Aquasol, and sterol synthesis was expressed as disintegrations per minute [^{14}C]-mevalonate incorporated per gram of tissue, after correction for recovery of internal standard.

HMG-CoA reductase activity in HepG2 cells

HMG-CoA reductase (EC 1.1.1.34) activity was measured by a modification of the method described by Shapiro *et al.* [20]. Cells (80–90% confluent) were preincubated with 10% FBS in DMEM for 24 hr prior to assay, washed three times with DMEM, and incubated with DMEM containing 10% heat-inactivated lipoprotein-deficient serum (HILPDS) in 60-mm culture dishes, in triplicate with or without test compound, for 8–24 hr. Cells were washed twice with ice-cold PBS, once with ice-cold buffer containing 100 mM NaCl–100 mM K_2HPO_4/KH_2PO_4 (pH 7.4) and scraped from the plate into 100 mM NaCl–100 mM K_2HPO_4/KH_2PO_4–10 mM EDTA (pH 7.4) and frozen in liquid N_2 in capped polypropylene tubes. Cell suspensions were thawed at room temperature and sonicated on ice for 10 sec. Fifty microliters of cell sonicate (50–150 μg protein) was preincubated for 25 min at 37°, and the reaction was started by the addition of 90 μL of assay mixture containing: 55 mM glucose-6-phosphate, 5.5 mM NADP, 55 mM NaCl, 55 mM potassium phosphate buffer (pH 7.4), 11 mM EDTA, 5.5 mM DTT and 0.7 unit of glucose-6-phosphate dehydrogenase. Ten microliters of 5 mM DL-[^{14}C]HMG-CoA (5000 dpm/pmol) was then added, and the reaction mixture was incubated at 37° for 30 min. The reaction was terminated by the addition of 25 μL of 10 N HCl, and 20 μL of mevalonolactone (2 mg) and 10 μL of [^{3}H]mevalonolactone (30,000 dpm) were added. The reaction product, [^{14}C]mevalonate, was converted to the lactone by incubation at 37° for 1 hr. Suspensions were then centrifuged at 10,000 *g* in an Eppendorf microfuge for 15 min at 4° to precipitate protein. Aliquots (100 μL) of the clear supernate were then fractionated by TLC on silica gel G plates

in acetone–toluene (1:1). After exposure to I_2 vapor, the region of the chromatogram corresponding to mevalonolactone ($R_f = 0.6$) was removed and counted in Aquasol liquid scintillation fluid using a dual-channel [^{3}H]/[^{14}C] program. HMG-CoA reductase activity was expressed as picomoles of [^{14}C]mevalonate formed per minute per milligram of microsomal protein. Recovery of the internal standard ranged from 50 to 90%, and the reaction was linear using 10–180 μg of protein for up to 40 min at 37°.

HMG-CoA reductase activity in hepatic microsomes

Hamsters (6–8 per group) were administered azalanstat (RS-21607), lovastatin or ketoconazole by oral gavage in 0.5 mL of propylene glycol once daily for 7 days. Cholestyramine (500 mg/kg) was administered in 0.5 mL of distilled water twice daily for 7 days. Animals were fasted overnight, then were bled by cardiac puncture, and serum was collected. The livers were excised and rinsed in ice-cold saline; then microsomes were prepared at 5° as follows: 1-g aliquots of each liver were chopped with scissors, placed in a 5:1 (w/v) medium containing 50 mM potassium phosphate buffer (pH 7.4) and homogenized with 4 strokes of the pestle in a Potter–Elvehjem glass–teflon homogenizer driven by a Wheaton Instruments motor set at 50% maximum speed. Each homogenate was then centrifuged at 11,000 *g* for 15 min. Supernates were decanted and centrifuged at 100,000 *g* for 80 min. Microsomal pellets were washed with fresh medium, recentrifuged, then dispersed in 2 mL of medium by passing twice through a 22-gauge needle, and frozen at −80°. Microsomal HMG-CoA reductase activity was measured by a modification of the method of Kempen *et al.* [21]. Microsomal protein (50–200 μg) was preincubated with 10 units of *E. coli* inorganic pyrophosphatase in order to hydrolyze inactive phosphorylated enzyme, in 90 μL of 20 mM imidazole-hydrochloride buffer (pH 7.4) for 1 hr at 37°. One hundred microliters of a solution containing 200 mM potassium phosphate buffer (pH 7.4), 40 mM glucose-6-phosphate, 5.4 mM NADP, 0.7 unit of glucose-6-phosphate dehydrogenase, 20 mM EDTA and 10 mM DTT was then added, and the mixture was incubated at room temperature for 10 min. The reaction was initiated by the addition of 10 μL of DL-[3-^{14}C]HMG-CoA (50 μM) and incubated for 1 hr at 37°; the reaction was terminated by the addition of 25 μL of 5 N HCl. Mevalonolactone (1.5 mg) and [^{3}H]mevalonolactone (20,000 dpm) were then added, and the solutions were incubated for 1 hr at 37° in order to convert [^{14}C]mevalonate to the lactone. Mevalonolactone was isolated by extraction with anhydrous ethyl ether and TLC on 250 μm silica gel G plates in toluene–acetone (1:1). The reaction product was visualized by exposure to I_2 vapor, removed from the plate, and counted in Aquasol scintillation fluid. After correction for recovery of internal standard, HMG-CoA reductase activity was expressed as nanomoles of [^{14}C]-mevalonate synthesized per milligram of microsomal protein per hour. Recoveries of internal standard ranged from 50 to 80%, and the reaction was linear using 25–150 μg of protein for up to 80 min at 37°. Protein concentrations were determined by the method of Bradford [22].

Cholesterol 7α-hydroxylase activity in hepatic microsomes

Incubations contained hepatic microsomes (2 mg protein), [^{3}H]cholesterol (diluted with 0.14 μmol endogenous substrate), NADPH (1 μmol), $MgCl_2$ (3 μmol), EDTA (0.1 μmol), cysteamine hydrochloride (20 μmol), and potassium phosphate buffer, pH 7.4 (100 μmol), in a total volume of 1 mL. After a 5-min preincubation period, the reactions were initiated by NADPH and agitated for 10 min at 37°. The reactions were terminated by the addition of 7.5 mL of acetone (containing 0.02% BHT). Following precipitation of protein and evaporation, the residues were dissolved in 200 μL isopropanol containing 0.02% BHT, sonicated for 5 min, filtered through 4.5 μm filters (Gelman Acrodisc 3CR) and analyzed by HPLC (20 μL injection volume). Separation of products was achieved with two consecutive 5-μm, 25-cm Beckman Ultrasphere Sil columns preceded by a Newguard silica guard column. The columns were eluted with hexane/isopropanol (1 mL/min) under the following conditions: 11.5 min isocratic at 97/3, 15 min with a linear gradient to 77/23, and 2 min isocratic at 77/23. Retention times for cholesterol and 7α-hydroxycholesterol were 9 and 30 min, respectively.

Measurement of LDL uptake by HepG2 cells

This was carried out by a modification of the method of Kempen *et al.* [21]. HepG2 cells were grown to 90% confluence in 16-mm diameter multiwell culture dishes in 10% heat-inactivated LPDS. Cells were washed with MEM and then incubated for 24 hr in MEM containing 20 mM HEPES (pH 7.4) in 9% LPDS plus 1% heat-inactivated LPDS plus 0.1 to 10 μM RS-21607 in ethanol (which was also added to control cultures). Cells were washed three times with ice-cold PBS, and LDL uptake studies were carried out in 0.5 mL MEM–20 mM HEPES (pH 7.4) containing 10% heat-inactivated LPDS. Human ^{125}I-LDL was added so that final concentrations of 5, 10, 20 and 40 μg/mL of isotopically labeled LDL were incubated with the cells for 3 hr at 37° in the presence and absence of a 50-fold excess of unlabeled human LDL. Then cells were washed five times with 1 mL of 50 mM Tris–150 mM NaCl (pH 7.4) containing BSA (2 mg/mL) and once with 50 mM Tris–150 mM NaCl (pH 7.4) and incubated with 1 mL of 0.3 M NaOH at 4° overnight. A solubilized cell suspension of 750 μL was removed for counting, and 50 μL was taken for protein determination by the method of Lowry *et al.* [23]. Specific cell uptake of ^{125}I-LDL was calculated for each concentration of ^{125}I-LDL, by subtracting nonspecific uptake in the presence of an excess of unlabeled LDL from total uptake in the absence of unlabeled LDL and expressed as nanograms of ^{125}I-LDL associated per milligram of cell protein in 3 hr.

Measurement of LDL binding by hamster hepatic membranes

This was based on the procedure described by Kovanen *et al.* [24]. Hepatic membranes were

isolated from the livers of male hamsters treated orally with azalanstat (RS-21607) (50 mg/kg/day in propylene glycol) for 7 days or lovastatin (0.1% of diet, equivalent to approximately 100 mg/kg) for 10 days. Animals were bled by cardiac puncture on days 7 or 10, and the livers were excised and placed in ice-cold saline containing 1 mM PMSF. One gram of each liver was homogenized with 20 strokes of a glass–teflon homogenizer in 10 mL of Buffer A [150 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris–HCl, 1 mM PMSF (pH 7.5)] on ice. The homogenate was centrifuged at 500 *g* for 5 min, and the supernate was centrifuged at 8000 *g* for 15 min at 5°. The 8000 *g* supernate was then centrifuged at 100,000 *g* for 1 hr at 5°, resuspended in 6 mL Buffer A by passing through a 22-gauge needle, and recentrifuged. The microsomal pellets were either assayed immediately or stored at −80°. Immediately prior to assay, the pellets were suspended in Buffer B [50 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris–HCl (pH 7.5) containing 1 mM PMSF] using a 22-gauge needle and then sonicated for 20 sec on ice. LDL binding was carried out in 80 μL of Buffer C [50 mM Tris–Cl, 80 mM NaCl, 1 mM $CaCl_2$, 20 mg/mL of BSA (pH 7.5)] containing 150–200 μg of membrane protein, hamster ^{125}I-LDL (2–160 μg/mL) or ^{125}I-VLDL (2–90 μg/mL) for 90 min at 0° with or without a 40- or 120-fold excess of unlabeled hamster LDL or VLDL, respectively. A 50-μL aliquot of the reaction mixture was then layered onto 150 μL of 10% FBS in a cellulose nitrate tube and centrifuged at 100,000 *g* for 30 min at 4°. The supernate was removed by aspiration, and the pellet was washed with 200 μL of 10% FBS. The pellet was then recentrifuged for 5 min at 100,000 *g* at 5°, the wash was removed, and the portion of the tube containing the pellet was sliced with a blade and counted in a Beckman gamma counter. The protein content of the membranes was determined by the method of Lowry *et al.* [23], and ^{125}I-LDL and ^{125}I-VLDL binding was expressed as nanograms of lipoprotein bound per milligram of cell protein.

Serum lipoprotein analysis

Total serum and plasma cholesterol levels were determined enzymatically using kits obtained from Sigma or Schiapparelli Biosystems Inc., Fairfield, NJ. HDL cholesterol was determined after precipitation of LDL and VLDL with sodium phosphotungstate and magnesium chloride. LDL cholesterol was determined by calculation as described previously [25], and total serum triglycerides were determined colorimetrically using a Sigma kit. Hamster apolipoprotein A-1 and B were determined by electro-immune assay using monospecific antibodies to hamster apo A-1 and B, respectively, as described before [25].

Methyl-sterol analysis by GC/MS

Two different methods have been used for the quantitation of methyl-sterols. During the exploratory phase of this program, gas chromatography with flame ionization detection (GC-FID) was used to estimate the quantities of the various methyl-sterols since this method is expected to give rise to less variations in the response factors of various sterols (Method A). The sterol fractions were then characterized by GC–MS using electron ionization (EI) detection and full scan mass spectral analysis of each GC peak. When it was established that the methyl-sterol fraction consisted primarily of DHL and LAN, quantitation of these two sterols was carried out by GC–MS using selected ion monitoring of the molecular ions of DHL, LAN and of an internal standard, [32,32,32-$^2H_3$]DHL (Method B).

Method A. Methyl-sterols were isolated from hamster tissues as described in Materials and Methods, under "Cholesterol synthesis from [2-^{14}C]-mevalonate *in vivo*." An antioxidant (4.5 mg of BHT) and 4.5 μg of 5β-cholan-3α-ol internal standard were added to each sample. The sterols were converted into trimethylsilyl derivatives by heating in a dry pyridine solution with excess *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) at 70° [26]. GC analyses were carried out on an HP-5890A gas chromatograph, equipped with an HP-7673 autosampler, using a 30 m × 0.25 mm DB-17 column (0.5 μm film thickness). The oven temperature was increased from 90° to 290° at a rate of 25°/min, with the injector and detector temperatures maintained at 290° and 320°, respectively. Each peak in the GC trace was characterized by full scan electron ionization (70 eV) mass spectrometric analysis, using an HP-5890A gas chromatograph, equipped with an HP-7673 autosampler, and interfaced with an HP-5970 mass selective detector. The GC conditions were the same as above. Identification of the methyl-sterols was based on comparison of their relative retention time and mass spectral characteristics with those of authentic reference samples. Quantitative estimation of the methyl-sterol contents was carried out by GC-FID peak area measurements. Response factors for the identified components were established from authentic reference samples. With unidentified steroidal components, the response factor of the nearest eluting reference standard was used.

Method B. The methyl-sterols were isolated from the tissues as described under Materials and Methods. Internal standard (1 μg of [$^2H_3$]DHL) was added to each sample, and the solvents were evaporated. The samples were reconstituted with 100 μL chloroform, diluted with 6 mL hexane and purified by adsorption onto 500 mg silica gel mini-cartridges. The analytes were eluted with ether/hexane and after evaporation of the solvents they were silylated in toluene solution as described in Method A. The resulting samples were analyzed using the GC–MS instrumentation described in Method A, using an HP-1 12 m × 0.2 mm GC column. The oven temperature was held at 180° for 1 min, heated to 250° at a rate of 30°/min, and then to 275° at 10°/min. The ions monitored corresponded to the molecular ions of the trimethylsilyl derivatives of LAN (*m/z* 498), DHL (*m/z* 500) and [$^2H_3$]DHL (*m/z* 503). Linear calibration curves were obtained in the range of 0.05 to 2.0 μg sterol/g tissue or μg sterol/mL serum for both DHL and LAN.

Measurement of hamster liver LDL receptor

This was carried out using western blots of liver

membranes with a polyclonal antibody to the conserved 15 residue carboxy-terminal peptide of the LDL receptor: Cys-Tyr-Pro-Ser-Arg-Gln-Met-Val-Ser-Leu-Gln-Asp-Val-Ala [27]. The peptide was conjugated to keyhole limpet hemocyanin, and antiserum was raised in New Zealand white rabbits by Multiple Peptide Systems (San Diego, CA). Hamster liver membranes equivalent to 1 g of liver were dispersed in 0.6 mL of 50 mM Tris-malate buffer (pH 6.0) containing 2 mM $CaCl_2$, 1% (w/v) Triton X-100 and a protease inhibitor mixture containing: soybean trypsin inhibitor (5 μg/mL), leupeptin (5 μg/mL), benzamidine (17.5 μg/mL) and EDTA (5 μM), by repeated passage through a 25-gauge needle, and the mixture was centrifuged at 100,000 *g* in a Beckman table top ultracentrifuge for 30 min at 4°. Aliquots of the membranes were then adjusted to a final concentration of 6.25 mM Tris-malate (pH 6.8), 2% SDS, 20% glycerol and 10% mercaptoethanol and boiled for 3 min. Twenty microliters of solubilized liver membrane (200 μg) was applied to 60-mm wells of a 1.5-mm thick 8% polyacrylamide gel slab containing 8% SDS, and electrophoresis was conducted for 18 hr at room temperature as described by Laemmli [28]. Pre-stained molecular weight standards were run in separate lanes to monitor separation. After electrophoresis, proteins migrating in the gel were electrophoretically transferred to Schleicher & Schuell nitrocellulose paper with a constant current of 0.1 amp for 18 hr, in 25 mM Tris–192 mM glycine buffer (pH 8.3) containing 20% methanol at 20°. After transfer, the nitrocellulose paper was incubated in 100 mL of 5% Carnation dried milk in PBS for 1 hr at room temperature with shaking. After incubation, the nitrocellulose sheet was removed and placed in 50 mL of PBS containing 10% FBS with 500 μL of LDL receptor peptide anti-serum (final dilution 1:100) and incubated for 3 hr at room temperature. The membrane was then washed for 10 min with PBS, for 10 min with PBS containing 0.05% Nonidet P-40, and finally for 10 min in PBS. The washed blot was preincubated with 50 mL of PBS containing 10% FBS and 50 μL of ^{125}I-labeled protein A (1:1000 dilution) for 1 hr at room temperature with shaking. The paper was then washed three times with PBS and PBS–Nonidet P-40 as described above and exposed to X-ray film for 6–24 hr at −80°. Exposed areas of the film were measured densitometrically using a Molecular Dynamics Personal densitometer, and data were expressed in densitometric units.

RESULTS

Inhibition of cholesterol biosynthesis from [^{14}C]-acetate in cultured cells by azalanstat

Azalanstat (RS-21607) inhibited the incorporation of [^{14}C]acetate into the chromatographically isolated demethyl-sterol cholesterol-containing fraction equally effectively in hamster hepatocytes and HepG2 cells with IC_{50} values of 10 and 6.5 nM, respectively. It was, however, more effective in inhibiting cholesterol synthesis in human fibroblasts, in which it possessed an IC_{50} of 0.2 nM. In each

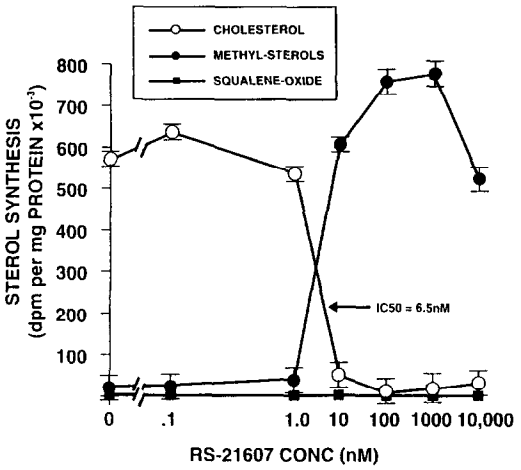

Fig. 1. Inhibition of cholesterol synthesis in HepG2 cells by azalanstat (RS-21607). HepG2 cells were preincubated in 10% LPDS in MEM for 24 hr in 60-mm culture dishes. They were incubated with RS-21607 at the final concentrations indicated in 95% air–5% CO_2 at 37° for 1 hr and then pulsed with 10 μCi [^{14}C]acetate per dish for 2 hr. After washing, carrier cholesterol (250 μg), lanosterol (250 μg) and [^{3}H]cholesterol (30,000 dpm) were added, and non-saponifiable, isotopically labeled sterols were isolated and counted as described in Materials and Methods. Each value represents the mean sterol synthesis ± SD for triplicate determinations after correction for recovery of internal standard.

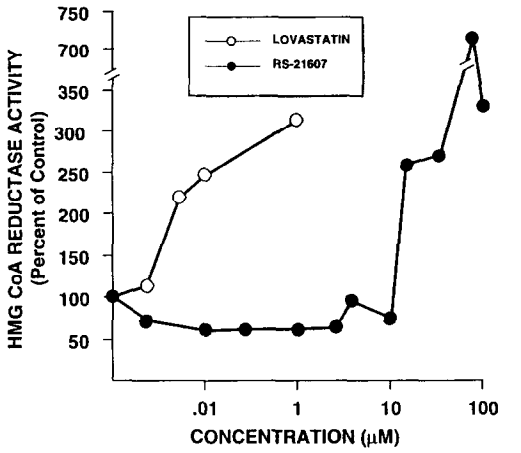

Fig. 2. Modulation of HMG-CoA reductase activity in HepG2 cells by RS-21607 and lovastatin. HepG2 cells were incubated in 10% heat-inactivated LPDS in DMEM containing azalanstat or lovastatin in 95% air–5% CO_2 at 37° for 20 hr. Cells were washed twice with ice-cold PBS and once with 100 mM potassium phosphate, 100 mM NaCl buffer (pH 7.4), harvested in the same buffer containing 10 mM EDTA, and frozen in liquid N_2. HMG-CoA reductase activity was determined on 50 μL of cell sonicates (50–100 μg protein), as described in Materials and Methods. Each value represents the mean of triplicate determinations for activity at each concentration of drug and is expressed as percent of vehicle control. Control HMG-CoA reductase activity (± SD) = 303 ± 20 pmol [^{14}C]mevalonate synthesized/mg protein/min.

cell type, inhibition of cholesterol synthesis was accompanied by the generation of isotopically labeled methyl-sterols, but there was no increase in isotopically labeled squalene-oxide in any of the cells treated with RS-21607 (Fig. 1).

Modulation of HMG-CoA reductase activity in HepG2 cells by azalanstat and lovastatin

Azalanstat (RS-21607) depressed HMG-CoA reductase activity in HepG2 cells in a biphasic concentration-dependent manner (Fig. 2). Low concentrations (0.01 to 1 μM) depressed reductase activity by approximately 40%, while higher concentrations (30–100 μM) stimulated activity by as much as 600%. Under the same conditions, the HMG-CoA reductase inhibitor lovastatin stimulated activity at all concentrations tested and led to a 200% increase in activity at a concentration of 1 μM. When HepG2 cells were exposed to heat-inactivated LPDS for 20 hr, washed with DMEM and then exposed to fresh LPDS, HMG-CoA reductase activity of the cells increased up to 8-fold over an 8-hr period, presumably due to the compensatory increase in synthesis of reductase protein resulting from the diminished intracellular levels of a regulatory sterol [29]. When the cells were incubated in the presence of 1 μM azalanstat under the same conditions, the increase in HMG-CoA reductase activity was suppressed by approximately 50% at all time points between 2 and 6 hr relative to control cells (data not shown).

Northern blots of mRNA isolated from HepG2 cells that had been incubated with azalanstat, lovastatin or 25-hydroxycholesterol, using a cDNA probe to the human HMG-CoA reductase mRNA (Fig. 3A), showed that incubation of cells in heat-

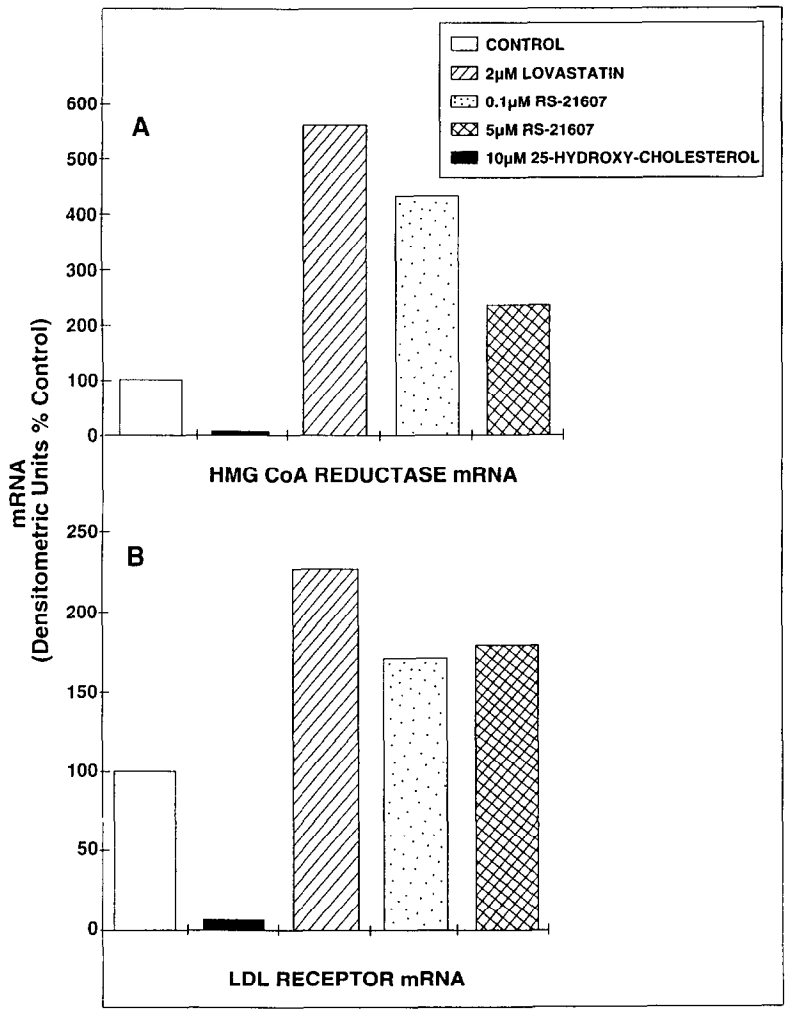

Fig. 3. Modulation of mRNA for HMG-CoA reductase and LDL receptor. HMG-CoA reductase (A) or LDL receptor (B) mRNA was isolated from HepG2 cells that had been incubated with lovastatin (2 μM) azalanstat (0.1 and 5 μM), or 25-hydroxycholesterol (10 μM) for 18 hr following exposure to LPDS for 24 hr as described in Materials and Methods. Autoradiographs of the northern blots were scanned densitometrically. Yields of mRNA were corrected for recovery of the loading control and expressed as percent of the signal given by untreated cells.

Table 1. Specific uptake of human ^{125}I-LDL by HepG2 cells

Additions	Uptake ^{125}I-LDL vs controls*
10 μM 25-Hydroxycholesterol	0.19 ± 0.05
2 μM Lovastatin	1.00 ± 0.32
0.1 μM RS-21607	1.13 ± 0.11
1 μM RS-21607	1.26 ± 0.10
10 μM RS-21607	1.24 ± 0.16

Test compounds were incubated in triplicate with HepG2 cells in 15-mm multi-well plates in the presence of 5, 10, 20 and 40 μg/mL of human ^{125}I-LDL. Specific cell uptake was determined by subtracting non-specific uptake in the presence of a 50-fold excess of unlabeled LDL from total uptake in the absence of LDL. Uptake is defined as ng^{125}I-LDL bound and internalized per mg cell protein per 3 hr at 37°. Values are means ± SD.

* Control = 1.0.

inactivated LPDS containing 0.1 or 5 μM azalanstat for 24 hr led to a 300 or 100% increase in HMG-CoA reductase mRNA, respectively, whereas incubation of cells with 2 μM lovastatin led to a 400% increase in mRNA. Incubation with 10 μM 25-hydroxycholesterol led to a 95% decrease in HMG-CoA reductase mRNA levels. This suggests that the down-regulation of HMG-CoA reductase activity brought about by azalanstat (Fig. 2) was occurring at a post-transcriptional step because mRNA levels for this enzyme were reduced in this concentration range of 0.1 to 5 μM.

Effect of azalanstat on modulation of ^{125}I-LDL uptake by HepG2 cells

Azalanstat (RS-21607) modestly enhanced specific uptake of ^{125}I-LDL by HepG2 cells by approximately 25% relative to lovastatin (Table 1). Under identical conditions, the HMG-CoA reductase inhibitor lovastatin did not increase LDL uptake by these

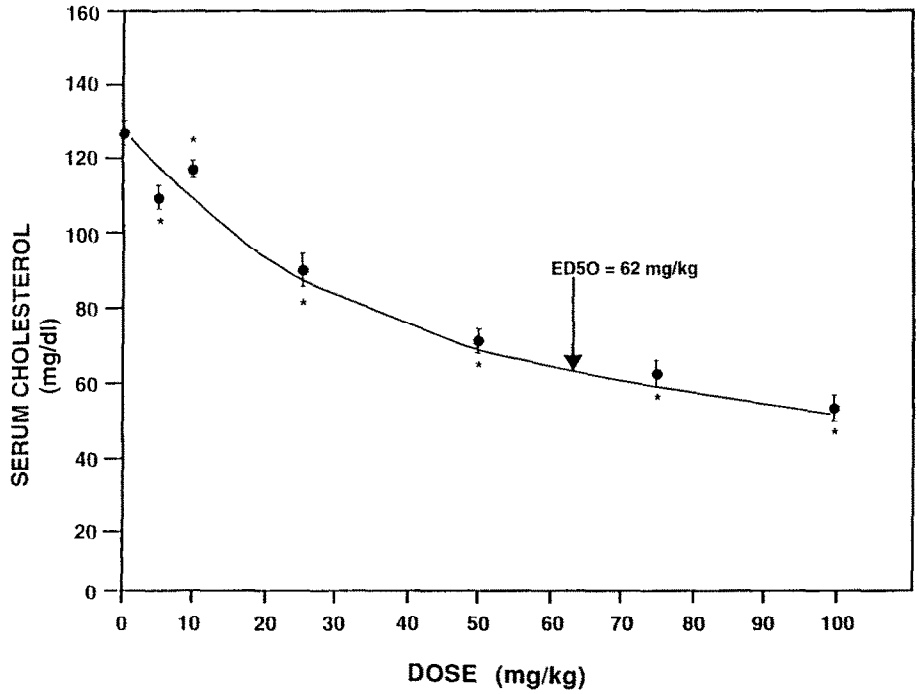

Fig. 4. Dose-dependent reduction of serum cholesterol levels in chow-fed hamsters by azalanstat. Male Golden Syrian hamsters (8 per group) in each treatment group were administered azalanstat orally by gavage in 0.5 mL propylene glycol, 9 hr into the light cycle, once daily for 14 days. Animals were bled by cardiac puncture under Nembutal anesthesia on day 15, 3 hr into the light cycle. Serum was isolated and analyzed for total cholesterol as described in Materials and Methods and compared with crupted under certain pharmacoloontrols, using Dunnett's test. Key: (*) $P < 0.05$ vs vehicle-treated controls. Values are means ± SEM.

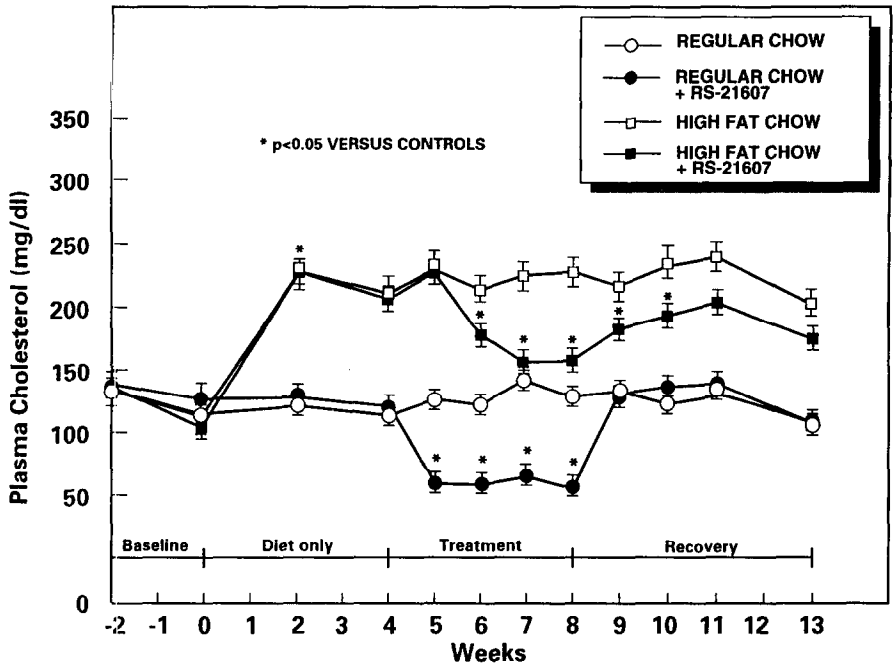

Fig. 5. Cholesterol lowering by azalanstat in hamsters fed low and high fat diets. Male hamsters (40 per group) were fed regular chow containing 0.027% cholesterol or a high fat diet containing 0.1% cholesterol, 5 9% butter fat and 6% peanut oil for 4 weeks. Half the animals in each diet group were then administered azalanstat (50 mg/kg/day) by oral gavage for 4 weeks, and the other half were administered vehicle (propylene glycol) only for 4 weeks. Animals were bled from the retro-orbital sinus, under light ether anesthesia on the weeks indicated, and plasma cholesterol was compared to pre-dose levels using a parametric two-way repeated measures analysis of variance test. Values are means ± SEM.

cells, in contrast to published reports for the HMG-CoA reductase inhibitor compactin [30], and 25-hydroxycholesterol decreased uptake of ^{125}I-LDL by HepG2 cells by 81%. Northern blots of mRNA isolated from HepG2 cells that had been incubated with azalanstat, lovastatin or 25-hydroxycholesterol, using a cDNA probe to the human LDL receptor (Fig. 3B), showed that both 0.1 and 5 μM azalanstat led to a 70% increase in mRNA for the LDL receptor. Under the same conditions, 2 μM lovastatin led to a 120% increase in mRNA levels, whereas 10 μM 25-hydroxycholesterol resulted in a 95% decrease in mRNA levels for the LDL receptor.

Effect of azalanstat on circulating cholesterol levels in chow-fed and cholesterol-fed hamsters

Oral administration of azalanstat (RS-21607) to chow-fed hamsters for 14 days resulted in a dose-dependent lowering of serum cholesterol (ED_{50} = 62 mg/kg, Fig. 4). When RS-21607 was administered orally to chow-fed hamsters at a dose of 50 mg/kg for 4 weeks, plasma cholesterol was reduced by 50% after 1 week and remained at this level for the rest of the dosing period, returning to control levels 1 week after dosing stopped (Fig. 5). This indicates that the cholesterol lowering brought about by azalanstat was not only rapid and sustained but reversible. When these animals were given a diet comparable to a typical Western diet, containing 0.1% cholesterol and 11.9% saturated fat, cholesterol levels were increased 2-fold (Fig. 5). Azalanstat reduced plasma cholesterol in these animals by 37% ($P < 0.05$) in a period of 2 weeks to levels that were still significantly lower than those of control animals 2 weeks after treatment had terminated, indicating that it lowered plasma cholesterol somewhat more slowly in cholesterol and saturated fat-fed animals and that these levels returned to baseline more slowly when dosing stopped.

Effect of azalanstat on HDL and Apo B containing lipoproteins

Cholesterol reduction in hamsters treated with azalanstat resulted principally in a reduction of the LDL fraction of serum (Table 2). HDL was also reduced but to a lesser extent, and the HDL/TC ratio was elevated significantly at all doses. Serum Apo B levels were depressed to a greater extent than Apo A-1 levels in azalanstat-treated hamsters leading to significant increases in the Apo A-1/Apo B ratio at all doses. Serum triglyceride levels were reduced inconsistently, and this only reached statistical significance at the 50 and 100 mg/kg doses.

Inhibition of 14α-Demethylation of dihydrolanosterol and lanosterol by azalanstat in vivo

When orally administered to hamsters for 2 weeks, azalanstat (50 mg/kg) inhibited the incorporation of [^{14}C]mevalonate into [^{14}C]cholesterol-containing demethyl-sterol fraction in all hamster tissues tested

Table 2. Effect of RS-21607 on hamster serum lipoproteins and apolipoproteins A-1 and B

Dose (mg/kg, p.o.)	N	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	HDL/TC (%)	Triglyceride (mg/dL)	Apo A-1 (mg/dL)	Apo B (mg/dL)	Apo A-1/Apo B (%)
Vehicle	8	130.5 ± 5.0	67.0 ± 1.1	42.2 ± 5.8	51.9 ± 2.2	272.0 ± 17.0	116.1 ± 5.2	30.4 ± 2.9	3.9 ± 0.3
25	8	91.2 ± 5.2*	57.8 ± 3.3*	13.2 ± 1.2*	63.4 ± 0.9*	257.6 ± 28.0	112.2 ± 7.8	17.3 ± 0.5*	6.5 ± 0.4*
50	8	71.8 ± 3.3*	47.0 ± 1.5*	9.9 ± 1.8*	66.3 ± 1.7*	185.8 ± 14.9*	102.8 ± 5.0	15.4 ± 1.3*	6.8 ± 0.3*
75	7	66.5 ± 4.2*	47.3 ± 2.3*	3.5 ± 1.1*	71.4 ± 1.3*	201.7 ± 19.2	89.5 ± 4.8*	9.1 ± 0.7*	10.0 ± 0.6*
100	7	60.7 ± 4.2*	40.8 ± 1.6*	6.1 ± 2.0*	68.2 ± 3.0*	177.4 ± 18.0*	92.6 ± 2.6*	11.7 ± 0.9*	8.2 ± 0.8*

Male hamsters (90–110 g) were administered azalanstat (RS-21607) by oral gavage in 0.5 mL propylene glycol once daily (9 hr into the light cycle) for 14 days. They were bled by cardiac puncture (3 hr into the light cycle) on day 15, and lipoproteins were analyzed as described in Materials and Methods. Data represent the mean values of 7–8 animals per group ± SEM. Doses were based on the mean weights of animals in each group and were adjusted for weight gain during the experiment.

* $P < 0.05$ (Dunnett's test).

and led to an enhanced generation of isotopically labeled methyl-sterols (Table 3). Cholesterol (demethyl-sterol) synthesis was inhibited by 98% in the liver, 95% in the kidney, 87% in the spleen, 38% in the adrenals, 93% in the testes and 95% in the small intestine, indicating that the inhibition was not tissue-selective. When the methyl-sterol fraction was isolated from the non-saponifiable lipid fraction of the tissues and analyzed by GC/MS, DHL was the principal methyl-sterol detected in the liver, gall bladder, adrenals, spleen, kidney and serum with smaller amounts of LAN and some unidentified methyl-sterols, whereas LAN was the most abundant methyl-sterol detected in the testes and small intestine (Table 4). DHL and LAN levels were elevated to a greater extent in the liver (approximately 100-fold) than in other tissues (2 to 38-fold), whereas the concentration of DHL in the serum of treated animals was 0.78 mg/dL (mean of two experiments, Table 4). It is noteworthy that although the tissue levels of DHL were elevated markedly in treated hamsters, the serum levels were elevated minimally, representing only approximately 1% of the total serum cholesterol levels. This indicates that DHL does not replace cholesterol in the circulation as was reported for the late-stage inhibitor triparanol [9].

Modulation of hepatic microsomal HMG-CoA reductase activity in hamsters by azalanstat

When azalanstat (RS-21607) was administered by oral gavage to hamsters maintained on a regular light–dark cycle at doses of 25–100 mg/kg for 2 weeks, it suppressed total hepatic microsomal HMG-CoA reductase activity in a dose-related manner with an ED_{50} of 31 mg/kg (Fig. 6). Serum cholesterol levels in these animals were highly correlated with hepatic HMG-CoA reductase activity ($r = 0.97$, $P < 0.01$), suggesting that cholesterol lowering may be related to suppression of HMG-CoA reductase activity in the liver.

Effect of azalanstat upon cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase (EC 1.14.13.17) is thought to be the rate-limiting step in the degradation of cholesterol to bile acids [31], and compounds that induce this activity, such as the bile sequestrant cholestyramine, are effective cholesterol-lowering agents. Azalanstat induced hepatic cholesterol 7α-hydroxylase activity 1.5- to 5-fold in hamsters following oral administration of 50 and 75 mg/kg, for 1–2 weeks (Table 5). In contrast, lovastatin decreased cholesterol 7α-hydroxylase activity by 50%. Oral administration of 50 mg/kg of azalanstat in combination with a chow diet containing 4% cholestyramine for 1 week was more effective in inducing cholesterol 7α-hydroxylase activity and lowering serum cholesterol than either agent administered alone (Table 5).

Treatment of hamsters with cholestyramine plus azalanstat

When azalanstat (30 mg/kg) was administered orally once daily to hamsters for 7 days (Table 5), serum cholesterol was reduced by 44% ($P < 0.05$).

Table 3. Incorporation of [^{14}C]mevalonate into hamster tissue lipids

Tissue	Demethyl-sterols (dpm/g × 10^{-3})	Methyl-sterols (dpm/g × 10^{-3})	Squalene-oxide (dpm/g × 10^{-3})	Squalene (dpm/g × 10^{-3})
Liver	0.83 ± 1*	67.6 ± 9.7*	0.31 ± 0.05*	0.74 ± 0.1*
Control	53.8 ± 18	13.3 ± 4.9	0.71 ± 0.11	5.75 ± 1.0
Kidney	17.2 ± 3.4*	1198 ± 308*	11.6 ± 1.2	4.4 ± 0.8
Control	332.2 ± 128.5	190 ± 60.6	12.6 ± 3.9	4.5 ± 1.4
Spleen	1.36	11.6	0.26	0.33
Control	10.6	2.44	0.24	0.39
Adrenals	6.6	5.7	1.37	1.58
Control	10.6	1.8	0.53	0.89
Testes	0.43	12.0	0.09	0.18
Control	6.7	1.97	0.13	0.25
Gall bladder	1.62 ± 0.26*	17.51 ± 14.7	2.49 ± 2.12	0.26 ± 0.11
Control	3.91 ± 0.18	5.62 ± 4.7	2.35 ± 2.16	0.69 ± 0.15
Small intestine	2.1 ± 0.2*	34.3 ± 2.2*	0.7 ± 0.1	2.0 ± 0.3
Control	41.6 ± 2.6	11.2 ± 1.3	1.1 ± 0.1	2.4 ± 0.2
Serum	0.30	3.43	0.16	0.1
Control	11.8	1.48	0.20	1.06

Hamsters (8 per group) were administered RS-21607 at a dose of 50 mg/kg once daily by oral gavage (9 hr into the light cycle) for 14 days. On day 15, they were given the last dose (3 hr into the light cycle) and after 1 hr were administered 10 μCi [^{14}C]mevalonate i.p. and killed after 1.5 hr. Tissue lipids were isolated after saponification as described in Materials and Methods. Livers, kidneys, gall bladders and small intestines were processed individually, and data represent the mean value ± SEM for tissues from 8 animals in each group. Spleens, adrenals, testes and serum were processed as two groups of four pooled tissues, and data represent the mean value for the two groups. Statistics were applied only to individually processed tissues (N = 8). Data represent the amount of sterol in dpm/g of tissue × 10^{-3}.

* $P < 0.05$ vs controls treated with vehicle.

The bile acid resin cholestyramine (500 mg/kg), administered orally twice daily for 7 days, resulted in 15% ($P < 0.05$) cholesterol lowering. When both agents were administered simultaneously to hamsters, serum cholesterol was reduced by 58% ($P < 0.05$), indicating that the cholesterol-lowering activity of these two agents was additive. Azalanstat significantly depressed hepatic HMG-CoA reductase activity in these animals (Table 5) by 56% ($P < 0.01$), whereas cholestyramine stimulated reductase activity by 174% ($P < 0.01$). When the two agents were co-administered orally to hamsters, the increase in hepatic HMG-CoA reductase activity brought about by cholestyramine was attenuated significantly by azalanstat, and it increased by only 71% over control levels ($P < 0.01$).

Table 4. Quantitation of sterols in hamster tissues and serum

	Sterols (μg/g tissue or μg/mL serum)							
	Cholestanol		Dihydrolanosterol		Lanosterol		Other(s)	
Tissue	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Serum	0.4	0.7	(0.2)	6.7 (8.9)	BQL	0.2 (0.2)	0.3	1.0
Adrenals	2.0	4.9	(1.6)	40.4	BQL	15.5	4.4	21.2
Spleen	2.2	3.8	(2.6)	36.1	BQL	BQL	4.9	12.3
Testes	0.6	1.3	(0.2)	7.6	2.4	12.9	5.0	15.0
Kidneys	1.2	1.8	(1.2)	16.5	(0.6)	BQL	1.1	16.5
Gall bladder	BQL	BQL	8.5 (2.4)	20.7 (96)	3.5	BQL	11.4	4.3
Liver	4.4	13.7	3.1 (1.2)	334 (127)	0.2 (0.9)	8.6 (11.1)	10.2	43.5
Small intestine			(0.7)	(56)	(4.5)	(83.9)		

Quantitative determinations were carried out by GC (Method A), or by GC–MS analysis using a selected ion monitoring technique (Method B, figures in parentheses). In Method A, the identity of cholestanol, dihydrolanosterol (DHL) and lanosterol (LAN) was established by relative retention time, and the identifiers were confirmed by full scan analysis in all cholesterol measurements when DHL and LAN were greater than 3 μg/mL. Compounds listed under "Other(s)" include all unidentified sterols eluting between cholestanol and the end of the GC run (10 min beyond LAN). Quantitative estimates from two injections per sample were within 0.1 μg, except for the DHL values of serum and kidney, which showed somewhat larger variations (0.34 and 0.16 μg, respectively) due to the presence of interfering peaks. BQL is < 0.1 μg/g tissue or 0.05 μg/mL serum.

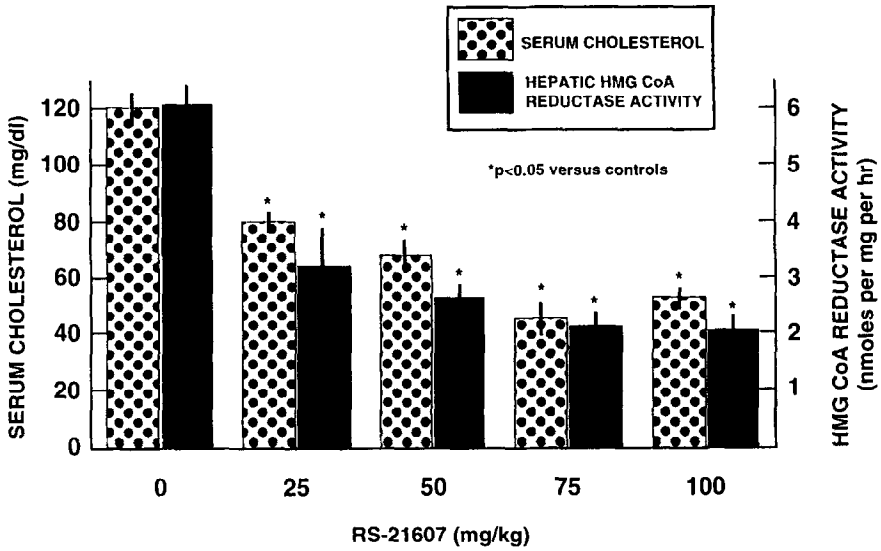

Fig. 6. Dose-dependent reduction of hepatic microsomal HMG-CoA reductase activity and serum cholesterol in azalanstat-treated hamsters. Male Syrian hamsters (8 per group) were exposed to a 12-hr light–dark cycle beginning at 6:00 a.m. and administered azalanstat in 0.5 mL of propylene glycol 9 hr into the light cycle once daily for 14 days. Animals were fasted overnight and bled by cardiac puncture 3 hr into the light cycle on day 15. The livers were excised, and HMG-CoA reductase activity of hepatic microsomes was measured as described in Materials and Methods. Activity (nmol [^{14}C]-mevalonate synthesized/mg protein/hr) of treated hamsters was compared with that of vehicle-treated control animals using a non-parametric *t*-test. Serum cholesterol levels (mg/dL) were determined as described in Materials and Methods and compared with vehicle-treated controls using Dunnett's test. Values are means ± SEM.

Effect of azalanstat on uptake of ^{125}I-LDL and ^{125}I-VLDL by hamster liver membranes

Hamster ^{125}I-LDL and ^{125}I-VLDL were both bound in a saturable and specific manner by hamster liver membranes, although VLDL was taken up more avidly than LDL (data not shown), indicating that the hamster, like the dog [24], possesses functional hepatic LDL receptors. When hamsters were administered azalanstat by oral gavage at a dose of 50 mg/kg for 7 days (Table 5), specific binding of ^{125}I-LDL by isolated hepatic membranes was reduced significantly by 62% ($P < 0.01$), whereas ^{125}I-VLDL binding was not affected (data not shown). Serum cholesterol was lowered significantly in these animals by 41% ($P < 0.05$). Lovastatin administered at a dose of approximately 100 mg/kg (0.1% of feed) for 10 days did not affect significantly ^{125}I-LDL binding in contrast to its effect in the dog [24].

To confirm the ligand binding data, hepatic membranes from hamsters treated with RS-21607 and lovastatin were solubilized and subjected to western blotting using an antibody to the conserved 15 residue carboxy-terminal peptide of the LDL receptor [27]. As can be seen (Table 5), expression of LDL receptor protein was reduced significantly after treatment with azalanstat by 49% ($P < 0.01$) and serum cholesterol levels were reduced by 53% ($P < 0.05$). Lovastatin (0.1% of diet) reduced expression of the LDL receptor protein in hamster liver membranes by 53% ($P < 0.01$) and lowered serum cholesterol levels by 73% ($P < 0.05$).

DISCUSSION

The cholesterol-lowering activity of azalanstat in hamsters appears to result from inhibition of intracellular cholesterol biosynthesis in the principal cholesterologenic tissues, such as the liver and intestine. Studies with hamsters that had been treated with azalanstat and then exposed intraperitoneally to [^{14}C]mevalonate showed that it inhibited incorporation of isotope into tissue cholesterol deposits by up to 98%. The fraction designated "cholesterol" in these studies undoubtedly contains other demethyl-sterols isolated from the tissues by TLC, so that the inhibition of true cholesterol synthesis may have been somewhat underestimated. However, it was clearly high, and earlier studies indicated that the principal demethyl-sterol in this fraction was, in fact, cholesterol. Azalanstat led to an increased incorporation of isotope into the methyl-sterol (MS) fraction of these tissues (Table 3), implicating a block in cholesterol biosynthesis between methyl-sterols and cholesterol. When the methyl-sterols were isolated and characterized by GC/MS analysis, it was shown that DHL was the principal MS formed in most tissues with lower amounts of LAN and some unidentified MS (Table 4). This implicates LDM as the molecular target [32] and not lanosterol Δ-24 reductase, the enzyme blocked by the late stage inhibitor MER-29 (triparanol) [33]. Tissue levels of squalene-oxide were not elevated in tissues of treated animals (Table 3), indicating that RS-21607 does not inhibit the enzyme squalene-oxide

Table 5. Pharmacological activity of azalanstat (RS-21607) in hamsters

Treatment of animals	Serum cholesterol* (% control)	Hepatic cholesterol 7α-hydroxylase activity† (% control)	Hepatic HMG-CoA reductase activity‡ (% control)	Specific binding of ^{125}I-LDL to hepatic membranes§ (% control)	Expression of LDL receptor protein¶ (% control)
Azalanstat (50 mg/kg, 2 weeks)	36¶	235¶			
Azalanstat (50 mg/kg, 10 days)	47¶				51**
Azalanstat (50 mg/kg, 1 week)	59¶	149¶		38**	
4% Cholestyramine (1 week)	97	197¶			
Azalanstat (50 mg/kg + 4% cholestyramine (1 week)	47¶	275¶			
Azalanstat (75 mg/kg, 2 weeks)	62¶	501¶			
Lovastatin (50 mg/kg, 2 weeks)	82¶	50¶			
Lovastatin (100 mg/kg, 2 weeks)	27¶				
Lovastatin (100 mg/kg, 10 days)				74	47**
Azalanstat (30 mg/kg, 1 week)	56¶		44**		
Cholestyramine (500 mg/kg, b.i.d., 1 week)	85¶		274**		
Azalanstat (30 mg/kg, 1 week) + cholestyramine (500 mg/kg, b.i.d., 1 week)	42¶		171**,††		

* Serum cholesterol was measured enzymatically as described in Materials and Methods and is expressed as a percent of vehicle-treated controls. Control values for the different experiments ranged from 82.2 ± 3.9 to 146.1 ± 5.1 mg/dL.

† Cholesterol 7α-hydroxylase activity was measured by HPLC detection of 7α-hydroxycholesterol synthesized per mg of protein in hepatic microsomes of treated animals and is expressed as a percent of the vehicle-treated control values. Control values for the different experiments ranged from 0.111 ± 0.022 to 0.182 ± 0.024% conversion of [^{3}H]cholesterol to product.

‡ HMG-CoA reductase activity was measured in male hamsters (8 per group) administered azalanstat (30 mg/kg daily) by gavage in propylene glycol 9 hr into the light cycle and cholestyramine (500 mg/kg) was administered by gavage twice daily in aqueous suspension for 1 week. Animals were fasted overnight and bled by cardiac puncture 3 hr into the light cycle on day 8. Livers were excised and then rinsed with ice-cold saline; microsomal HMG-CoA reductase was measured as described in Materials and Methods. Activity is expressed as a percent of control activity. Control values for the different experiments ranged from 1.08 ± 0.15 to 12.03 ± 2.41 nmol [^{14}C]mevalonate synthesized/mg protein/hr.

§ Specific uptake of ^{125}I-LDL in hepatic membranes was determined in hamsters (6 per group) administered azalanstat (50 mg/kg for 1 week), 9 hr into the light cycle or lovastatin administered in the feed for 10 days (final dose approximately 100 mg/kg body weight daily). Animals were killed and bled by cardiac puncture 3 hr into the light cycle 1 day after dosing had stopped. Hepatic membranes were prepared as described in Materials and Methods, and ^{125}I-LDL ligand binding studies were carried out with freshly isolated membranes. The data were determined in nanograms ^{125}I-LDL specifically bound per milligram of microsomal protein and are expressed as a percent of that bound in vehicle-treated control animals. Control values ranged from 153 ± 25 to 1656 ± 573 ng/mg protein.

‖ Expression of LDL receptor protein was measured in male hamsters (6 per group) that had been administered azalanstat (50 mg/kg) for 10 days, 9 hr into the light cycle, or lovastatin (0.1% of feed equivalent to approximately 100 mg/kg body wt/day) for 10 days. Animals were fasted overnight, then bled by cardiac puncture 3 hr into the light cycle, and the livers were excised. LDL receptor protein was measured by western blots with an antibody to the conserved 15 residue carboxy-terminal peptide of the LDL receptor, as described in Materials and Methods. The data were determined in densitometric units of LDL receptor protein on autoradiogram films of liver membranes from treated animals and are expressed as a percent of the vehicle-treated control values. Control values ranged from 8.9 ± 0.9 to 17.4 ± 0.7 densitometric units.

¶ $P < 0.05$ (Dunnett's test).

** $P < 0.01$ (non-parametric t-test).

†† $P < 0.01$ vs cholestyramine-treated animals.

cyclase unlike certain other imidazoles [34] and ketoconazole [35], and this was confirmed by the *in vitro* studies in human fibroblasts and HepG2 cells (Fig. 1). Although the levels of DHL were found to be highest in the liver, gall bladder and small intestine of treated hamsters, serum levels were relatively low (0.78 mg/dL) compared with total serum cholesterol levels in these animals (55 mg/dL), suggesting that DHL was fairly effectively cleared from the circulation via the liver and gall bladder, as has been demonstrated by Miettinen in patients treated with the 14α-demethylase inhibitor ketoconazole [13]. Inhibition of cholesterol synthesis by azalanstat was not hepato-selective at doses of 50 mg/kg (Table 3) or 3 mg/kg (data not shown), and in this respect it resembles the HMG-CoA reductase inhibitor lovastatin rather than pravastatin, which is a more selective inhibitor of cholesterol synthesis [36].

The correlation between the cholesterol-lowering activity of azalanstat and the inhibition of hepatic HMG-CoA reductase activity (Fig. 6) suggests that cholesterol lowering by this agent may be accomplished by suppressing the activity of two enzymes involved in cholesterol biosynthesis: LDM and HMG-CoA reductase. It is interesting that cotreatment of hamsters with azalanstat and the bile acid sequestrant cholestyramine, an agent known to lead to up-regulation of HMG-CoA reductase activity in the liver [37], resulted in additive cholesterol lowering in hamsters. The compensatory increase in hepatic microsomal HMG-CoA reductase activity brought about by cholestyramine was attenuated significantly by this agent (Table 5), providing a rationale for co-therapy with these agents in humans. The biphasic concentration-dependent modulation of HMG-CoA reductase activity by azalanstat in HepG2 cells is similar to that reported for ketoconazole in intestinal epithelial cells [38] and found by us in HepG2 cells (data not shown), but differs from the effect of lovastatin, which led to a large compensatory increase in reductase activity. The inhibition of HMG-CoA reductase activity brought about by azalanstat at low concentrations was not the result of a direct inhibition of enzyme activity by residual drug, because incubation of HepG2 cell sonicates with 100 μM azalanstat did not inhibit activity (data not shown). The inhibition of reductase activity in hamster liver probably reflects the inhibition occurring at low pharmacological attainable doses of azalanstat in HepG2 cells. This appears to occur at a post-transcriptional step because mRNA levels for the enzyme were elevated in the concentration range where activity was inhibited (unlike the LDL receptor which appeared to be regulated at a transcriptional step). This is in contrast to the transcriptional regulation of HMG-CoA reductase brought about by cholesterol metabolites such as 26-hydroxycholesterol [39]. It has been suggested that sterols derived from epoxycholesterol [40] or 3β-hydroxylanost-8-en-32-ol (lanostenal), the oxysterol generated during lanosterol 14α-demethylation [41], may regulate HMG-CoA reductase activity, and it is possible that a metabolite of lanosterol (or dihydrolanosterol) generated by the azalanstat inhibition of LDM may down-regulate reductase activity. In support of this hypothesis, we have demonstrated that 3β-hydroxylanost-8-en-32-ol accumulates in rat microsomes incubated with azalanstat [16], and there is a recent report that the synthetic oxysterol 15α-fluorolanost-7-en-3β-ol down-regulates HMG-CoA reductase activity at a post-transcriptional step in Chinese hamster ovary cells [42].

Inhibition of cholesterol synthesis by HMG-CoA reductase inhibition has been reported to lead to up-regulation of hepatic LDL receptor activity in certain animal models [24] and in humans [43]. It was surprising, therefore, to find that neither azalanstat nor lovastatin resulted in enhanced ligand binding of ^{125}I-LDL or ^{125}I-VLDL by isolated hamster liver membranes, even though serum cholesterol levels were reduced significantly in these animals. We also found no increase in LDL receptor mRNA in azalanstat-treated animals, and there was actually a significant reduction in expression of LDL receptor protein (Table 5). It is recognized that rodents such as the rat and hamster may not be ideal species for demonstrating LDL receptor modulation, unlike the dog [24], although we were able to demonstrate an increase in LDL receptor mRNA in lovastatin-treated hamsters (data not shown) in agreement with the data of Ma *et al.* [44]. It has been assumed for many years that HMG-CoA reductase and the LDL receptor are co-ordinately regulated [45]; however, the data presented here and a recent report by Larsen *et al.* [46], who showed that an oxysterol analogue was able to down-regulate HMG-CoA reductase expression in HepG2 cells without concomitantly regulating the LDL receptor, indicate that the co-ordinate regulation can be disrupted under certain pharmacological conditions.

The increase in cholesterol 7α-hydroxylase activity brought about by azalanstat in the livers of treated hamsters is interesting, and it is possible that it may interfere with cholesterol absorption and biliary cholesterol secretion, as has been reported by Miettinen for ketoconazole [13], leading to altered bile acid synthesis. This may also explain the elevated activity of hepatic cholesterol 7α-hydroxylase in livers of animals treated with azalanstat. Hepatic cholesterol depletion may have resulted from diminished newly synthesized cholesterol and reduced cholesterol absorption leading to suppressed secretion of VLDL cholesterol by the liver and serum cholesterol lowering. The data presented here show that azalanstat (RS-21607) lowers serum cholesterol levels in hamsters by mechanisms related to the inhibition of LDM, HMG-CoA reductase and cholesterol 7α-hydroxylase and, therefore, may be an effective cholesterol-lowering agent in humans. Preliminary studies have shown that azalanstat is well tolerated and effective in lowering LDL cholesterol levels in moderately hyperlipidemic males.*

* Schwartz K, personal communication. Cited with permission.

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