HMG-CoA reductase regulation: use of structurally diverse first half-reaction squalene synthetase inhibitors to characterize the site of mevalonate-derived nonsterol regulator production in cultured IM-9 cells

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Abstract **The activity of HMG-CoA reductase (HMGR) is tightly regulated, in part through post-transcriptional mechanisms that are mediated by nonsterol products of mevalonate metabolism. Previous reports have suggested that these mediators are derived from farnesyl pyrophosphate (FPP). Recent studies have implicated FPP hydrolysis products (e.g., farnesol), the squalene synthetase (SQS) reaction products presqualene pyrophosphate (PSQPP) and squalene, or their metabolites. To distinguish among these possible mediators, we evaluated the ability of HMGR and SQS inhibitors to induce compensatory increases in HMGR activity in cultured IM-9 cells. Mevinolin (HMGR inhibitor) produced predicted increases in HMGR activity that were related to the degree of cholesterolgenesis inhibition (e.g., 4-fold, 9-fold, and 17-fold increases relative to 50%, 76%, and 90% inhibition, respectively). By contrast, a variety of structurally distinct reversible, competitive, first half-reaction SQS inhibitors all reduced cholesterolgenesis by up to 90% with no appreciable increases in HMGR activity. These observations strongly suggest that nonsterol-mediated post-transcriptional mechanisms regulating HMGR activity remain intact after SQS first half-reaction inhibition, indicating that nonsterol regulator production is independent of SQS action and ruling out PSQPP, squalene and their metabolites as possible mediators. Unexpectedly, the SQS mechanism-based irreversible inactivator, zaragozic acid A (ZGA) exhibited the greatest degree of HMGR modulation, producing 5-fold, 11-fold, and 40-fold increases in HMGR activity at concentrations that produced 25%, 50%, and 75% cholesterolgenesis inhibition, respectively. The markedly greater magnitude of HMGR stimulation by ZGA versus mevinolin at similar levels of cholesterolgenesis inhibition suggests that ZGA may directly interfere with the production or action of the nonsterol regulator.**—Petras, S. F., S. Lindsey, and H. J. Harwood, Jr. **HMG-CoA reductase regulation: use of structurally diverse first half-reaction squalene synthetase inhibitors to characterize the site of mevalonate-derived nonsterol regulator production in cultured IM-9 cells.** ***J. Lipid Res.*** **1999.** 40: **24–38.**

Supplementary key words cholesterol synthesis • HMG-CoA reductase • squalene synthetase • enzyme inhibition • metabolic regulation • cultured cells • mevinolin • zaragozic acid • farnesol • nonsterol reductase regulator

HMG-CoA reductase (HMGR; E.C. 1.1.1.34) catalyzes the rate-determining reaction in cholesterol and polyisoprenoid biosynthesis, the conversion of HMG-CoA to mevalonic acid (1). HMGR thus plays a critical role in controlling the various metabolic pathways that utilize isoprenoids or isoprenoid derivatives, such as those producing cholesterol and other sterols, steroid hormones, bile acids, dolichols, ubiquinones, isopentenyl-tRNAs, heme A, and farnesylated and geranylgeranylated proteins, that play important roles in the regulation of cellular growth and metabolism (2–4).

The activity of HMGR is tightly regulated through a variety of mechanisms that alter either the intracellular concentration of the enzyme, the catalytic efficiency of the enzyme, or both. Processes that modulate HMGR catalytic efficiency include reversible phosphorylation (5), reversible thiol-disulfide formation (6), allosteric activation by NAD(P)H (7), and alterations in membrane fluidity (8). Processes that alter intracellular HMGR concentration include those that modulate HMGR transcription (2, 9, 10), translation (11–15), and protein stability (14–17). Both sterol and nonsterol products of mevalonate metabolism are required for full suppression of HMGR expression (3, 15, 16). For example, sterols are thought to mediate HMGR transcriptional repression (9); nonsterol meva-

Abbreviations: SQS, squalene synthetase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGR, HMG-CoA reductase; PFT, protein farnesyltransferase; FPP, farnesyl pyrophosphate; PSQPP presqualene pyrophosphate; GGPP, geranylgeranyl pyrophosphate; NSRR, nonsterol reductase regulator; ZGA, zaragozic acid A; FXR, farnesoid-activated receptor; RXR, retinoid-activated receptor; PPAR, peroxisome proliferator-activated receptor; HI-FBS, heat-inactivated fetal bovine serum.

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lonate metabolites are thought to mediate HMGR translational repression (13, 15); and both sterols and nonsterol mevalonate metabolites are thought to synergystically enhance HMGR degradation (15, 16, 18).

Although much effort has been directed toward characterizing these nonsterol regulators of HMGR activity (NSRRs), neither the identities of these mevalonate metabolites nor the precise location along the pathway from which these metabolites are produced has been unequivocally established. For example, previous reports have demonstrated that these NSRRs are derived from farnesyl pyrophosphate (FPP) but are not FPP itself. These reports have also indicated that these NSRRs are neither dolichol, ubiquinone, isopentenyl adenine, geranylgeranyl pyrophosphate (GGPP), nor post-squalene cholesterolgenic metabolites (3, 11, 12, 18–21). More recent studies have implicated FPP hydrolysis products (e.g., farnesol, farnesyl phosphate, farnesoic acids; 11, 19, 21, 22), the squalene synthetase (SQS) reaction products presqualene pyrophosphate (PSQPP) and squalene (18, 20, 23), and their respective metabolites, as putative NSRRs, although the existence of specific prenylated proteins that function as mediators of these effects has not been ruled out (2, 24).

SQS (E.C. 2.5.1.21) catalyzes the head to head condensation of two molecules of FPP to form squalene (25, 26). The reaction is unique when compared to other FPP-utilizing enzymes, and proceeds in two distinct steps, both of which are thought to involve the formation of carbocationic transition-state intermediates (25, 26). In the first half reaction, two molecules of FPP react to form the stable cyclopropylcarbinyl diphosphate intermediate, PSQPP, with concomitant release of a proton and a molecule of inorganic pyrophosphate (25, 26). In the second half reaction, PSQPP undergoes heterolysis, isomerization, and reduction with NADPH to form squalene (25, 26).

The recent discovery of a number of structurally distinct classes of SQS inhibitors (reviewed in 27) has made it possible to further define the location along the sterol and polyisoprenoid pathways from which the NSRRs are produced. Indeed, inhibitors of SQS that compete with FPP to reversibly inhibit the enzymatic first half-reaction and that show specificity for SQS inhibition relative to inhibition of other FPP-utilizing enzymes (e.g., the prenyltransferases) have the potential to differentiate among the above-mentioned nonsterol products of mevalonate metabolism, by preventing the production of PSQPP, squalene, and their metabolites, without interfering with the production of other FPP metabolites produced through reactions independent of the action of SQS. In cultured cells or experimental animals, such inhibitors would lead to compensatory induction of HMGR activity, an observation indicative of blocked NSRR production if either PSQPP, squalene, or their metabolites were the true NSRRs. However, post-transcriptional regulation of HMGR activity would remain intact and induction of HMGR activity would not be observed if the true NSRRs were produced as a result of non-SQS-mediated FPP metabolism (e.g., FPP hydrolysis to farnesol, protein prenylation, etc.).

In this regard, we have evaluated the mechanisms of action of a number of structurally distinct classes of SQS inhibitors that have appeared in the scientific and patent literature, and have identified a variety of series whose members function as reversible, competitive, first half-reaction SQS inhibitors that show a high degree of selectivity for SQS inhibition relative to inhibition of the FPP-utilizing enzyme, protein farnesyltransferase (PFT), and relative to inhibition of post-squalene cholesterolgenic enzymes. In addition, we have utilized representative members of these classes of SQS inhibitors to interfere with the production of PSQPP, squalene, and their metabolites in cultured cells, and have asked whether such inhibition leads to compensatory induction of HMGR activity.

EXPERIMENTAL PROCEDURES

Chemicals

[1-^{3}H]FPP (22.5 Ci/mmol), [2-^{14}C]mevalonolactone (54.1 mCi/mmol), [3-^{14}C]HMG-CoA (55 mCi/mmol), [5-^{3}H]mevalonolactone (24 Ci/mmol), and [2-^{14}C]acetate (56 mCi/mmol) were from New England Nuclear (Boston, MA). Unlabeled FPP was from American Radiolabeled Chemicals (St. Louis, MO). Heat-inactivated fetal bovine serum (HI-FBS) for growth of IM-9 and HepG2 cells was from Intergen (Purchase, NY). HI-FBS for growth of CaCo-2 cells and for maintenance of rat primary hepatocytes was from HyClone Laboratories (Logan, UT). Lipoprotein-deficient HyClone HI-FBS for use in stimulating CaCo-2 cell and rat primary hepatocyte cholesterol synthesis was prepared as previously described (27). Recombinant H-ras was from Oncogene Sciences (Uniondale, NY). ZGA was prepared from the fungal species ATCC #20986 as described (28). All other chemicals were from previously listed sources (27, 29, 30).

Buffers and solutions

PMED buffer = 50 mm K_xPO_4 (pH 7.4), 5 mm $MgCl_2$, 1.0 mm EDTA, 5.0 mm DTT. TEDK buffer = 50 mm Tris (pH 7.5), 1.0 mm EDTA, 5.0 mm DTT, 70 mm KCl. TDZ buffer = 50 mm Tris-HCl (pH 7.5), 5 mm DTT, 20 μm $ZnCl_2$. Supplemented DMEM-A = 10% HI-FBS, 2 mm l-glutamine, 40 μg/mL gentamicin in DMEM. Supplemented DMEM-B = 20% HI-FBS, 2 mm l-glutamine, 40 μg/mL gentamicin in DMEM.

Growth of HepG2 cells

HepG2 cells grown in T-75 flasks and released by trypsin treatment as previously described (27), were seeded in 24-well plates at a density of 1.2×10^5 cells/well and maintained in 1.0 mL of supplemented DMEM-A for 7 days in a 37°C, 5% CO_2 incubator with medium changes on days 3 and 5. At this time, cultures reached 80–90% confluency and maintained a >90% cell viability (Trypan blue dye exclusion). On day 8, the medium was removed and replaced with fresh supplemented DMEM-A containing 1% DMSO ± effector compounds.

Growth of CaCo-2 cells

CaCo-2 cells grown in T-75 flasks and released by trypsin treatment as previously described (27) were seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well and maintained in 1.0 mL of supplemented DMEM-B for 6 days in a 37°C, 10% CO_2 incubator with medium changes on days 2 and 4. At this time, cultures reached 80% confluency and maintained a >90% cell viability (Trypan blue dye exclusion). On day 6, the medium was removed and replaced with 1.0 mL of fresh supplemented DMEM-B

that contained 20% lipoprotein-deficient HI-FBS. After 18 h of incubation in this medium to maximize rates of cholesterol synthesis, the medium was removed and replaced with 1.0 mL of fresh medium containing 0.4% DMSO ± effector compounds.

Growth of IM-9 cells

IM-9 cells were maintained in suspension culture at 37°C in 1-L spinner flasks containing 500 mL of RPMI-1640 supplemented with 10% HI-FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (27, 31). Every 3 days, resulting late-log phase cultures whose cell viability was >90% (Trypan blue dye exclusion) were separated into two equal portions. One portion was diluted to a concentration of 5×10^5 cells/mL with fresh medium and maintained at 37°C for culture continuation. The second portion was apportioned in 2.5-mL aliquots to 12 × 75-mm plastic culture tubes, sedimented by centrifugation at 900 *g* for 5 min, and resuspended to a density of 2.5×10^6 cells/mL with 1.0 mL culture medium containing 0.4% DMSO ± effector compounds.

Isolation and culture of primary rat hepatocytes

Morphologically intact, rat hepatic parenchymal cells were obtained from anesthetized rats after in situ liver perfusion with oxygenated HBSS containing 1 mg/mL collagenase and 2 mm $CaCl_2$ as previously described (27). Greater than 90% of the final cell preparation consisted of intact parenchymal cells that excluded Trypan blue dye. Cells were seeded in 6-well plates at a density of 5×10^5 cells/well in 1.0 mL DMEM/F12 containing 10% HI-FBS, 2 mm l-glutamine, 40 μg/mL gentamicin, and 1 μg/mL insulin, and incubated for 3 h in a 37°C, 5% CO_2 incubator to allow cell attachment. The medium was then removed, 1.0 mL of fresh medium containing lipoprotein-deficient HI-FBS was added, and cells were incubated for an additional 24 h to maximize rates of cholesterol synthesis. The medium was then removed and replaced with 1.0 mL of fresh medium containing 1% DMSO ± effector compounds.

Isolation of hepatic microsomes

Hepatic microsomes were prepared from male Sprague-Dawley rats (approx. 150 g) as previously described (29, 30). For preparations to be used for SQS measurements, microsomal pellets were resuspended in 1.0 mL PMED buffer per gram liver by 5 strokes of a Potter-Elvehjem pestle and stored frozen in liquid N_2. For preparations to be used for SQS solubilization, microsomal pellets were stored in liquid N_2 without resuspension.

Preparation of trypsin-solubilized SQS

SQS was solubilized from the microsomal membrane by limited trypsinolysis as described (27, 29) and was stored frozen in liquid N_2 in 5-mL aliquots. Just prior to use, aliquots were thawed and diluted with PMED buffer to a protein concentration of 1.0 mg/mL.

Isolation of rat brain cytosol

Rat brain cytosol was prepared from male Sprague-Dawley rats (150–200 g) as previously described (30) and was stored frozen in liquid N_2 in 2-mL aliquots. Just prior to use, aliquots were thawed and diluted with TDZ buffer to a protein concentration of 3.0 mg/mL.

Isolation of IM-9 cell microsomes

IM-9 cells were sedimented by centrifugation at 900 *g* for 5 min at 4°C, washed twice in TEDK buffer, and resuspended in 0.5 mL TEDK buffer per 10^8 cells (27, 31). Cell suspensions were frozen in liquid N_2 for a minimum of 60 min, then rapidly thawed to room temperature, homogenized first using 15 strokes of a ground glass pestle in a 2-mL Potter-Elvehjem tissue homogenizer and subsequently, after transfer to a 5-mL Potter-Elvehjem tissue homogenizer, using 5 strokes of a motor-driven Teflon pestle. Homogenates were centrifuged at 6000 *g* for 10 min at 4°C and resultant supernatants were centrifuged at 172,000 *g* for 90 min at 4°C. Microsomal pellets were resuspended in 0.2 mL TEDK buffer per 10^8 cells and stored frozen in liquid N_2.

Measurement of SQS activity

SQS activity was evaluated by measuring conversion of [1-^{3}H]FPP to [^{3}H]squalene under anaerobic conditions, using an ascorbate/ascorbate oxidase oxygen consumption system [final assay concentrations: 48 mm K_xPO_4 (pH 7.4), 4.8 mm $MgCl_2$, 0.33 mm EDTA, 1.67 mm DTT, 258 μm $NADP^+$, 2.1 mm glucose-6-phosphate, 0.94 U glucose-6-phosphate dehydrogenase, 9.4 mm NaF, 50 mm sodium ascorbate, 1.5 U ascorbate oxidase, 4% DMSO, 5.1 μm [1-^{3}H]FPP (sp. act. ≈250dpm/pmol), 0.33 mg/mL microsomal protein] as previously described (29). SQS activity is expressed as pmol squalene formed per min of incubation at 37°C per mg microsomal protein, based on the stoichiometry of the reaction whereby two moles of FPP react to form one mole of squalene and half of the radiolabel is lost from the C-1 position of the prenylating FPP due to 1-*pro*-S hydrogen release.

Measurement of SQS first half-reaction activity

SQS first half-reaction activity was evaluated by measuring proton release from C-1 of the prenylating FPP during condensation of 2 molecules of [1-^{3}H]FPP to form [^{3}H]PSQPP through equilibration of the expelled tritium with the primary hydroxyl of a fixed volume of added MeOH as previously described (27), using reaction conditions identical to those described above for squalene production. SQS first half-reaction activity is expressed as pmol proton released per min of incubation at 37°C per mg protein based on the relationship whereby dpm tritium released is equal to 6.25 times the dpm of the assessed MeOH for the sample and MeOH volumes utilized.

Measurement of PFT activity

PFT activity was evaluated by measuring the covalent incorporation of the farnesyl moiety of [1-^{3}H]FPP into H-ras [final assay concentrations: 0.5 μm [1-^{3}H]FPP (sp. act. ≈13,000 dpm/pmol), 5 mm $MgCl_2$, 20 μm $ZnCl_2$, 4 μm H-ras, 50 mm Tris-HCl (pH = 7.5), 5 mm DTT, 20 mm KCl, 1.2% DMSO, 1.2 mg/mL rat brain cytosolic protein] as previously described (30). PFT activity is expressed as pmol H-ras farnesylated per min of incubation at 37°C per mg cytosolic protein.

Measurement of HMGR activity

HMGR activity was evaluated by measuring conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonic acid in the presence of NADPH [final assay concentrations: 33.3 mm Tris (pH = 7.5), 3.3 mm DTT, 33.3 mm KCl, 210 μm $NADP^+$, 1.7 mm glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 66.7 μm [^{14}C]HMG-CoA (sp. act. ≈10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6–1.2 Ci/ mmol), 60.7 mm EDTA, 0.67 mg/ml microsomal protein] as previously described (32). HMGR activity is expressed as pmol of mevalonate formed from HMG-CoA per min of incubation at 37°C per mg of microsomal protein.

Measurement of cholesterol synthesis

Cholesterolgenesis was evaluated by measuring incorporation of [2-^{14}C]acetate into cholesterol and related sterols as described (27). Immediately after addition of media containing DMSO ± effector compounds to cultures of HepG2 cells, CaCo-2 cells, IM-9 cells, or rat primary hepatocytes, 25 μl of media containing 4 μCi of [2-^{14}C]acetate was added to each well or culture tube. Plates were then sealed with parafilm, culture tubes were capped,

and cells were incubated at 37°C for 6 h with gentle shaking. Samples were then saponified by addition of 1 mL of 5 N KOH in MeOH, followed by incubation first for 2 h at 70°C then overnight at room temperature. Mixtures were then transferred to glass conical tubes and extracted three times with 4.5 mL hexane. The pooled organic fractions were dried under nitrogen, resuspended in 25 μl chloroform, and applied to 1 × 20-cm channels of Silica Gel 60C TLC plates. Channels containing nonradioactive cholesterol, lanosterol, and squalene were included on selected TLC plates as separation markers. TLC plates were developed in hexane–diethyl ether–acetic acid 70:30:2, air dried, and assessed for radioactivity using a Berthold Linear Radioactivity Analyzer that reports peak location and integrated peak area. Cholesterol synthesis is expressed as dpm [^{14}C]acetate incorporated into cholesterol during the 6-h incubation at 37°C.

Measurement of protein concentration

Protein concentration was measured using the BCA Protein Assay Reagent and bovine serum albumin as standard.

RESULTS

Selection of structurally distinct SQS inhibitors for evaluation

For use in distinguishing between FPP hydrolysis products and their metabolites and SQS-catalyzed reaction products and their metabolites, as potential NSRRs, SQS inhibitors were required to satisfy four criteria of suitability for evaluation. First, compounds were required to inhibit the SQS-catalyzed first half-reaction and thereby prevent the formation not only of squalene but also of PSQPP. Second, to assure that effects observed after SQS inhibition could not be attributed to concomitant inhibition of protein prenylation, compounds were required to exhibit a high degree of specificity for SQS inhibition relative to protein prenyltransferase inhibition. Third, to assure that effects observed were not due to secondary inhibition of post-squalene cholesterolgenic enzymes, compounds were required to show greater than 90% inhibition of cholesterol synthesis in cultured cells without producing significant accumulation of post-squalene cholesterol precursors. Fourth, to assure that alterations in HMGR regulation were a result of SQS inhibition per se and not an idiosyncratic phenomenon specific to a single class of inhibitors, compounds were required to be members of structurally distinct classes of SQS inhibitors, whose effects, if similar, could not be attributed to a compound class effect.

Of the various SQS inhibitors reported in the scientific and patent literature, we selected representative members of five structurally distinct classes, the structures of which are shown in **Fig. 1**. P-3622 (3-(4-chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-A-pentenonitrile) and CP-210172 (3-[4-phenylbenzyloxy]-1-azabicyclo[2.2.2]octane) are respective representatives of the substituted diethylaminoethoxystilbene class (27) and substituted quinuclidine class (29) of cationic SQS inhibitors thought to function as carbocationic transition state mimetics. CP-295697 ([4-(4-phenylphenyl)-butyl]-1,1-bisphosphonate) is a representative member of the substituted bisphosphonate class of

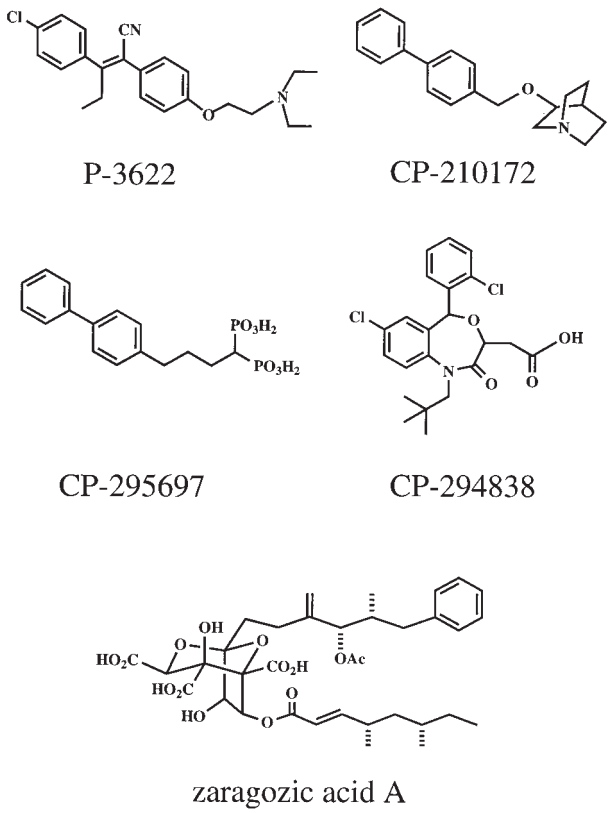

Fig. 1. The structures of P-3622, CP-210172, CP-295697, CP-294838, and zaragozic acid A.

polyanionic SQS inhibitors (33) thought to function as FPP mimetics. CP-294838 (7-chloro-5-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-acetic acid) is a representative member of the substituted benzoxazepinone class of monoanionic SQS inhibitors (34) thought to mimic portions of one or both of the farnesyl residues condensing in the SQS-catalyzed first half-reaction and/or the stable reaction intermediate, PSQPP. ZGA (a.k.a. squalastatin I) is the prototype of a class of natural product polyanionic SQS inhibitors (28) thought to mimic FPP when inserted in one orientation and the stable reaction intermediate PSQPP when inserted in a different orientation.

Inhibition of SQS by P-3622, CP-210172, CP-295697, CP-294838, and ZGA

In the presence of K_m concentrations of FPP (5.2 μm; 29) and in buffer containing 50 mm Pi, P-3622, CP-210172, CP-295697, and CP-294838, all inhibited rat liver microsomal SQS in a log-linear manner (**Fig. 2**), exhibiting IC_{50} values of 0.75 ± 0.2 μm (SD, n = 4), 0.86 ± 0.11 μm (SD n = 6), 13.5 ± 6.5 nm (DOM, n = 2), and 110 ± 16 nm (SD, n = 5), respectively. Under these experimental conditions, ZGA also inhibit rat liver microsomal SQS in a log-linear manner with and IC_{50} of 1.8 ± 1.0 nm (SD, n = 22). All five compounds also inhibited human microsomal SQS, exhibiting IC_{50} values similar to those obtained using the rat liver enzyme (27, 34). Furthermore, all five compounds inhibited the human and rat liver enzymes after solubilization from

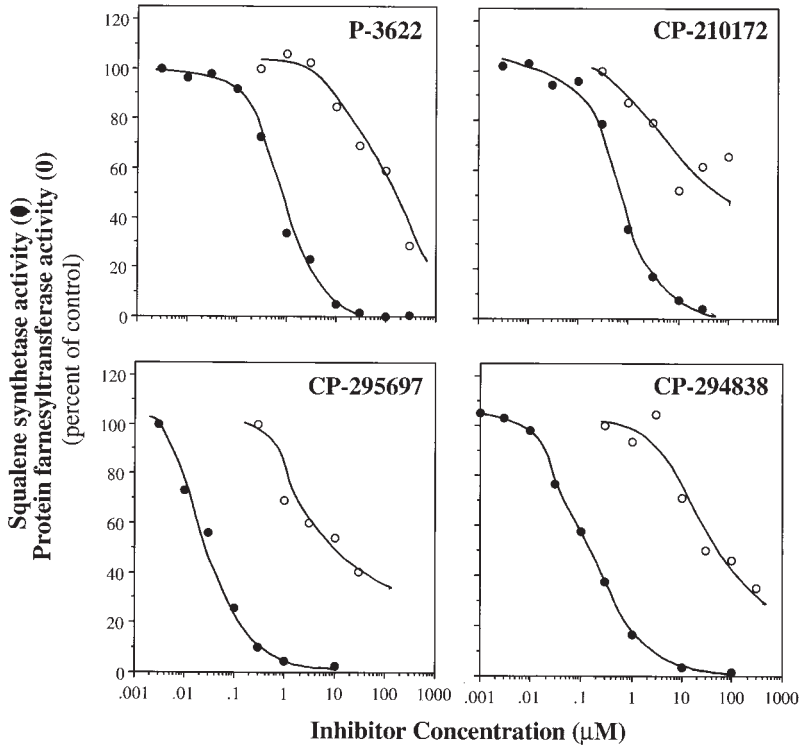

Fig. 2. Inhibition of SQS and PFT by P-3622, CP-210172, CP-295697, and CP-294838. For assessing SQS inhibition, 25 μg rat liver microsomal protein was incubated for 30 min at 37°C in a final volume of 75 μL of PMED buffer containing 5.1 μm [^{3}H]FPP (sp. act. 253 dpm/pmol), 4% DMSO, the indicated inhibitor concentrations, and the remaining SQS substrates and cofactors as described under Experimental Procedures. For assessing PFT inhibition, 30.5 μg rat brain cytosolic protein was incubated for 30 min at 37°C in a final volume of 25 μL of TDZ buffer containing 0.5 μm [^{3}H]FPP (sp. act. 12770 dpm/pmol), 5 mm $MgCl_2$, 4 μm H-ras, 20 mm KCl, 1.2% DMSO, and the indicated inhibitor concentrations. After incubation, squalene production and H-ras farnesylation were assessed as described under Experimental Procedures. Shown are the percentage of control SQS (●) and PFT (○) activities as a function of inhibitor concentration. Control SQS activity averaged 142 ± 21 (SD; n = 3) pmol FPP converted to squalene per min per mg microsomal protein. Control PFT activity averaged 0.31 ± 0.20 (SD; n = 4) pmol FPP incorporated into H-ras per min per mg cytosolic protein.

the microsomal membrane by limited trypsinolysis (27, 34), indicating that inhibition of SQS was a consequence of direct interference with the SQS catalytic machinery rather than a consequence of membrane modification leading to reduced enzyme specific activity.

Reversible, competitive inhibition of SQS by P-3622, CP-210172, CP-295697, and CP-294838

In the presence of 5.2 μm FPP and 50 mm P_i, and within the inhibitor concentration range in which inhibition was log-linear, P-3622, CP-210172, CP-295697, and CP-294838 all inhibited SQS competitively with respect to FPP (**Fig. 3**), exhibiting K_i values of 0.7 μm, 0.49 μm, 34 nm, and 170 nm, respectively (**Table 1**). Inhibition by all four compounds was reversible, as judged by the intersection at the origin of a series of lines generated for various inhibitor concentrations in plots of remaining enzymatic activity versus enzyme concentration (**Fig. 4**), and also by the lack of effect of enzyme concentration or 37°C incubation duration on enzyme inhibition (not shown). By contrast, inhibition of SQS by ZGA was the consequence of irreversible inactivation (29), as characterized by 37°C incubation duration and enzyme concentration-dependent enzyme inhibition that could not be reversed by centrifugation or dilution (29), and also by intersection of such lines at various points on the X-axis whose distance along the X-axis was proportional to the amount of enzyme titrated by ZGA (29).

First half-reaction inhibition of SQS by P-3622, CP-210172, CP-295697, CP-294838, and ZGA

Consistent with their actions as competitive inhibitors with respect to the first half-reaction substrate FPP, P-3622, CP-210172, CP-295697, and CP-294838 all inhibited the SQS-catalyzed first half-reaction to the same extent as they inhibited the overall reaction leading to squalene production (**Fig. 5**). In the absence of inhibitor, proton release from the C1 position of the donor FPP molecule, which is a measure of PSQPP formation in the enzymatic first half-reaction, closely paralleled squalene production. When evaluated in the presence of P-3622, CP-210172, CP-295697, or CP-294838, proton release was inhibited dose-dependently exhibiting log-linear inhibition between 0.1 μm and 10 μm (Fig. 5). Inhibition of proton release paralleled inhibition of squalene formation measured in the same reaction vessel (Fig. 5), with both exhibiting identical IC_{50} values (Table 1). Similar first half-reaction inhibition that was identical to overall reaction inhibition was also observed with ZGA (data not shown).

That no additional inhibition of the second half-reaction was noted for these inhibitors (independent inhibition of both half-reactions would result in greater inhibition of the overall reaction than the first half-reaction) suggests that inhibition of the first half-reaction is sufficient to inhibit the overall reaction. This observation is consistent with previous findings that both half-reactions occur either at a single site within the enzyme active center or at separate sites that are either spatially overlapping or highly interactive (25), such that none of these compounds is able to interact only with the second half-reaction center without at the same time interacting with the first half-reaction center. Indeed, that ZGA has been shown to inhibit the second half-reaction when PSQPP is used as substrate (35), but shows no greater inhibition of the overall reaction than of the first half-reaction when FPP is utilized as substrate, further supports this concept.

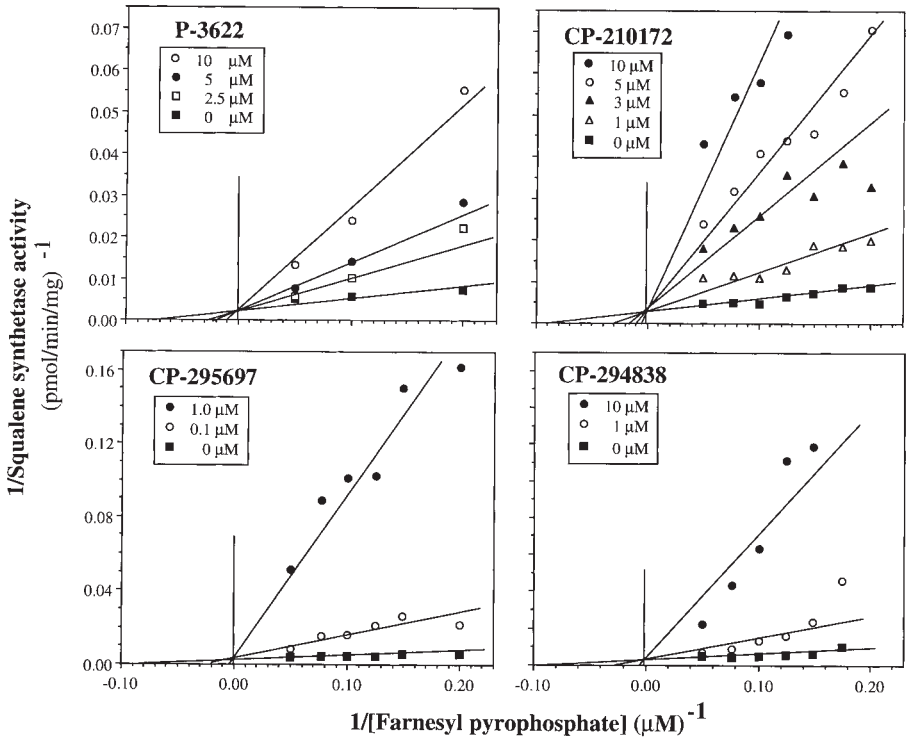

Fig. 3. Competitive inhibition of SQS by P-3622, CP-210172, CP-295697, and CP-294838 with respect to FPP. Rat liver microsomes (25 μg protein; 199 pmol/min/mg) were incubated for 30 min at 37°C in a final volume of 75 μL of PMED buffer containing 4% DMSO, the indicated inhibitor concentrations, the indicated concentrations of [^{3}H]FPP (sp. act. 253 dpm/pmol), and the remaining SQS substrates and cofactors as described under Experimental Procedures. After incubation, squalene production was assessed as described under Experimental Procedures. Data are the average of duplicate determinations and are expressed as reciprocal SQS activity as a function of reciprocal FPP concentration.

Characteristics of SQS inhibition by P-3622, CP-210172, CP-295697, CP-294838, and ZGA

Taken together, these results indicate that P-3622, CP-210172, CP-295697, and CP-294838 are all reversible competitive first half-reaction SQS inhibitors that satisfy the first criteria of suitability for use in evaluating the nature of NSRR. ZGA, which exhibits irreversible inactivation kinetics (29), also demonstrated first half-reaction inhibition characteristics and therefore was subjected to further evaluation.

TABLE 1. Inhibition kinetics of SQS inhibitors

Compound	Inhibition of SQS First Half-reaction (proton release)	Inhibition of SQS Overall Reaction (squalene formation)		Type of Inhibition
	IC_{50}	IC_{50}	K_i	
	μM	μM		
P-3622	1.80	1.80	0.70	R, C
CP-210172	0.70	0.65	0.49	R, C
CP-295697	0.10	0.10	0.034	R, C
CP-294838	0.12	0.11	0.17	R, C
Zaragozic acid A	0.002	0.002	0.00008	I

Characteristics of SQS inhibition by P-3622, CP-210172, CP-295697, and CP-294838 are based on the inhibitor, substrate, and enzyme concentration-dependency profiles shown in Figs. 3–5. The data for SQS inhibition by ZGA is that of Lindsey and Harwood (29). K_i values were calculated from the data of Fig. 3 using Trinity Software (Campton, NH) Enzyme Kinetics program (ISBN #0-927365-25-1), which calculates K_i as the negative x-intercept from secondary plots of K_m/V_{max} versus inhibitor concentration. R, reversible inhibition; C, competitive inhibition; I, irreversible inactivation.

Specificity of P-3622, CP-210172, CP-295697, CP-294838, and ZGA for SQS inhibition versus PFT inhibition

The ability of P-3622, CP-210172, CP-295697, and CP-294838 to function as farnesyl mimics and the competitive nature of their inhibition with respect to FPP suggest that these inhibitors might also inhibit other FPP-utilizing enzymes. Indeed, P-3622, CP-210172, CP-295697, CP-294838 all inhibited PFT activity (Fig. 2). However, in studies in which SQS and PFT were evaluated at their respective K_m FPP concentrations to eliminate differences in inhibition resulting from differing degrees of FPP saturation, all four compounds exhibited a >150-fold and up to 1900-fold specificity for SQS inhibition (**Table 2**). ZGA also demonstrated a 1500-fold specificity for SQS inhibition (Table 2). Thus, P-3622, CP-210172, CP-295697, CP-294838, and ZGA all show specificity for SQS inhibition versus PFT inhibition sufficient to satisfy the second criteria of suitability for use in evaluating the nature of NSRR.

Inhibition of cholesterolgenesis in primary rat hepatocytes and in HepG2, CaCo-2, and IM-9 cells by P-3622, CP-210172, CP-295697, CP-294838, and ZGA

As a consequence of SQS inhibition, P-3622, CP-210172, CP-295697, CP-294838, and ZGA all inhibited

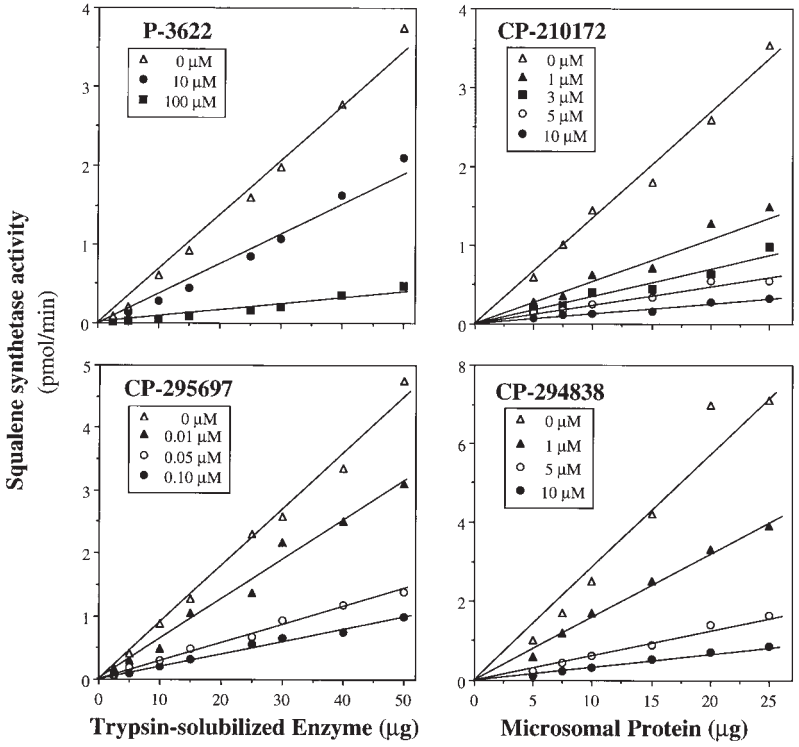

Fig. 4. Reversible inhibition of SQS by P-3622, CP-210172, CP-295697, and CP-294838. Aliquots containing the indicated amounts of rat liver microsomal or trypsin-solubilized microsomal protein were incubated for 30 min at 37°C in a final volume of 75 μL of PMED buffer containing 20.1 μm [^{3}H]FPP (sp. act. 380 dpm/pmol), 4% DMSO, the indicated inhibitor concentrations, and the remaining SQS substrates and cofactors as described under Experimental Procedures. After incubation, squalene production was assessed as described under Experimental Procedures. Shown is SQS activity as a function of protein concentration in the presence or absence of the indicated concentrations of inhibitor.

cholesterolgenesis in a variety of human cell lines (HepG2, CaCo-2, IM-9) and in primary rat hepatocytes, exhibiting IC_{50} values that represent a composite of intrinsic potency and cell penetrability (**Table 3**). Inhibitory profiles of individual compounds were similar in all cell lines evaluated, except for the polyanionic compounds CP-295697 and ZGA, which were considerably more potent in primary hepatocytes (Table 3), an observation attributed to the existence of polyanion transporters operational in primary hepatocytes but not in these cultured cells (36).

Inhibition of cholesterol synthesis by up to 90% in HepG2 cells (**Table 4**), IM-9 cells (Table 4), CaCo-2 cells (not shown) and primary rat hepatocytes (not shown) occurred for each compound evaluated without significant accumulation of post-squalene cholesterol precursors (except as noted below), indicating that cholesterolgenesis inhibition occurred prior to the formation of squalene, consistent with their mechanism of action as SQS inhibitors. By contrast, the lanosterol 14α-demethylase inhibitor, ketoconazole (27), and the oxidosqualene cyclase inhibitor, UK-124,617 (27), produced the predicted accumulation of lanosterol and of squalene oxide and squalene dioxide, respectively (Table 4), consistent with their post-squalene cholesterolgenic enzyme inhibition. Similar accumulation of cholesterol precursors would have been anticipated had post-squalene cholesterolgenic enzyme inhibition occurred after treatment with P-3622, CP-210172, CP-295697, CP-294838, or ZGA.

At concentrations that produced >90% inhibition of cholesterolgenesis, P-3622 and CP-210172 did show secondary inhibition at the site of oxidosqualene cyclase (Table 4), an enzyme whose catalytic mechanism also proceeds through carbocationic reaction intermediates (37). In addition, at concentrations of CP-295697, CP-294838, and ZGA that produced >90% cholesterolgenesis inhibition, a sizable peak of farnesol was noted (not shown).

Taken together, these results indicate that the cationic inhibitors, P-3622 and CP-210172, and the anionic inhibitors, CP-295697, CP-294838 and ZGA, inhibit cholesterolgenesis in HepG2 cells, IM-9 cells, CaCo-2 cells, and rat primary hepatocytes by greater than 70% and greater than 90%, respectively, without significant inhibition of any post-squalene cholesterolgenic enzymes, and thus satisfy the third criteria of suitability for use in evaluating the nature of the NSRR.

Choice of IM-9 cells for evaluating the effects of P-3622, CP-210172, CP-295697, CP-294838, and ZGA on HMGR induction

Studies evaluating the consequences to HMGR regulation of SQS inhibition were undertaken using cultured IM-9 cells (transformed but nonmalignant lymphocytes) as a representative cell line. This cell line was chosen because cholesterol synthesis and HMGR regulation in these cells is similar to that in hepatic cell lines (27, 31, 32; Table 3), and because these cells grow in suspension culture, thus allowing sufficient cell numbers to be obtained in a single culture vessel for both measurement of cholesterolgenesis inhibition and for microsomal isolation and HMG-CoA reductase activity determination.

Inhibition of cholesterol synthesis in IM-9 cells by the HMGR inhibitor, mevinolin, induces compensatory increases in HMGR activity

Numerous reports have demonstrated that inhibition of mevalonate production by the HMGR inhibitors limits production not only of regulatory sterols but also of

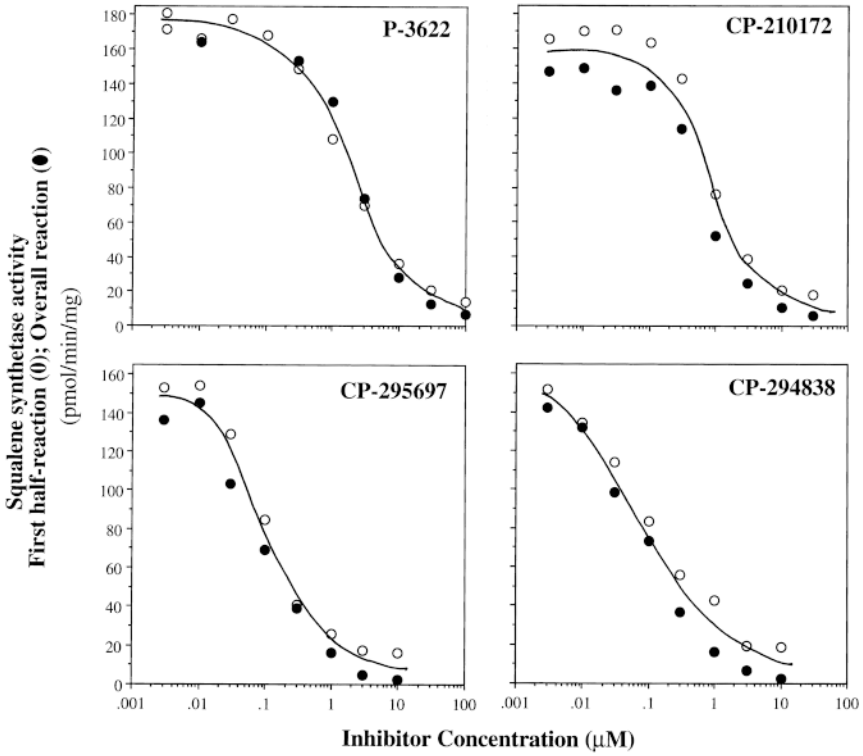

Fig. 5. SQS first half-reaction inhibition by P-3622, CP-210172, CP-295697, and CP-294838. Rat liver microsomes (75 μg protein; 180 pmol/min/mg) were incubated for 30 min at 37°C in a final volume of 225 μL PMED buffer containing 5.1 μm [^{3}H]FPP (sp. act. 270 dpm/pmol), 4% DMSO, the indicated inhibitor concentrations, and the remaining SQS substrates and cofactors as described under Experimental Procedures. After incubation, reactions were terminated by addition of 120 μL of 10 m NaOH. Aliquots, 115 μL, of terminated reaction mixtures were apportioned to 500-μL microfuge tubes for assessing squalene formation (overall reaction). The remaining 230 μl of each reaction mixture was diluted to 250 μL with water and assessed for proton release (first half-reaction). Shown are the SQS first half-reaction (○) and overall reaction (●) activities, expressed respectively, as pmol tritium released and pmol squalene formed from [1-^{3}H]FPP per min per mg protein, as a function of inhibitor concentration.

NSRRs and induces compensatory increases in HMGR activity (e.g., 17, 38, 39). Inhibition of SQS activity should also limit sterol formation, but should not alter NSRR production if the NSRR is formed through FPP metabolism independent of the action of SQS, and thus should not induce HMGR activity. Conversely, if the NSRR is produced from FPP metabolism that is dependent on the action of SQS (e.g., PSQPP, squalene or their metabolites), NSRR production should be reduced by SQS inhibition and

TABLE 2. Specificity of SQS inhibitors for SQS inhibition relative to PFT inhibition

Compound	Squalene Synthetase Inhibition (IC_{50})	Protein Farnesyl-transferase Inhibition (IC_{50})	Specificity (SQS IC_{50}/PFT IC_{50})
	μM	μM	
P-3622	0.7	100	143
CP-210172	0.9	150	167
CP-295697	0.007	13	1860
CP-294838	0.13	52	400
Zaragozic acid A	0.002	3	1500

IC_{50} values for SQS inhibition and PFT inhibition by P-3622, CP-210172, CP-295697, and CP-294838 are based on the concentration-dependency profiles shown in Fig. 2. The data for SQS inhibition and PFT inhibition by ZGA are that of Harwood (30).

TABLE 3. Inhibition of cholesterol synthesis in cultured cells

Compound	Inhibition of [^{14}C]acetate Incorporation into Cholesterol (IC_{50})			
	HepG2 Cells	Primary Rat Hepatocytes	CaCo-2 Cells	IM9 Cells
		μM		
Mevinolin	0.02	—	—	0.01
P-3622	0.4	0.9	1.4	1.0
CP-210172	2.3	—	—	3.8
CP-295697	20	0.05	20	30
CP-294838	10	9	2.0	1.4
Zaragozic acid A	20	0.02	>30	30

HepG2 cells, CaCo-2 cells, IM-9 cells, and rat primary hepatocytes, seeded and maintained in culture as described under Experimental Procedures, received 1.0 mL of their respective fresh medium containing either 1% DMSO (HepG2 cells, rat hepatocytes) or 0.4% DMSO (IM-9 cells, CaCo-2 cells) ± the indicated inhibitor concentrations. Immediately after media additions, 25 μl of respective media containing 4 μCi of [2-^{14}C]acetate was added to each well or culture tube. After incubation at 37°C for 6 h with gentle shaking, samples were saponified, the nonsaponifyable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated as described under Experimental Procedures.

TABLE 4. Specificity of SQS inhibitors for SQS inhibition versus post-squalene cholesterolgenic enzyme inhibition

Compound	Concentration	Cholesterol Synthesis Inhibition	Distribution of Sterols				
			Cholesterol	Lanosterol	Squalene dioxide	Squalene oxide	Squalene
		μM	%		% of control cholesterol		
Hep-G2 cells							
Control	0	0	100	0	2	0	1
CP-295697	60	59	41	0	0	0	1
	200	87	13	0	0	0	0
CP-294838	30	68	32	4	2	0	0
	100	87	13	0	2	0	0
P-3622	0.3	58	42	1	4	5	2
	1	74	26	0	9	8	0
CP-210172	3	67	33	3	5	7	0
	10	83	17	0	11	11	0
ZGA	20	42	58	0	0	0	1
	200	92	8	0	0	0	2
Ketoconazole	0.5	69	31	45	0	0	0
UK-124617	10	89	11	4	18	27	0
IM-9 cells							
Control	0	0	100	2	0	1	0
CP-295697	20	50	50	0	0	3	0
	200	83	17	2	4	8	0
CP-294838	10	74	26	0	1	2	0
	30	90	11	0	0	3	0
P-3622	1	52	33	1	0	2	0
	10	91	9	0	11	9	0
CP-210172	3	49	51	5	6	13	0
	30	88	12	0	19	44	0
ZGA	30	43	57	1	1	1	0
	60	82	19	2	2	2	0

Cells seeded and maintained in culture as described under Experimental Procedures were administered 1.0 mL of fresh medium containing 1% (HepG2 cells) or 0.4% (IM-9 cells) DMSO ± the indicated inhibitor concentrations. Immediately after media additions, 25 μl of medium containing 4 μCi of [^{14}C]acetate was added to each well or culture tube. After incubation for 6 h at 37°C with gentle shaking, samples were saponified, and the nonsaponifiable lipids were extracted with hexane, separated by silica gel TLC, and quantitated as described under Experimental Procedures. Radioactivity incorporated into cholesterol (R_f = 0.27) and the cholesterol precursors lanosterol (R_f = 0.34), squalene dioxide (R_f = 0.42), squalene oxide (R_f = 0.65), and squalene (R_f = 0.88) is expressed as a percentage of the radioactivity incorporated into cholesterol by control cells.

compensatory induction of HMGR, similar to that noted for the HMGR inhibitors, should be observed.

To assess the effect of cholesterol synthesis inhibition via HMGR or SQS inhibition on induction of HMGR activity in IM-9 cells, evaluation was conducted in two stages. First, inhibition of cholesterolgenesis was measured in an aliquot of cells immediately after compound addition. Compensatory increases in HMGR activity induced by cholesterolgenesis inhibition were then assessed 24 h after compound addition in the remainder of the suspension culture. This two-stage strategy was used to assure that assessment of the degree of cholesterolgenesis inhibition required to induce HMGR was not compromised by the ensuing HMGR induction, as would be the case if both measurements were made simultaneously after 24 h incubation with compound.

As shown in **Fig. 6**, the HMG-CoA reductase inhibitor, mevinolin, produced the predicted increases in HMG-CoA reductase activity that were dose-dependently related to the degree of reduction in cholesterol synthesis. A 4-fold increase in HMG-CoA reductase activity was noted at concentrations of mevinolin that produced 50% inhibition of cholesterol synthesis, with 9-fold and 17-fold increases in enzymatic activity noted after 75% and 90% inhibition of cholesterolgenesis, respectively (Fig. 6).

Inhibition of cholesterol synthesis in IM-9 cells by P-3622, CP-210172, CP-295697, and CP-294838 without compensatory increases in HMGR activity

In contrast to increases in HMGR activity that occur in response to treatment with mevinolin (Fig. 6), the reversible competitive first half-reaction cationic SQS inhibitors P-3622 and CP-210172 did not increase HMGR activity even at concentrations that produced greater than 90% inhibition of cholesterol synthesis (**Fig. 7**, top panels). Similarly, the reversible competitive first half-reaction anionic SQS inhibitors CP-295697 and CP-294838 did not increase HMGR activity at concentrations that produced up to 90% inhibition of cholesterol synthesis, and exhibited only modest increases in HMGR activity at concentrations that inhibited

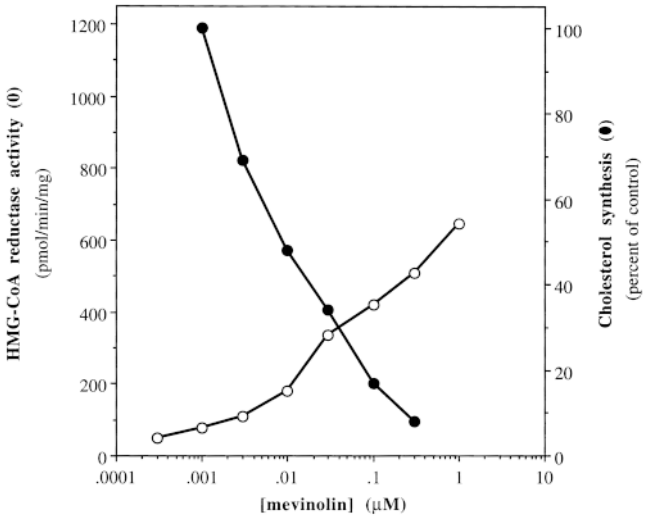

Fig. 6. Cholesterol synthesis inhibition by mevinolin induces compensatory increases in HMGR activity. IM-9 cells maintained in culture as described under Experimental Procedures were resuspended to a density of 10^6 cells/mL in fresh medium and apportioned to 50-mL plastic culture tubes in 20-mL aliquots. To each tube was added 40 μL of DMSO ± the indicated concentrations of mevinolin. Cell suspensions were incubated for 1 h at 37°C with gentle shaking, then 1-mL aliquots were removed, placed in 12 × 75-mm plastic culture tubes together with 25 μL of medium containing 2 μCi of [^{14}C]acetate, and incubated with gentle shaking for 6 h at 37°C. After incubation, samples were saponified, the nonsaponifiable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated as described under Experimental Procedures. The remaining 19 mL of each cell suspension was incubated at 37°C for an additional 23 h, at which time cells were harvested, microsomes were isolated, and HMGR activity was determined as described under Experimental Procedures. Shown are the averages of duplicate determinations of cholesterol synthesis (●) and HMGR activity (○) as a function of mevinolin concentration.

cholesterolgenesis by greater than 90% (Fig. 7, bottom panels). Similar results were also obtained for CP-216011, a member of the substituted quinuclidine class of cationic SQS inhibitors, and CP-312279, CP-320524, CP-320628, and CP-340868, members of the substituted benzoxazepinone class of monoanionic SQS inhibitors (not shown).

Taken together, these observations are consistent with maintenance of post-transcriptional regulatory control of HMGR activity after cholesterol synthesis inhibition via SQS inhibition, strongly suggesting that the NSRR is neither PSQPP, squalene, nor their metabolites, but rather must be generated from FPP through pathways that are independent of the action of SQS. These results also indicate that this effect is a mechanistic effect rather than a compound or class-specific effect as multiple representatives of all four classes of structurally distinct SQS inhibitors produce similar effects.

Inhibition of cholesterol synthesis in IM-9 cells by ZGA leads to greater compensatory increases in HMGR activity than does mevinolin

In contrast to the results noted for all of the reversible competitive first half-reaction SQS inhibitors evaluated, the SQS mechanism-based irreversible inactivator, ZGA, markedly induced HMGR activity (**Fig. 8**), exhibiting an even greater induction of HMGR than mevinolin at similar levels of cholesterol synthesis inhibition (Fig. 6). Indeed, ZGA induced 5-fold, 11-fold, and 40-fold increases in HMGR activity at concentrations that produced 25%, 50%, and 75% inhibition of cholesterol synthesis, respectively (Fig. 8), whereas mevinolin did not significantly induce HMGR activity at concentrations that inhibited cholesterol synthesis by 25% and produced only 4-fold, 9-fold, and 17-fold increases in HMGR activity at concentrations that yielded 50%, 76%, and 90% inhibition of cholesterol synthesis, respectively (Fig. 6). These observations suggest that ZGA may directly interfere with either the production or action of the NSRR (see below).

DISCUSSION

Previous studies have demonstrated that nonsterol products of mevalonate metabolism are important post-transcriptional regulators of HMGR activity, functioning both as translational repressors and as stimulators of protein degradation (3, 11–18). The majority of these studies have concluded that farnesol, derived from FPP hydrolysis, or metabolites of farnesol are likely NSRR candidates (10, 11, 19, 21, 22). However, several studies have questioned the importance of farnesol in HMGR regulation or have implicated the stable SQS reaction intermediate, PSQPP, or its metabolites as the true NSRR (18, 20, 23).

In this report we have demonstrated that, unlike direct HMGR inhibition, which induces compensatory increases in HMGR activity after >25% inhibition of cholesterolgenesis, SQS first half-reaction inhibition produces only minimal increases in HMGR activity at concentrations that inhibit cholesterolgenesis by up to 90%, suggesting *1*) that normal post-transcriptional HMGR regulation is maintained after SQS first half-reaction inhibition, presumably through continued NSRR formation, and *2*) that NSRR formation occurs prior to the action of SQS, thus precluding PSQPP and its metabolites as potential NSRRs. These observations are thus consistent with farnesol or one of its metabolites as the true NSRR.

Much evidence in the literature supports the role of farnesol or farnesol metabolites as putative NSRRs. First, accelerated HMGR degradation can be induced in permeabilized CHO (20, 22) and met-18b-2 (18, 19) cells by administration of mevalonate (18, 20), FPP (19, 20) or farnesol (19, 22), but not by a nonhydrolyzable FPP analog (20, 22), suggesting that FPP action is due to its hydrolysis to farnesol. Second, FPP-mediated HMGR degradation can be prevented by FPPase inhibition (19, 22), and this inhibition can be overcome by addition of farnesol (22), further suggesting a requirement for FPP hydrolysis for activity. Third, a lag of 1–2 h before induction of HMGR degradation in permeabilized met-18b-2 cells by FPP (19) or in intact cells by mevalonate (18), but not by farnesol (19) suggests a more direct role for farnesol. Fourth, the reduction in HMGR half-life induced by farnesol in CHO

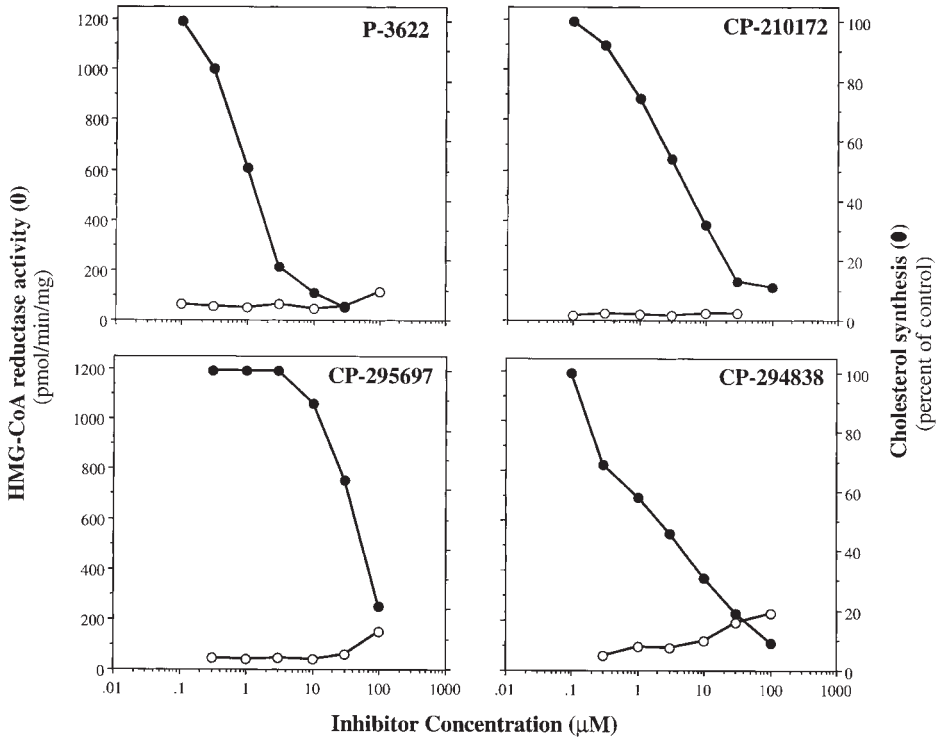

Fig. 7. Cholesterol synthesis inhibition by P-3622, CP-210172, CP-295697, and CP-294838 does not lead to compensatory increases in HMGR activity. IM-9 cells maintained in culture as described under Experimental Procedures were resuspended to a density of 10^6 cells/mL in fresh medium and apportioned to 50-mL plastic culture tubes in 20-mL aliquots. To each tube was added 40 μL of DMSO ± the indicated inhibitor concentrations. Cell suspensions were incubated for 1 h at 37°C with gentle shaking, then 1-mL aliquots were removed, placed in 12 × 75-mm plastic culture tubes together with 25 μL medium containing 2 μCi of [^{14}C]acetate and incubated with gentle shaking for 6 h at 37°C. After incubation, samples were saponified, the nonsaponifiable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated as described under Experimental Procedures. The remaining 19 mL of each cell suspension was incubated at 37°C for an additional 23 h, at which time cells were harvested, microsomes were isolated, and HMGR activity was determined as described under Experimental Procedures. Shown are the averages of duplicate determinations of cholesterol synthesis (●) and HMGR activity (○) as a function of inhibitor concentration.

and met-18b-2 cells is similar in magnitude to that noted for mevalonate under identical experimental conditions (19, 21). Fifth, compounds that inhibit mevalonate-mediated HMGR degradation (e.g., the protease inhibitor ALNN; the endoplasmic reticulum Ca^{++}ATPase inhibitor thapsigargin) also prevent farnesol-mediated HMGR degradation in CHO cells (21). Sixth, SQS-deficient CHO cells, which exhibit impaired mevalonate-induced HMGR degradation in the absence of added 25-hydroxycholesterol (21, 40), also demonstrate impaired farnesol-induced HMGR degradation in the absence of added 25-hydroxycholesterol, but show synergistic effects in combination with 25-hydroxycholesterol (21). Seventh, intracellular farnesol levels were increased after mevalonate addition and reduced after HMGR inhibitor addition to CHO cells (21), conditions that respectively increase and reduce HMGR degradation in these cells (17, 21). Finally, addition of farnesol, farnesyl acetate, or ethyl farnesyl ether to CHO cells (11, 21), tocotrienols (farnesylbenzopyrans) to Hep-G2 cells (41), or farnesol or geranylgeranol to permeabilized met-18b-2 cells (19) all reduce HMGR translation and/or stimulate HMGR degradation, whereas geraniol, geranyl acetate, 3-methyl-buten-1-ol, geranylbenzopyran, geranylgeranylbenzopyran, phytylbenzopyran, geranyl pyrophosphate, GGPP, squalene, all-*cis*-farnesol, and nerolidol are without effect (11, 19, 41). Together, these observations provide compelling evidence that farnesol is a key component in the post-transcriptional control of HMGR translation and degradation.

It should be noted, however, that in at least one study (23), increases in hepatic farnesol levels after mevalonate or farnesol administration to rats did not result in accelerated HMGR degradation. While this observation appears not to be consistent with farnesol as the NSRR, it is conceivable that farnesol levels in control animals are sufficient to saturate NSRR-mediated regulatory responses (42) such that further increases (e.g., by mevalonate or farnesol administration) would not further enhance HMGR degradation. Consistent with this suggestion are the observations that in animals pretreated with high doses of mevinolin, to deplete endogenous NSRRs, farnesol levels are reduced and accelerated HMGR degradation

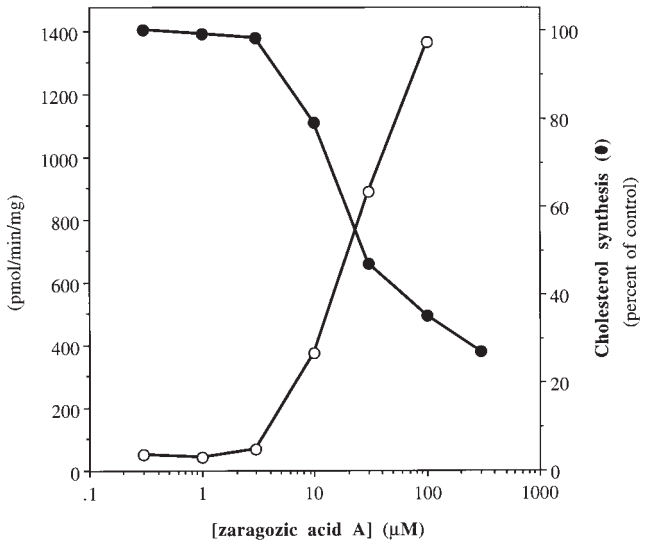

Fig. 8. Cholesterol synthesis inhibition by ZGA leads to greater compensatory increases in HMGR activity than does mevinolin. IM-9 cells maintained in culture as described under Experimental Procedures were resuspended to a density of 10^6 cells/mL in fresh medium and apportioned to 50-mL plastic culture tubes in 20-mL aliquots. To each tube was added 40 μL of DMSO ± the indicated concentrations of ZGA. Cell suspensions were incubated for 1 h at 37°C with gentle shaking, then 1-mL aliquots were removed, placed in 12 × 75-mm plastic culture tubes together with 25 μL of medium containing 2 μCi of [^{14}C]acetate and incubated with gentle shaking for 6 h at 37°C. After incubation, samples were saponified, the nonsaponifiable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated as described under Experimental Procedures. The remaining 19 mL of each cell suspension was incubated at 37°C for an additional 23 h, at which time cells were harvested, microsomes were isolated, and HMGR activity was determined as described under Experimental Procedures. Shown are the averages of duplicate determinations of cholesterol synthesis (●) and HMGR activity (○) as a function of ZGA concentration.

is prevented (43), but can be restored by subsequent farnesol administration (10). Indeed, many of the studies described above that demonstrate farnesol-mediated control of HMGR in cultured cells were conducted after such pretreatment (11, 12, 18–22, 44). Thus, under physiological conditions, NSRR levels may be near saturating and pathways leading to post-transcriptional HMGR regulation may be maximally activated. In this regard, it is noteworthy that HMGR activity was not reduced to below baseline after SQS inhibition in our studies in which cells were not pretreated to deplete endogenous NSRRs.

Although the above observations indicate that farnesol is a key component in the post-transcriptional control of HMGR activity, whether farnesol per se or one of its metabolites is the true NSRR remains to be determined. In this regard, it has been suggested that the true NSRR is a prenylated protein (2, 24). This possibility is unlikely for the following reasons. *1*) The lag in induction of HMGR degradation in cultured cells by FPP but not farnesol (19) is inconsistent with a need to convert farnesol to FPP prior to its use in protein prenylation reactions. *2*) The ability of FPPase inhibitors to prevent the NSRR-like actions of FPP (19, 22), when FPP and GGPP levels would be expected to rise with such treatment, is also inconsistent with a role for protein prenylation in NSRR formation. *3*) The nonhydrolyzable farnesol analogs ethyl farnesyl ether and the tocotrienols cannot be converted to FPP and GGPP and utilized in protein prenylation reactions, but can induce both HMGR degradation and translational repression in cells (11). *4*) The PFT inhibitor BZA-5B cannot prevent mevalonate-mediated HMGR degradation in CHO cells even at concentrations (22) that completely block protein farnesylation in that cell line (45). *5*) Geranylgeraniol can but GGPP cannot repress HMGR translation or induce HMGR degradation in cultured cells (19) even though only the latter can be utilized directly in protein prenylation reactions.

While it is not known how farnesol or one of its metabolites signals for the translational repression and accelerated degradation of HMGR, a variety of possible mechanisms exist. For example, farnesol or a farnesol metabolite could intercalate into the endoplasmic reticulum membrane and have direct effects on HMGR to render it more susceptible to degradation (19). This possibility appears unlikely, however, as neither geraniol, geranyl acetate, all-*cis*-farnesol, nerolidol, prenylbenzopyrans, nor nonhydrolyzable FPP analogs enhanced HMGR degradation (11, 19, 22, 41), all of which would be expected to exhibit effects similar to farnesol if the actions of farnesol were simply due to nonspecific membrane intercalating properties. Indeed, the size specificity of the various farnesol analogs and the stereochemical specificity of the farnesol isomers are consistent with specific binding to a protein possessing a farnesoid binding site that is highly selective for all-*trans*-farnesol.

Recent reports, however, have implicated a heat-stable, dialyzable farnesol metabolite in the regulation of choline phosphotransferase activity in CEM-C1 cells (46), suggesting that this metabolite may alter choline metabolism and phospholipid biosynthesis, and thereby alter membrane fluidity, potentially rendering HMGR more susceptible to degradation. The ability of farnesyl acetate, farnesyl bromide, farnesyl methyl ether, and nerolidol to mimic the actions of farnesol in inhibiting choline incorporation into cellular lipids (46), whereas gerainol, 3-methylbut-2-en-1-ol, juvenile hormone, and squalene are all without significant effect (46), is in close agreement with the analog specificity noted for NSRR-mediated control of HMGR (see above) and supports this suggestion. Whether these actions play an important role in the farnesol-mediated control of HMGR, however, remains to be determined.

Alternatively, farnesol or a farnesol metabolite could exert an effect on the activity or expression of a protease, protease effector protein, or HMGR binding protein that facilitates HMGR degradation. Indeed, regulated HMGR degradation has been shown to require production of a short-lived protein (17, 47). However, farnesol reduces HMGR translational efficiency in addition to inducing its degradation, and these actions occur or do not occur in parallel for all farnesol analogs evaluated (11), suggesting

a common mechanism. Such a protease activation mechanism for regulating HMGR degradation would necessitate a second but coupled mechanism for exerting translational control.

In this regard, farnesol or a farnesol metabolite could associate with a specific regulatory protein that directly or indirectly affects HMGR translational efficiency and at the same time induces a protease, protease effector protein or HMGR binding protein that facilitates HMGR degradation. Indeed, recent studies have identified an orphan nuclear receptor, termed the farnesoid X-activated receptor (FXR) which is activated by farnesol, juvenile hormone III, and closely related isoprenoids (48). FXR possesses structural and functional similarities to the ecdysone receptor of insects, exhibiting similar heterodimer formation with RXR and similar activation by various farnesoid ligands (48). Interestingly, FXR is expressed in tissues known to possess significant flux through the mevalonate pathway (48), and exhibits a farnesoid specificity for activation (e.g., strongly activated by all-*trans*-farnesol, weakly activated by farnesal, farnesyl acetate, and geranylgeraniol, not activated by geraniol, farnesoic acid, squalene, or post-squalene cholesterolgenic intermediates; 48) that shows a striking similarity to the farnesoid specificity for NSRR-mediated HMGR regulation (see above). A more recent study has also indicated that all-*trans*-farnesol and related farnesoids can induce transcriptional events associated with PPARα activation (49), with farnesoic acid exhibiting approximately twice the potency of farnesol (49). Whether farnesoids induce regulatory signals controlling both HMGR translation and degradation through activation of either FXR or PPARα remains an exciting possibility that warrants further evaluation.

The unexpected observation that ZGA produces greater increases in HMGR activity than mevinolin at equivalent degrees of cholesterolgenesis inhibition has afforded both additional insight into the nature of the NSRR and also an explanation for the controversy in the literature associated with establishment of farnesol as the NSRR. Indeed, these observations suggest that ZGA directly interferes with NSRR-mediated control of HMGR. Thus, whereas induction of HMGR activity by mevinolin is self-limiting and occurs only to the extent that it returns NSRR production to pretreatment levels, HMGR induction by ZGA cannot normalize NSRR action and hence HMGR activity is maximally induced. Such uncontrolled HMGR induction, in combination with blocked SQS activity, is presumably responsible for the substantial accumulation of farnesol and its metabolites reported by Bergstrom et al. (28) and Bostedor et al. (50) and others (23, 51, 52) after administration of ZGA to experimental animals.

Further support for a direct interference by ZGA of NSRR-mediated control of HMGR activity can be inferred from studies in cultured cells (18, 20, 44) and in experimental animals (53) in which HMGR degradation is decreased and HMGR production is increased after ZGA administration, even though farnesol levels are vastly elevated (23, 28, 51, 52). In addition, the inability of mevalonate to decrease ZGA-induced increases in HMGR in animals (42) or to restore rapid HMGR degradation in ZGA-treated cells (18) is also consistent with direct interference of NSRR-mediated HMGR regulation by ZGA.

Several mechanisms exist whereby ZGA could interfere with NSRR-mediated control of HMGR. First, this effect could be due to irreversible SQS inhibition, inducing a greater block in cholesterol formation, leading to transcriptional derepression that overrides maintenance of NSRR-mediated translational and degradational control. This is unlikely, however, as HMGR induction by such a mechanism should not be greater than that noted with mevinolin, should not be noted at levels of cholesterolgenesis inhibition for which mevinolin does not induce HMGR, and should not be observed until cholesterolgenesis inhibition severely limits sterol availability. Indeed, observations similar to those noted for the squalene epoxidase inhibitor, NB-598 (16, 18, 20), and for SQS inhibitors that induce HMGR activity only when cholesterol synthesis is inhibited by >90% (this report), would be anticipated if the actions of ZGA were simply a consequence of irreversible SQS inhibition.

Second, ZGA could interact with HMGR in the endoplasmic reticulum membrane to render it less susceptible to NSRR-mediated degradation. However, as other farnesoid mimics that would be expected to exhibit similar nonspecific effects do not antagonize NSRR action (see above), it is unlikely that an effect of ZGA at the endoplasmic reticulum membrane is responsible for this antagonism.

Third, ZGA could directly interfere with production of the NSRR. Such a mechanism is attractive as it would not only explain the marked induction of HMGR activity at low levels of cholesterol synthesis inhibition, but would also explain the greater induction of HMGR activity by ZGA than by mevinolin. Direct interference with production of the NSRR would also explain the disparate observations *1*) that ZGA blocks accelerated HMGR degradation and induces HMGR formation while at the same time producing vast quantities farnesol and farnesoic acids, in both cultured cells (18, 20, 44) and experimental animals (53), and *2*) that mevalonate is unable to reverse the ZGA-induced loss of NSRR-mediated HMGR regulation in cultured cells (18) and experimental animals (42). However, as farnesol levels are markedly increased after treatment with ZGA in both cultured cells (52) and in experimental animals (23, 28, 50, 51), such a mechanism would preclude farnesol per se as the NSRR, and would imply that the NSRR is a farnesol metabolite whose production is prevented by ZGA.

Fourth, ZGA could interfere with NSRR action by directly interacting with the enzymes, receptors, or regulatory proteins proposed above as potential facilitators of NSRR-mediated HMGR regulation. Indeed the similarity between the specificities with which farnesyl mimics elicit their NSRR action (11, 19) and their choline phosphotransferase inhibition (46) or nuclear receptor activation (48, 49), together with the structural similarity between ZGA and the putative NSRRs is supportive of this possibility.

In conclusion, the results of these studies have provided further evidence that the NSRR is either farnesol or a farnesol metabolite. The results of these studies have also de-

fined actions of ZGA that are distinct from those of other SQS inhibitors, suggesting possible side-mechanisms of action for ZGA that might be useful as tools for identifying both the farnesol metabolite that functions as the NSRR and the protein(s) through which the NSRR exerts its effects. Finally, recent studies in marmosets have suggested a greater cholesterol-lowering efficacy for SQS inhibitors than HMGR inhibitors (54, 55). Whether these differences are a consequence of diminished induction of HMGR after SQS inhibition or are due to other factors (e.g., pharmacokinetic differences, etc.) remains to be more thoroughly evaluated, but should be conducted using SQS inhibitors other than ZGA.

We thank Dr. Robert J. Aiello for his guidance in the preparation and maintenance of primary rat hepatocytes, Ms. Faan Wen Bangerter for supplying the CaCo-2 cells used in these studies, Dr. Chris A. Hitchcock for supplying the squalene epoxide cyclase inhibitor, UK-124617, used in these studies, and Dr. Takushi Kaneko and coworkers for preparing the ZGA, Dr. Ernest S. Hamanaka and coworkers for preparing the CP-210172 and CP-294838, and Dr. Roger B. Ruggeri and coworkers for preparing the CP-295697 used in these studies.

Manuscript received 16 February 1998 and in revised form 18 August 1998.

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