Cholesterol synthesis inhibition distal to squalene upregulates biliary phospholipid secretion and counteracts cholelithiasis in the genetically prone C57L/J mouse

G A Clarke, G Bouchard, B Paigen, M C Carey

Background and aims: Newly synthesised cholesterol contributes poorly to biliary lipid secretion but may assume greater importance when the rate limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is upregulated. As this occurs in the gall stone susceptible C57L/J inbred mouse, we employed two cholesterol biosynthesis inhibitors, Tu 2208 and Ro 48-8071, potent inhibitors of squalene epoxidase and oxidosqualene-lanosterol cycloase, respectively, to assess their potential in preventing cholesterol cholelithiasis in the C57L/J mouse strain. Mice were fed a lithogenic diet comprising a balanced nutrient intake with 15% dairy fat, 1% cholesterol, and 0.5% cholic acid added.

Methods: We determined gall stone phenotype, HMGR activity, biliary lipid secretion rates, and counterregulatory events in male C57L/J mice and gall stone resistant AKR treated with Tu 2208 (30–60 mg/kg/day) or Ro 48-8071 (30–100 mg/kg/day), while ingesting chow or the lithogenic diet.

Results: Both agents reduced the gall stone prevalence rate from 73% to 17% in C57L/J mice, inhibited HMGR activity, and decreased hepatic cholesterol concentrations without appreciably influencing biliary cholesterol secretion. In C57L as well as AKR mice, both agents increased biliary phospholipid (which is mostly phosphatidylcholine) secretion rates and at the highest doses effectively reduced the biliary cholesterol saturation index.

Conclusions: Cholesterol biosynthesis inhibitors acting distally to squalene do not reduce biliary cholesterol secretion rates despite reductions in cholesterol biosynthesis and hepatocellular levels. However, they effectively prevent gall stone formation through stimulation of pathways that lead to enhanced biliary phospholipid secretion.

D e novo biosynthesis, plasma lipoproteins, and dietary intake plus resorbed biliary cholesterol as chylomicron remnants, comprise the sources of hepatic cholesterol molecules for biliary secretion.1 Cholesterol that is taken up or newly synthesised by the hepatocyte may be secreted directly into bile, esterified by acyl coenzyme A: cholesterol O-acyltransferase 2,2 or used as substrate for bile salt progenesis, or newly synthesised by the hepatocyte may be secreted as the predominant substrate for bile salt transport.3 The preponderance of current evidence points towards diet as the principal source of biliary cholesterol and between basal and cholesterol hypersecretory—that is, lithogenic states.

Because of the complexity of hepatic metabolism of cholesterol and the consequent difficulties in its quantitation, the respective contributions of each cholesterol source to biliary secretion remain unsettled. Moreover, these contributions may differ between laboratory animals and humans and between basal and cholelithogenic hypersecretory—that is, lithogenic states.

In previous work, we demonstrated relative hypersecretion of biliary cholesterol compared with the other biliary lipids in the gall stone susceptible C57L/J mouse.4 Accordingly, when fed a lithogenic diet high in cholesterol and cholic acid, C57L/J mice exhibit an approximately 80% gall stone prevalence rate.5 Moreover, compared with gall stone resistant mouse strains such as AKR/J, C57L/J mice exhibit an apparent increase in hepatic uptake of high density lipoprotein cholesterol,6 augmentation of intracellular cholesterol transport,7 and failure to downregulate cholesterol biosynthesis.8 All of these factors could promote biliary cholesterol hypersecretion and lithogenicity. Despite the theoretical effect of reducing de novo cholesterol synthesis, which might affect biliary cholesterol secretion by depleting hepatic cholesterol stores,9–15 it has been impossible hitherto to test the physiological impact of inhibiting cholesterol biosynthesis in mice. This is occasioned because rodents respond to “statins”, the benchmark class of cholesterol synthesis inhibitors, in a paradoxical manner by increasing transcription of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) to such an extent that overall cholesterol biosynthesis is actually increased.16–18 In fact, in hamsters, statins may induce lithogenic bile and gall stones.19 Furthermore, statins also inhibit formation of isoprenoid precursors, which are important products of the cholesterol synthesis pathway with protean physiological roles (fig 1).

For these reasons we explored Tu 2208 (Yoshitomi Pharmaceuticals Ltd, Osaka, Japan), an inhibitor of squalene epoxidase (fig 1), as inferred from the properties of its antecedent analogue, Tu 2708,20 and Ro 48-8071 (F Hoffmann-La Roche Ltd, Basel, Switzerland), an inhibitor of oxidosqualene-lanosterol cycloase,21 shown to effectively suppress de novo cholesterol synthesis. In contrast with the statins, these agents inhibit enzymes that act on substrates formed distally to the synthesis of isoprenoid precursors (fig 1). By administering these cholesterol synthesis inhibitors

Abbreviations: LD, lithogenic diet; Mdr2, multidrug resistance protein, isoform 2 (where italicised in lowercase indicates the corresponding murine gene); bsep, bile salt export protein; ABC, ATP binding cassette (class and number are suffixed for official designations—that is, B4 and B11 for mdr2 and bsep, respectively); Lif, murine cholesterol gall stone alleles; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SREBP, sterol regulatory element binding proteins; PC, phosphatidylcholine (biliary phospholipid); CSI, cholesterol saturation index
to C57L gall stone prone mice, we tested the hypotheses that decreasing de novo biosynthesised cholesterol would influence biliary lithogenicity and cholesterol gall stone prevalence rates. We found that neither agent reduced biliary cholesterol secretion despite depletion of hepatic cholesterol stores and inhibition of HMGR. None the less, both agents caused bile to become desaturated with cholesterol and prevented gall stone formation, principally by increasing biliary phospholipid secretion rates. These findings indicate that cholesterol biosynthesis inhibitors acting distally to HMGR can prevent cholesterol gall stone formation but this effect is achieved by upregulating biliary phospholipid secretion most likely through counterregulatory mechanisms.

**MATERIALS AND METHODS**

Tu 2208 was a gift of Dr T Yamauchi (Yoshitomi Pharmaceuticals) and Ro 48-8071 was a gift of Dr Olivier Morand (F Hoffmann-La Roche, Ltd). Pure crystalline simvastatin was received from Merck, Sharp and Dohme (Ireland, Ltd). Protein dye binding substrates and standards were obtained from Biorad Inc. (Hercules, California, USA). Precast polyacrylamide gels, sample, running and transfer buffers were obtained from Novex-Invitrogen (Carlsbad, California, USA). The primary monoclonal antibody, MC204, recognising murine multidrug resistance protein, isoform 2 (Mcdr2), was obtained from Kamiya Biomedical Company (Seattle, Washington, USA). A polyclonal antibody raised against the C terminus of rat bile salt export protein (Bsep) was a generous gift of Dr Bruno Stieger (University Hospital, Zurich, Switzerland). Antibodies were detected using appropriate horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) with a chemiluminescence kit (New England Nuclear Inc., Boston, Massachusetts, USA). A "Quantity One" densitometer (Biorad Inc.) was used to measure expression density.

**Inbred mice**

Six week old male C57L and AKR mice were supplied by the Jackson Laboratory (Bar Harbor, Maine, USA). They were acclimatised for two weeks in the animal research facility of Harvard Medical School and maintained under normal 12 hour alternating light-dark conditions, with water available ad libitum. All experiments were executed according to the accepted criteria for the care and experimental use of laboratory animals, and euthanasia was consistent with recommendations of the American Veterinary Medical Association. All protocols were approved by the Institutional Animal Care and Use Committee of Harvard University.

**Drug administration and dosage**

Previously, we had quantified the daily weight consumption of the lithogenic diet (LD) (prepared in the diet kitchen of the Jackson Laboratory) by C57L and AKR inbred mouse strains and thereby calculated the concentrations of drug necessary to compound with the daily feeds. Chow and LDs (plus drug at each dosage level) were compounded in the diet kitchen of the Jackson Laboratory. Eight week old male C57L mice were fed LD plus Tu 2208 30 mg/kg/day (group Tu 30, n = 14) and 60 mg/kg/day (group Tu 60, n = 6), Ro 48-8071 30 mg/kg/day (group Ro 30, n = 15), 60 mg/kg/day (group Ro 60, n = 6), and 100 mg/kg/day (group Ro 100, n = 6). Contemporaneous controls (n = 12) were fed LD alone or LD plus simvastatin 10 mg/kg/day. AKR mice were fed LD alone (n = 11) or LD plus simvastatin (n = 5). While on diet, animals were sacrificed at 56 days using pentobarbital sodium 4.5 mg/100 g body weight for anaesthesia (Nembutal; Abbott Laboratory, Chicago, Illinois, USA).
Gall bladder phenotypes, and collection of hepatic bile and blood
To determine gall stone prevalence, an upper midline laparotomy was performed following an overnight fast, the cystic duct was ligated and transected, and a cholecystectomy performed. The liver was removed immediately, weighed, snap frozen in liquid N₂, and stored at −70°C. Gall bladder volume was determined by water displacement,24 and polarising microscopy was performed on bile that was digitally expressed from the perforated fundus of each gall bladder (n = 5–15) to determine the presence of mature stones, sandy stones, crystals, liquid crystals, and mucin gel, as described by Wang and colleagues.2 Blood was aspirated from the left ventricle, plasma prepared, and stored at −70°C.

Biliary lipid secretion
In a separate study, eight week old male C57L mice were fed LD plus Tu 60 mg/kg/day (group Tu 60), Ro 30, 60, or 100 mg/kg/day (group Ro 30, 60, or 100, respectively), or LD plus simvastatin 10 mg/kg/day (group simva); control animals (given no drug) were fed LD alone. To obviate the possibility that observed changes in biliary lipid secretion might be idiosyncratic to mutant murine cholesterol gall stone alleles (Lith alleles), AKR controls were also employed and fed LD alone, LD plus simvastatin 10 mg/kg/day, and LD plus Ro 100 mg/kg/day. Mice were anaesthetised as described above, after 14 days on diet. The cystic and distal common bile ducts were ligated after midline laparotomy. An oblique cut was made in the mid common bile duct using a microscissors, permitting cannulation with a polyethylene catheter of 0.61 mm external diameter (Beckton Dickinson Inc., Sparks, Maryland, USA), which was then sutured in position. Hepatic bile was collected for one hour into a tared container, with the animal’s temperature maintained at 37°C under a heating lamp. Six animals were included in each group but in a small number, bile duct cannulation was not possible and hence the adjusted n values are detailed in the results section.

Analyses
Total plasma cholesterol was measured using standard enzymatic techniques.22 Biliary phospholipid compositions were determined using the Bartlett assay.24 We verified that the Bartlett assay was reporting phospholipid as opposed to other phosphorylated compounds by positively correlating its results with a commercially available cholamine assay (Wako Chemicals USA, Inc., Richmond, Virginia, USA). Bile salts were assayed using the 3α-hydroxysteroid dehydrogenase assay,24 and biliary cholesterol, as well as biliary steroid molecular species, by high performance liquid chromatography.25 Cholesterol saturation indices (CSIs) were determined using critical tables26 derived for taurocholate, the principal murine bile salt on the LD. As it has been shown that HMGR activity exhibits a positive correlation with the absolute rate of hepatic cholesterol biosynthesis, as measured by incorporation of [14C]-octanoate into sterols,27 we assayed HMGR activity ex vivo in the presence of [14C]-HMG-CoA, glucose 6-phosphate, NADP, and glucose 6-phosphatase using hepatic microsome fractions of standardised protein concentration according to the methods described by Doerner and colleagues.27 Protein concentrations were determined using the Bradford assay.28 Following homogenisation and lipid extraction using standard techniques, total hepatic cholesterol was determined by a cholesterol oxidation/esterase assay (Sigma Chemical Co., St Louis, Missouri, USA), as described previously.11 Unesterified cholesterol was measured by high performance liquid chromatography.25 The results are expressed in mg per gram of wet hepatic tissue.

Immunoblotting of membrane transporters
For western blots, 5 ml of crude liver homogenate, each containing 2.9 mg/ml protein concentration (by Bradford assay with appropriate dilution), were mixed with 25 ml of a commercially available sample buffer lithium dodecyl sulphate (Invitrogen Corporation, Carlsbad, California, USA), 60 ml H₂O, and 10 ml of 100% 2-mercaptoethanol. Hence the protein concentrations in each sample were identical (0.145 mg/ml). Samples were denatured at 70°C for 10 minutes. Equal volumes of each sample were loaded onto a polyacrylamide gel, and electrophoresis was performed under reducing conditions. Proteins were then transferred to a nitrocellulose membrane and Ponceau S staining was performed to validate equal protein transfers. Membranes were “blocked” using a mixture of milk, bovine serum albumin, and Tween 20. The primary antibodies were diluted in Tris buffered saline, pH 7.4, and detected by chemiluminescence using appropriate horseradish peroxidase conjugated secondary antibodies.

For mdr2, the primary antibody was diluted at a ratio of 1:5000 in Tris buffered saline and incubated for two hours at room temperature. For bsep, the primary antibody was diluted at a ratio of 1:2000 and incubated for two hours at room temperature.

Statistics
Observed and expected intergroup frequencies of non-parametric data were compared using the χ² method. Means of parametric data sets were compared using an independent sample Student’s t test; confidence intervals were 5% and 95%. A p value of less than 0.05 was considered to be statistically significant. Correlations were determined using linear regression analysis. Values are expressed as mean (SEM).

RESULTS
In vitro HMGR activity, hepatic cholesterol stores, and plasma cholesterol levels
Mean weight gain over the experimental time frame (3.5 (1.5) g) and food consumption (3 (1) g/day) were similar in all groups. There was no mortality on diet alone or in the treatment groups, and all animals remained healthy. Figure 2 shows that the inhibitors downregulated the rate limiting enzyme in hepatic microsomal
fractions, as inferred by the in vitro HMGR assay. As listed in table 1, this effect was associated with a marked decrease in total hepatic cholesterol concentrations, albeit in a much more marked fashion with Ro (41% and 76% of controls in the Ro and Tu groups, respectively). Neither Ro nor Tu however induced a significant decrease in hepatic free cholesterol concentration (69% and 94% of controls, respectively), as evidenced by decreases in the esterified/free cholesterol ratio. Although a trend was apparent, no significant differences in plasma total cholesterol between inhibitor groups (152 (6) mg/dl (Ro) 158 (6) mg/dl (Tu)) and controls (165 (7) mg/dl) were found (56 days).

### Cholesterol gall stone prevalence rates

Figure 3 shows that gall stone prevalence rates at 56 days decreased with increasing dose of Ro (respectively, 69%, 57%, and 17% at 30, 60, and 100 mg/kg/day). Gall stone prevalence rates also decreased (respectively, 29% and 17% with Tu 30 and 60 mg/kg/day). Stone prevalence in contemporaneous control animals fed LD alone was 73%, with significant reductions in Ro 100 and Tu 30 and 60 treated animals. AKR mice fed LD alone exhibited a gall stone prevalence rate of 0% at the time point studied whereas those fed LD plus simva exhibited a prevalence rate of 40%. This represents a statistical trend (p = 0.10, $\chi^2$) (fig 3).

#### Biliary lipid secretion, lipid coupling, and cholesterol saturation indices

Quantitation of biliary lipid secretion rates in hepatic bile employing an acute (one hour) fistula is shown in table 2. Overall, volumes of secreted bile did not show any significant changes between groups. Cholesterol secretion rates were not significantly decreased in any group receiving the cholesterol synthesis inhibitors; on the contrary, there were statistically significant increases in cholesterol secretion rates in C57 Ro 30 and Ro 60 groups compared with controls (LD alone). In the case of AKR mice, no statistically significant changes were observed in Ro or simva fed animals. Bile salt secretion rates were unaffected by either drug at any dose level compared with control mice fed the LD diet alone. None the less, Ro treated mice exhibited significantly higher bile salt secretion rates than mice receiving Tu. However, with respect to biliary phosphatidylcholine (PC) secretion, both drugs resulted in significant increases in PC secretion rates compared with control mice. The response of LD diet fed AKR mice to Ro 100 was identical to that observed in the C57L strains with the same dose. Mice exhibited unchanged total bile salt secretion rates (314 (59) in Ro 100 v 381 (153) in controls), cholesterol secretion rates (20 (3) in Ro 100 v 18 (5) in controls, respectively) but significantly higher biliary PC outputs (58 (9) in Ro 100 v 42 (10) in controls, respectively). This confirms that high Ro doses uncouple PC secretion from that of bile salt secretion, irrespective of the presence or absence of gall stone susceptible (Lith) alleles (table 2).

We verified the changes in phospholipid content of bile (as determined by the Bartlett method) by quantifying the choline moiety of phospholipids after cholinate oxidase hydrolysis. Our data (not shown) are consistent with our previous determination and establish that the increases in phospholipid secretory rates are not artefacts. In most samples, Bartlett and choline assays were within 10% of each other; however, in one analysis of an Ro treated animal (at a dose of 30 mg/kg/day), the results were divergent. In this case, the Bartlett assay yielded an appreciably higher value for phospholipid secretion and employing TLC with

### Table 1 Influence of inhibitors of cholesterol synthesis on hepatic cholesterol concentrations and esterified to free cholesterol ratio

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total (mg/g liver)</th>
<th>Free (mg/g liver)</th>
<th>Esterified to free ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 LD</td>
<td>13.1 (9)</td>
<td>0.86 (0.21)</td>
<td>15.6 (3.1)</td>
</tr>
<tr>
<td>C57 simvastatin</td>
<td>21.8 (1.7)</td>
<td>3.18 (0.24)</td>
<td>22.2 (4)</td>
</tr>
<tr>
<td>Ro 30</td>
<td>4.9 (1.0)</td>
<td>0.51 (0.04)</td>
<td>8.0 (1.3)</td>
</tr>
<tr>
<td>Ro 60</td>
<td>6.0 (1.7)</td>
<td>0.76 (0.22)</td>
<td>7.7 (2)</td>
</tr>
<tr>
<td>Ro 100</td>
<td>5.2 (1.4)</td>
<td>0.52 (0.07)</td>
<td>9.0 (2.2)</td>
</tr>
<tr>
<td>Tu 30</td>
<td>10.6 (1.0)</td>
<td>0.79 (0.08)</td>
<td>13.6 (2.4)</td>
</tr>
<tr>
<td>Tu 60</td>
<td>9.4 (0.8)</td>
<td>0.83 (0.07)</td>
<td>10.9 (1.3)</td>
</tr>
</tbody>
</table>

*Analytes carried out at eight weeks on the lithogenic diet (LD).
†Hepatic cholesterol concentration assays were performed as described in the methods section.
‡Significantly different compared with the C57 controls fed the LD.

Hepatic cholesterol homeostasis was markedly changed by Ro and Tu. Striking reductions in total cholesterol were noted with all doses of Ro; much less marked reductions were effected by Tu. As expected, simvastatin caused hepatic cholesterol levels to increase. Levels of unesterified cholesterol remained substantially unchanged.

#### Figure 3

Gall stone prevalence rates expressed as a percentage in male C5/LJ mice fed a lithogenic diet (LD) with the addition of incremented doses of the Tu 220B or Ro 48 8071 agents (see methods), and both C57 and AKR controls fed LD alone or LD plus simvastatin (simva). Statistically significant differences in prevalence rates were observed between Tu 30, Tu 60, and Ro 100 and both C57 control groups. There was no significant difference in gall stone prevalence between either AKR group. Bars and arithmetic values indicate prevalence rates; *p<0.05 compared with both controls.
appropriate standards, an unknown polyphosphated organic compound was present at the origin but its chemical nature was not pursued further. As expected from the aforementioned data, biliary lipid coupling ratios were modified by Ro and Tu administration. Cholesterol:bile salt ratios were significantly higher in the Ro 30 (251%, p = 0.004), Ro 60 (212%, p = 0.03), and Tu 60 (192%, p = 0.05) groups than in controls. Although the cholesterol:bile salt ratios did not differ in a statistically significant manner, the trend was towards higher values in the Ro 30 and Ro 60 groups. However, individual PC:bile salt ratios were all significantly increased compared with controls following Ro 30 (177%, p = 0.001) treatment. CSI of hepatic bile (table 2) revealed values diminishing with escalating Ro dose, thereby coinciding with the cholesterol gall stone prevalence rates (fig 3). At the time of measurement in the morning, unsaturated bile was secreted only by Ro 100 and Tu 60 fed mice.

**Hepatic cholesterol concentration**

Hepatic cholesterol homeostasis was markedly changed by Ro and Tu, as shown in table 1. Striking reductions in total cholesterol were noted with all doses of Ro; much less marked reductions were effected by Tu. As expected, simvastatin caused hepatic cholesterol levels to increase. Levels of unesterified cholesterol remained substantially unchanged (table 1).

**Hepatic expression of canalicular transporters**

Bar graphs quantifying expression of Mdr2 (ATP binding cassette B4—ABCB4), the canalicular PC transmembrane translocator (PC “flipase”), and bsep, the bile salt export pump (ABCB11), by immunoblotting are shown in fig 4. Ro but not Tu significantly increased Mdr2 expression compared with controls fed LD alone. However, bsep expression was unchanged between all drug groups and controls.

### DISCUSSION

This study examined the in vivo effects of inhibitors of cholesterol synthesis, acting distally to oxidation of squalene (fig 1), in inbred mouse strains that possesses variant *Lith* alleles. To allow the gall stone phenotype to become manifest, the model requires dietary administration of large amounts of cholesterol as well as cholic acid, ensuring a predominant change in the bile salt pool to that of sodium

**Figure 4** Results of digitally quantified multidrug resistance protein, isofor 2 (*mdr2*) and bile salt export protein (*bsep*) immunoblot expression in murine livers. In animals fed Ro 48-8071, *mdr2* expression increased with escalating dose. In contrast, the results were variable in the case of Tu 2208. In both inhibitor groups, *bsep* expression did not change compared with controls (lithogenic diet (LD) alone). Histogram indicates means of n = 3–5 provided as a percentage of changes in expression density compared with controls. Inset demonstrates a representative immunoblot for *mdr2*, with labelling as per the X axis.

<table>
<thead>
<tr>
<th>Treatment group [Na]</th>
<th>BS †</th>
<th>PC †</th>
<th>Chol †</th>
<th>CSI †</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57L-LD</td>
<td>6</td>
<td>64 (10)</td>
<td>19 (4)</td>
<td>106 (14)</td>
</tr>
<tr>
<td>Ro 30</td>
<td>6</td>
<td>824 (79)</td>
<td>138 (6)*</td>
<td>58 (60)*</td>
</tr>
<tr>
<td>Ro 60</td>
<td>5</td>
<td>766 (156)</td>
<td>111 (18)*</td>
<td>43 (7)*</td>
</tr>
<tr>
<td>Ro 100</td>
<td>4</td>
<td>692 (29)</td>
<td>94 (80)*</td>
<td>26 (2)</td>
</tr>
<tr>
<td>Tu 60</td>
<td>5</td>
<td>520 (47)</td>
<td>104 (5)*</td>
<td>28 (3)</td>
</tr>
<tr>
<td>AKR-LD</td>
<td>6</td>
<td>381 (153)</td>
<td>42 (10)</td>
<td>18 (5)</td>
</tr>
<tr>
<td>AKR-simva</td>
<td>3</td>
<td>415 (89)</td>
<td>64 (10)*</td>
<td>26 (4)</td>
</tr>
<tr>
<td>C57-simva</td>
<td>5</td>
<td>480 (48)</td>
<td>67 (9)</td>
<td>18 (2)</td>
</tr>
<tr>
<td>AKR-Ro 100</td>
<td>5</td>
<td>314 (59)</td>
<td>58 (9)*</td>
<td>20 (3)</td>
</tr>
</tbody>
</table>

LD, lithogenic diet; BS, bile salt; PC, phosphatidylcholine (biliary phospholipid); Chol, cholesterol; CSI, cholesterol saturation index.

†Values are expressed as nmol/min/×100 g (body weight).

‡Dimensionless values.

*p* 0.05 v controls.

C57L mice fed Ro displayed higher BS secretion rates than controls whereas the single Tu fed group showed lower levels. Higher Chol secretion rates were observed in Ro 30 and Ro 60 fed animals compared with controls. Statistically significant elevations in biliary phospholipid secretion rates compared with controls were demonstrated in all Ro and Tu drug groups in both C57L and AKR mice. Chol and BS secretion were not changed significantly in AKR mice. CSI of murine hepatic bile in the fed state were calculated using critical tables for taurocholate, the principal murine bile salt in the LD. Chol desaturation of bile was evident in the Ro 100 and Tu 60 treatment groups as well as in AKR mice on LD alone. All other groups displayed supersaturated and lithogenic bile, as evidenced by the study.
taurocholate. Not only does this increase the mass of cholesterol absorbed from the gut but it also promotes biliary secretion of cholesterol and facilitates a solid crystalline phase transition within gall bladder bile. Therefore, given this state of augmented mass movement of cholesterol from the gut to the liver and secretion into bile, any change in the rates of de novo cholesterol biosynthesis may be commensurately difficult to discern. However, they may be inferred from two measures; firstly, hepatic HMGAR activity (reduced threefold in Ro 30, 100, and Tu 30 groups, and fivefold in the Tu 60 group); and secondly, hepatic cholesterol concentration (halved in Ro fed animals and reduced by one third in Tu 60), which is perhaps the best yardstick of cholesterol homeostasis in any of the experimental animals.

Although we do not know if circadian changes in biliary secretion occur in mice fed a lithogenic diet, the possibility of such circadian changes exists. However, to obviate any possibility that circadian factors might have interfered with our study, we administered the pharmacological agents admixed in the diet (thereby providing a sustained, usually nocturnal, intake) and secondly, by performing our surgical protocols at the same hour (and in random fashion) daily, we controlled for such effects.

We found that despite significantly reduced HMGAR activities (fig 2) and hepatic cholesterol concentrations (table 1), neither Ro 48-8071 nor Tu 2208 decreased biliary cholesterol secretion, irrespective of the presence or absence of mutant 

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HMGR (fig 1). However, both caused accumulation of cholesterol synthesis intermediaries to occur which are believed to directly or indirectly inhibit HMGR.

Simvastatin associated upregulation of cholesterol synthesis increased gall stone prevalence rates in resistant AKR animals but not in the susceptible C57L strain. This is probably an effect of the variant Lith alleles, as our previous work has shown that LD causes downregulation of Hmgar in the AKR but not in the C57L strain.

Despite evidence that de novo synthesised cholesterol in rodents may make a marked contribution to bile salt synthesis and bile secretion, neither of the inhibitors tested exhibited significant effects on bile salt secretion. On the contrary, a tendency towards increased bile salt secretion was observed in groups receiving Ro (table 2). LD is a potent downregulator of Cyp7a1, as previous work has shown that the latter is suppressed by cholic acid present in the LD and by deoxycholic acid, its bacterial metabolite, via the farnesoid X receptor. To account for increased bile salt secretion, it is also tempting to speculate that Ro 48-8071 may increase hepatic uptake and secretion of cholic acid metabolites. Although we did not find increased hepatic 

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density lipoprotein receptor, SR-B1.

cholesterol diet.

decreased gallstone formation in alipoprotein E-deficient mice fed a high-

secretion.

cholesterol saturation in the hamster and rat by manipulation of the pools of

physical-chemistry of gallbladder bile.

impairment of the degree of biliary lipid secretion.

by squalene epoxidase inhibitor avoids apoptotic cell death in L6 myoblasts.

formation in hamsters.

of hydroxymethylglutaryl coenzyme A reductase, on cholesterol gallstone

hypocholesterolemic effects of pravastatin sodium, a 3-hydroxy-3-

methylglutaryl coenzyme A reductase inhibitor, in rats.

homozygous expression of the multidrug resistance-2 P-glycoprotein leads to a complete absence of

cholesterol from bile and to liver disease.

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