# Effect of β-muricholic acid on the prevention and dissolution of cholesterol gallstones in C57L/J mice[1]

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**Abstract** **This study investigated whether β-muricholic acid, a natural trihydroxy hydrophilic bile acid of rodents, acts as a biliary cholesterol-desaturating agent to prevent cholesterol gallstones and if it facilitates the dissolution of gallstones compared with ursodeoxycholic acid (UDCA). For gallstone prevention study, gallstone-susceptible male C57L mice were fed 8 weeks with a lithogenic diet (2% cholesterol and 0.5% cholic acid) with or without 0.5% UDCA or β-muricholic acid. For gallstone dissolution study, additional groups of mice that have formed gallstones were fed chow with or without 0.5% β-muricholic acid or UDCA for 8 weeks. One hundred percent of mice fed the lithogenic diet formed cholesterol gallstones. Addition of β-muricholic acid and UDCA decreased gallstone prevalence to 20% and 50% through significantly reducing biliary secretion rate, saturation index, and intestinal absorption of cholesterol, as well as inducing phase boundary shift and an enlarged Region E that prevented the transition of cholesterol from its liquid crystalline phase to solid crystals and stones. Eight weeks of β-muricholic acid and UDCA administration produced complete gallstone dissolution rates of 100% and 60% compared with the chow (10%). We conclude that β-muricholic acid is more effective than UDCA in treating or preventing diet-induced or experimental cholesterol gallstones in mice.** —Wang, D. Q-H., and S. Tazuma. **Effect of β-muricholic acid on the prevention and dissolution of cholesterol gallstones in C57L/J mice.** ***J. Lipid Res.*** **2002.** 43: **1960–1968.**

**Supplementary key words** bile flow • phospholipid • intestinal cholesterol absorption • phase diagram

Cholesterol gallstones are a major public health problem in all developed countries. In the United States, approximately 10–15% of the adult population suffers from cholesterol gallstones (1, 2), which constitutes one of the most common and most costly digestive diseases (3). Long-term administration of ursodeoxycholic acid (UDCA), a hydrophilic bile acid, has been shown to promote the dissolution of cholesterol gallstones (4) and to prevent the recurrence of gallstones after extracorporeal shock wave lithotripsy (5). Therapeutic mechanisms of UDCA include decreasing biliary secretion and intestinal absorption of cholesterol (6, 7), both of which could contribute to a decrease in bile cholesterol saturation. However, UDCA constitutes only ~50% of the biliary bile acid pool in patients with cholesterol gallstones (8, 9). The human intestinal bacteria can transform UDCA to lithocholic acid (10, 11) that is shown to be a hepatotoxic bile acid to laboratory animals (12). β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) is a natural trihydroxy hydrophilic bile acid, which is a major bile acid biosynthesized by rat (13) and mouse (14) liver and found in their bile. Because of the presence of a hydroxy group in the 6β position of the steroid ring, β-muricholic acid (15) is more hydrophilic than UDCA. In vitro studies (15) have demonstrated that β-muricholic acid can dissolve solid cholesterol monohydrate crystals via formation of a liquid crystalline mesophase, suggesting that it may be used to dissolve cholesterol gallstones. However, Cohen et al. (16) found that feeding low (0.1%) dose of β-muricholic acid for 6 weeks does not dissolve preexisting gallstones in prairie dogs or hamsters. Moreover, feeding 0.1% β-muricholic acid inhibits gallstone formation in hamsters, but fails to prevent gallstone formation in prairie dogs (16). Therefore, in this study we compared physical-chemical properties of β-muricholic acid with UDCA and investigated *i*) whether β-muricholic acid acts as a po-

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Abbreviations: CSI, cholesterol saturation index; HPLC, high performance liquid chromatography; UDCA, ursodeoxycholic acid.

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tent biliary cholesterol-desaturating agent to prevent cholesterol gallstone formation in gallstone-susceptible C57L mice carrying *Lith* genes (17); *ii*) whether it facilitates the dissolution of cholesterol gallstones; and *iii*) how it regulates hepatic and biliary cholesterol and bile salt metabolism. Our results showed that β-muricholic acid is more effective than UDCA in preventing cholesterol gallstones through inhibiting intestinal cholesterol absorption, decreasing biliary cholesterol secretion, and retarding phase separation from vesicular cholesterol to crystalline cholesterol monohydrate in bile of C57L mice. Also, it is more successful than UDCA in promoting the dissolution of cholesterol gallstones through a greater capacity to form a liquid crystalline phase. Therefore, we conclude that β-muricholic acid could be a potential cholelitholytic agent for preventing or treating diet-induced or experimental cholesterol gallstones in mice.

## MATERIALS AND METHODS

### Chemicals

Cholic acid, UDCA, taurocholate, tauroursodeoxycholate, and cholesterol were purchased from Sigma Chemical (St. Louis, MO), and grade I egg yolk lecithin was from Lipid Products (South Nutfield, Surrey, UK). Sodium tauro-β-muricholate and β-muricholic acid were obtained from Tokyo Tanabe (Tokyo, Japan), and its purity was >98% as determined by high performance liquid chromatographic (HPLC) and thin layer chromatographic analyses. Intralipid (20%, w/v) was purchased from Pharmacia (Clayton, NC), and medium-chain triglyceride was from Mead Johnson (Evansville, IN). Radioisotope [1,2-$^{3}$H]cholesterol, [4-$^{14}$C]cholesterol, DL-[5-$^{3}$H]mevalonolactone, and DL-[3-$^{14}$C]HMG-CoA were purchased from NEN Life Science Products, (Boston, MA), and [5,6-$^{3}$H]sitostanol was from American Radiolabeled Chemicals (St. Louis, MO). For HPLC analyses of bile salt species and cholesterol, all reagents were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals and solvents were American Chemical Society (ACS) or reagent grade quality (Fisher Scientific, Medford, MA).

### Animals and diets

Male C57L/J mice, 6–8 weeks old, were purchased from The Jackson Laboratory, Bar Harbor, ME. C57L strain is homozygous for susceptible *Lith* alleles (17). All animals were maintained in a temperature-controlled room (22 ± 1°C) with 12-h day cycles (6 AM-6 PM), and were allowed to adapt to the environment for 2-weeks prior to the experiments, and were provided free access to water and normal mouse chow containing trace cholesterol (<0.02%) (The Mouse Diet 1401, St. Louis, MO). To make semisynthetic diets, cholesterol and bile acids were added to powdered chow in ethanol, blended thoroughly with a mechanical mixer, and dried on trays at 50°C for 48 h. For gallstone prevention study, mice were divided into three groups (n = 20 each) fed a lithogenic diet (2% cholesterol and 0.5% cholic acid) with or without 0.5% UDCA or 0.5% β-muricholic acid for 8 weeks. In our initial study, we have observed that at week 8 of 1% cholesterol and 0.5% cholic acid feeding, 80% of male C57L mice formed gallstones, and 2% cholesterol plus 0.5% cholic acid induces 100% of C57L mice forming cholesterol gallstones. For gallstone dissolution study, additional groups (n = 20 each) of mice that have formed cholesterol gallstones due to the 8-week feeding of 2% cholesterol and 0.5% cholic acid, were fed chow (control) with or without 0.5% UDCA or 0.5% β-muricholic acid for 8 weeks. All experiments were executed according to 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 Committees of Harvard University and Hiroshima University.

### Collection of gallbladder biles and gallstones and microscopic studies

At week 8 of the semisynthetic diets (see above) feeding, nonfasted animals were weighed and anesthetized with an ip injection of 35 mg/kg pentobarbital. After cholecystectomy, gallbladder volume was measured by weighing the whole gallbladder and equating gallbladder weight (including stones) with gallbladder volume (17). Gallbladders were then opened, and 5 μl of fresh gallbladder bile were examined for mucin gel, solid and liquid crystals, and gallstones, which were defined according to previously established criteria (14, 17). After pooled gallbladder biles were centrifuged at 100,000 *g* for 30 min at 37°C and filtered through a preheated (37°C) Swinnex-GS filter (0.22 μm) assembly (Millipore Products Division, Bedford, MA), samples were frozen and stored at −20°C for further lipid analyses (see below).

### Cannulation of the common bile duct and collection of hepatic biles

Additional groups of mice (n = 5 each) fed the semisynthetic diets were used for biliary lipid secretion study (18). In brief, after cholecystectomy, the common bile duct was cannulated via a PE-10 polyethylene catheter. Hepatic bile was collected by gravity. The first hour collection of hepatic biles was used to study biliary lipid outputs. To determine the circulating bile salt pool sizes, 8-h biliary "washout" studies were performed according to previously described methods (18). After fresh hepatic biles were examined by polarizing light microscopy and their volumes were determined by weighing, samples were frozen and stored at −20°C for further lipid analyses. During surgery and hepatic bile collection, mouse body temperature was maintained at 37 ± 0.5°C with a heating lamp and monitored with a thermometer.

### Measurement of intestinal cholesterol absorption

Cholesterol absorption was determined by a plasma dual isotope ratio method (14, 19) in mice (n = 10 per group) fed chow, or chow containing 0.5% (by weight) of UDCA, cholic, or β-muricholic acids for 7 days. In brief, non-fasted mice were anesthetized by ip injection of 35 mg/kg pentobarbital. One hundred microliters of Intralipid containing 2.5 μCi of [$^{3}$H]cholesterol was injected (iv) into the jugular vein. Following this procedure, the animal was given by gavage an intragastric (ig) dose of 1 μCi of [$^{14}$C]cholesterol mixed with 150 μl of medium-chain triglyceride. After dosing, mice were returned to individual cages with wire mesh bottoms, where they were free to eat chow or the appropriate semisynthetic diets for an additional 3 days. Mice were then anesthetized as described above, and were bled from the heart into heparinized microtubes. Ten microliters of EcoLite (ICN Biomedicals, Costa Mesa, CA) were mixed with 100-μl portions of plasma and the original dosing mixture, respectively. The vials were counted in a liquid scintillation spectrometer (Beckman Instruments, San Ramon, CA). The ratio of the two radiolabels in plasma was used for calculating the percent cholesterol absorption:

$$\%\ \text{Cholesterol absorption} = \left(\frac{\text{Percent of IG dose } [^{14}\text{C}]\text{cholesterol per ml plasma}}{\text{Percent of IV dose } [^{3}\text{H}]\text{cholesterol per ml plasma}}\right) \times 100 \qquad \textit{(Eq. 1)}$$

### Determination of activities of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase

Liver samples were harvested from non-fasted mice (n = 5 per group) at week 8 of feeding of chow or the semisynthetic diets. To minimize diurnal variations of hepatic enzyme activities, all procedures (20) were performed between 8 AM and 9 AM. Microsomal activities of HMG-CoA reductase were determined by measuring the conversion rate of [$^{14}$C]HMG-CoA to [$^{14}$C]mevalonic acid using a radiochemical assay (21). Products were quantified by liquid scintillation counting with [$^{3}$H]mevalonolactone as internal standard. Protein concentration was determined by the assay of Bradford (22). Hepatic activities of cholesterol 7α-hydroxylase were determined by the HPLC-based assay system of Hylemon et al. (23).

### Phase boundaries of ternary lipid systems

Based on expected micellar phase and crystallization phase boundaries, supersaturated model bile systems were prepared with variable proportions of cholesterol, lecithin, and the mixtures of tauro-β-muricholate, taurocholate, and tauroursodeoxycholate in ratios of 25:75:0 (wt/wt/wt), 50:50:0 (wt/wt/wt), 85:15:0 (wt/wt/wt), and 0:20:80 (wt/wt/wt) with total lipid concentrations of 2.5 and 10 g/dl, which are similar to mouse hepatic and gallbladder biles. Model bile systems were made according to previously described methods (24, 25) and incubated at 37°C in a waterbath. Microscopic examination for crystalline and liquid-crystalline precipitates was performed at 1-day intervals using polarizing light microscopy and phase contrast optics (Nikon, Japan). After prolonged incubation (30 days), when no further changes were observed by microscopy, two-phase (micelles and either liquid or solid cholesterol crystal-containing) and three-phase (micelles, solid, and liquid crystal-containing) zones were defined. Micellar phase boundaries of the equilibrated model bile systems were determined according to published methods (24, 25), which were used for calculating the corrected cholesterol saturation indexes (CSI) (26). Also, the CSI values of gallbladder and hepatic biles were calculated from the critical tables (27). Relative lipid compositions of mouse gallbladder biles were plotted on condensed phase diagrams appropriate to their mean total lipid concentrations and to their predominant bile salt compositions.

### Lipid analyses

Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett (28). Total and individual bile salt concentrations, bile cholesterol, as well as cholesterol content in chow and gallstones were determined by HPLC (17). Hydrophobicity indexes of hepatic bile were calculated according to Heuman's method (29).

### Statistical methods

All data are expressed as means ± SD. Statistically significant differences among groups of mice fed chow or the semisynthetic diets were assessed by Student's *t*-test, Mann-Whitney U test, or Chi-square test. Analyses were performed with a SuperANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

## RESULTS

### Prevalence, chemistry, number and size of gallstones, as well as gallbladder volumes

At week 8 of feeding, 100% (20/20) of mice fed the lithogenic diet containing 2% cholesterol and 0.5% cholic acid formed cholesterol gallstones. However, the addition of 0.5% UDCA and 0.5% β-muricholic acid to the lithogenic diet significantly ($P < 0.001$) decreased gallstone prevalence to 50% (10/20) and to 20% (4/20), respectively. The gallstones were usually a round and solid sphere in shape, and pale yellow to white in color. The extracted sterols from these stones contained >99% cholesterol on a dry weight basis, as determined by HPLC. The frequency distribution of gallstones displayed that 65% of mice fed the lithogenic diet formed 7–9 stones, 20% with 1–3 stones, and 15% with 4–6 stones. In mice fed the lithogenic diet plus UDCA, 40% of mice formed 1–3 stones and 10% with 4–6 stones; however, 50% of mice was gallstones free. Most notably, the majority (80%) of mice fed the lithogenic diet plus β-muricholic acid was gallstones free, as well as 20% of mice formed only 1–3 stones. Furthermore, stone size was 0.68 ± 0.27 mm in diameter in mice fed the lithogenic diet, significantly ($P < 0.01$) bigger than those in the UDCA group (0.36 ± 0.18 mm) and in the β-muricholic acid group (0.34 ± 0.11 mm). In addition, gallbladder values were significantly ($P < 0.05$) larger in mice (41 ± 17 μl) fed the lithogenic diet than those in the UDCA group (33 ± 10 μl) and in the β-muricholic acid group (30 ± 7 μl).

### Biliary lipid compositions of gallbladder and hepatic biles

**Table 1** shows biliary lipid compositions of pooled gallbladder biles (n = 20 per group) and individual hepatic biles (n = 5 per group) at week 8 of the semisynthetic diet feeding. Mice fed the lithogenic diet displayed markedly higher mol% cholesterol, CSI value, and total lipid concentration (Table 1) compared with the mice fed the lithogenic diet plus UDCA or β-muricholic acid. Furthermore, the mean apparent CSI values and total lipid concentrations, as well as mole% cholesterol and phospholipid of hepatic biles, were significantly ($P < 0.05$) higher, but mole% bile salt significantly ($P < 0.001$) lower in the lithogenic diet-fed group compared with the lithogenic diet plus UDCA or β-muricholic acid feeding. Moreover, feeding the lithogenic diet plus β-muricholic acid and UDCA displayed significantly ($P < 0.05$) lower cholesterol/phospholipid and phospholipid/bile salt ratios of hepatic biles compared with the lithogenic diet (Table 1). These findings show that addition of β-muricholic acid and UDCA to the lithogenic diet mainly decreased the cholesterol and phospholipid compositions of mouse gallbladder and hepatic biles (Table 1) compared with the lithogenic diet feeding, and that solubility and phase separation of cholesterol were critically dependent on phospholipid/bile salt ratio and cholesterol concentration.

The HPLC analysis revealed that all bile salts were taurine conjugated and distributions of bile salt compositions were similar between hepatic and gallbladder biles. In mice fed the lithogenic diet, taurocholate (51.2 ± 4.8%), taurodeoxycholate (18.5 ± 5.5%), and taurochenodeoxycholate (18.0 ± 2.6%) were the predominant bile salt species. Tauro-β-muricholate (7.7 ± 1.5%), tauroursodeoxycholate (3.1 ± 0.5%), and tauro-ω-muricholate (1.5 ± 0.9%) were present in much smaller concentrations. In

TABLE 1. Biliary lipid compositions of gallbladder and hepatic biles after gallstone formation[a]

| Diet | Pooled Gallbladder Biles | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ch | PL | BS | Ch/PL | PL/BS | [TL] | CSI[b] | Corrected CSI[c] |
| | | *mole%* | | | | *g/dl* | | |
| LD | 13.22 | 18.96 | 67.81 | 0.70 | 0.28 | 10.31 | 1.95 | 2.41 |
| LD+UDCA | 7.28 | 17.10 | 75.62 | 0.43 | 0.23 | 9.88 | 1.20 | 1.48 |
| LD+β-MCA | 5.79 | 16.92 | 77.30 | 0.34 | 0.22 | 9.99 | 0.97 | 1.18 |
| | Individual Hepatic Biles | | | | | | | |
| LD | 10.26 ± 2.44 | 18.60 ± 1.83 | 71.14 ± 3.88 | 0.55 ± 0.10 | 0.26 ± 0.04 | 2.64 ± 0.77 | 2.02 ± 0.30 | 2.64 ± 0.50 |
| LD+UDCA | 3.92 ± 0.39[d] | 10.49 ± 0.84[d] | 85.59 ± 0.77[d] | 0.38 ± 0.06[f] | 0.12 ± 0.01[d] | 2.07 ± 0.37 | 1.29 ± 0.20[e] | 1.73 ± 0.17[e] |
| LD+β-MCA | 4.33 ± 1.20[d] | 11.49 ± 2.31[d] | 84.19 ± 3.38[d] | 0.39 ± 0.03[f] | 0.14 ± 0.03[d] | 2.64 ± 0.30 | 1.28 ± 0.17[f] | 1.73 ± 0.20[e] |

β-MCA, β-muricholic acid; BS, bile salts; Ch, cholesterol; CSI, cholesterol saturation index; LD, lithogenic diet containing 2% cholesterol and 0.5% cholic acid; PL, phospholipids; [TL], total lipid concentration; UDCA, ursodeoxycholic acid.

[a] Values were determined from pooled gallbladder biles (n = 20 per group) and five hepatic biles (the first hour of biliary secretion) per group.

[b] The cholesterol saturation index values of pooled gallbladder biles and individual hepatic biles were calculated from the critical tables (27), so they were estimates based on taurocholate.

[c] The corrected cholesterol saturation index values of pooled gallbladder biles and five hepatic biles were calculated (26) based on tauro-β-muricholate and taurocholate mixture in a ratio of 25:75 (wt/wt) (Materials and Methods).

[d] $P < 0.001$ compared with the LD group.

[e] $P < 0.01$ compared with the LD group.

[f] $P < 0.05$ compared with the LD group.

contrast, feeding the lithogenic diet plus 0.5% UDCA induced a significant ($P < 0.001$) increase in concentration of hydrophilic bile salts, tauro-β-muricholate (20.3 ± 1.9%), and tauroursodeoxycholate (6.0 ± 1.0%), as well as of a hydrophobic bile salt, taurochenodeoxycholate (30.2 ± 3.6%). However, there was a significant ($P < 0.001$) decrease in taurocholate (29.2 ± 3.7%). Moreover, no significant changes occurred in levels of tauro-ω-muricholate or taurodeoxycholate. Furthermore, mice fed the lithogenic diet plus 0.5% β-muricholic acid displayed significant ($P < 0.001$) increases in concentration of the hydrophilic bile salts, tauro-β-muricholate (27.6 ± 3.8%), and a significant ($P < 0.001$) decrease in taurochenodeoxycholate (0.9 ± 0.3%). Other bile salt compositions such as taurocholate, taurodeoxycholate, taurochenodeoxycholate, and tauro-ω-muricholate were not changed markedly compared with the lithogenic diet group. Biliary hydrophilicity indexes were +0.10 ± 0.01 in mice fed the lithogenic diet, and decreased significantly ($P < 0.01$) to –0.01 ± 0.02 by UDCA, and to –0.14 ± 0.04 ($P < 0.0001$) by β-muricholic acid.

The relative lipid compositions of pooled gallbladder biles (Table 1) in mice fed the semisynthetic diets are shown on condensed phase diagrams (**Fig. 1**). Top panel shows that the micellar phase boundary and cholesterol crystallization (Fig. 1, region A–E) pathways are appropriate for the taurocholate-rich bile, and bottom panel for the tauro-β-muricholate and taurocholate-rich bile. Other conditions are mean total lipid concentration = 10 g/dl and temperature = 37°C. Relative lipid compositions of pooled gallbladder biles from mice fed 2% cholesterol and 0.5% cholic acid fell in a central three-phase area denoted Region C (Fig. 1, top panel), where at equilibrium these biles are composed of solid cholesterol crystals, liquid crystals, and saturated micelles (24, 25). When bile salt hydrophobicity decreases (25), all physical states and crystallization pathways (Fig. 1, bottom panel) shift to the left, i.e., to lower phospholipid contents, and the micellar zone becomes smaller. Also, the enlarged Region E encapsulates physiological lipid compositions. Under these conditions, with the lithogenic diet plus β-muricholic acid feeding, relative lipid compositions of pooled gallbladder biles plotted in Region E in which at equilibrium only liquid crystals occur but never solid cholesterol crystals. However, lipid compositions of pooled gallbladder biles from mice fed the lithogenic diet plus UDCA fell on the crystallization path boundary *ab* between Regions C and E (Fig. 1, bottom panel).

### Bile flow and biliary lipid secretion rates

We observed changes in the mean bile flow rates in mice fed different diets for the first hour following interruption of the enterohepatic circulation. Compared with mice fed the lithogenic diet (93 ± 21 μl/min/kg), there were not significant differences in bile flow rates between groups of mice fed the lithogenic diet plus UDCA (91 ± 18 μl/min/kg) and the lithogenic diet plus 0.5% β-muricholic acid (96 ± 11 μl/min/kg).

**Figure 2** shows biliary cholesterol (top panel), phospholipid (middle panel), and bile salt secretion rates (bottom panel) during the first hour of biliary washout in mice fed different semisynthetic diets for 8 weeks. Feeding the lithogenic diet plus β-muricholic acid (11.6 ± 4.8 μmol/h/kg) or UDCA (15.4 ± 4.9 μmol/h/kg) significantly ($P < 0.01$) decreased biliary cholesterol outputs compared with the lithogenic diet (29.4 ± 6.9 μmol/h/kg). Also, biliary phospholipid outputs were reduced significantly ($P < 0.01$) by β-muricholic acid (30.1 ± 9.9 μmol/h/kg), but not by UDCA (41.3 ± 13.6 μmol/h/kg) compared with the lithogenic diet (53.6 ± 7.4 μmol/h/kg). Furthermore, no significant differences were found in biliary bile salt outputs (210.8–238.6 μmol/h/kg) and the

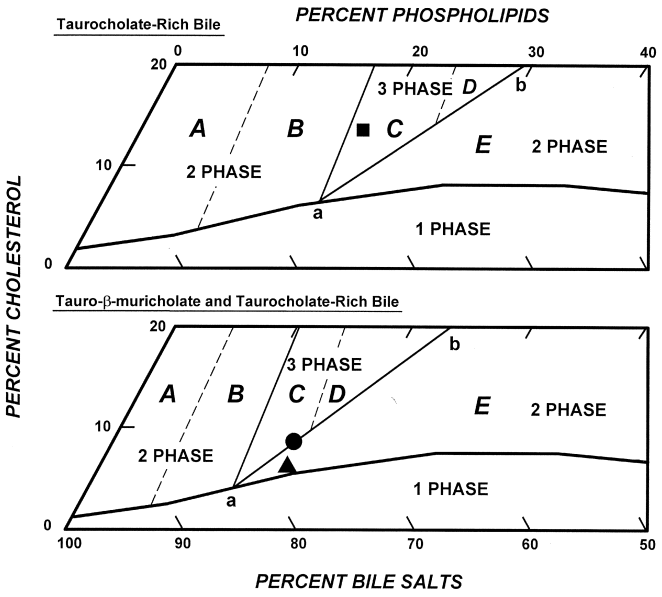


**Fig. 1.** Truncated phase diagrams of taurocholate-rich (top panel) and tauro-β-muricholate and taurocholate (1:3 ratio)-rich biles (bottom panel) are generated according to the average total lipid concentration (10 g/dl) of the pooled gallbladder biles (see Table 1). The one-phase micellar zone at bottom is enclosed by a solid curved line. Above it, two solid lines divide the two-phase zones from a central three-phase zone. Based upon the solid and liquid crystallization sequences present in the biles, the left two-phase and central three-phase regions are divided by dashed lines into Region A to E (25). Lipid compositions of pooled gallbladder biles from mice fed 2% cholesterol plus 0.5% cholic acid were located in the central three-phase zone (top panel), where the biles would be composed of solid cholesterol crystals, liquid crystals, and cholesterol saturated micelles at equilibrium. Of note is that because feeding 0.5% ursodeoxycholic acid (UDCA) and β-muricholic acid increased significantly the concentration of tauro-β-muricholate to 20-28%, all crystallization pathways shift to the left and the micellar zone becomes smaller. These changes generate a new condensed phase diagram with enlarged Region E (bottom panel). Therefore, relative lipid compositions of pooled gallbladder biles from mice fed the lithogenic diet plus 0.5% β-muricholic acid plotted in Region E where at theoretical equilibrium, the biles would be composed of liquid crystals and saturated micelles but never solid cholesterol crystals. Relative lipid compositions of the biles from mice fed the lithogenic diet plus 0.5% UDCA were located on the crystallization path boundary *ab* between Regions C and E. A square represents the lithogenic diet containing 2% cholesterol and 0.5% cholic acid; a circle represents the lithogenic diet plus 0.5% UDCA; and a triangle represents the lithogenic diet plus 0.5% β-muricholic acid at week 8 of feeding. See text for further description.

circulating bile salt pools (3.6–3.9 μmol) among the three groups of mice.

### Effect of bile acids on intestinal cholesterol absorption

The percentage of cholesterol absorption was 37 ± 6% in male C57L mice on chow containing trace (<0.02%) amounts of cholesterol. Feeding 0.5% cholic acid increased significantly ($P < 0.001$) percent cholesterol absorption to 62 ± 6% compared with the chow diet. In contrast, cholesterol absorption efficiency was decreased significant ($P < 0.01$) by 0.5% UDCA (19 ± 8%), and feeding 0.5% β-muricholic acid (11 ± 2%) displayed the lowest ($P < 0.001$) percentages of intestinal cholesterol absorption. Because similar amounts of food (3.6–4.0 g/day) were eaten by the mice, calculation of total cholesterol mass absorbed by the small intestine was ~0.25–0.30 mg/day in the chow diet group. On the cholic acid feeding, total cholesterol mass absorbed from the small intestine was increased to ~0.45–0.50 mg/day. In contrast, feeding β-muricholic acid and UDCA decreased total mass of cholesterol absorbed by the small intestine to ~0.08–0.09 mg/day and ~0.14–0.15 mg/day, respectively.

### Effect of bile acids on hepatic cholesterol and bile salt syntheses

The values for the activities of hepatic HMG-CoA reductase, while somewhat higher on the chow diet (55 ± 12 pmol/min/mg) compared with the other semisynthetic diet feeding (48–52 pmol/min/mg), were not significantly different among the three groups of mice. Feeding 0.5% UDCA (11.8 ± 2.9 pmol/min/mg) or β-muricholic acid alone (12.6 ± 3.4 pmol/min/mg) did not induce significant changes in activities of cholesterol 7α-hydroxylase compared with the chow diet (9.6 ± 2.4 pmol/min/mg). In contrast, the activities of cholesterol 7α-hydroxylase were decreased significantly ($P < 0.01$) to 3.0 ± 1.2 pmol/min/mg by the 2% cholesterol plus 0.5% cholic acid feeding. The addition of 0.5% UDCA and β-muri-

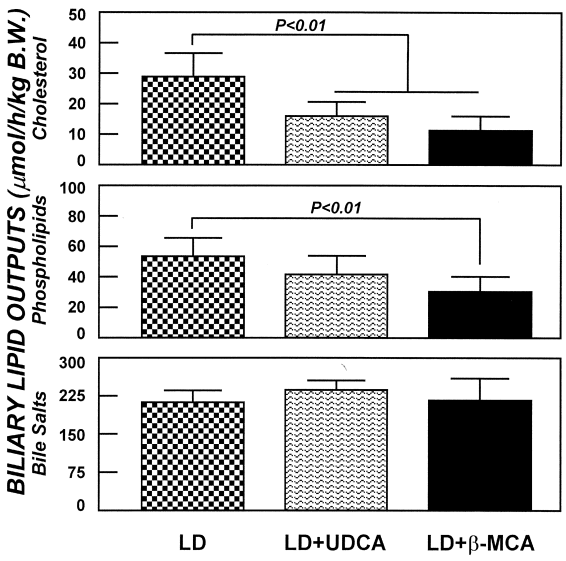


**Fig. 2.** Biliary cholesterol, phospholipid, and bile salt outputs (μmol/h/kg body weight) during the first hour of interruption of the enterohepatic circulation in mice (n = 5 per group) fed different semisynthetic diets for 8 weeks. The addition of 0.5% UDCA and 0.5% β-muricholic acid to the lithogenic diet significantly ($P < 0.01$) decreased biliary cholesterol outputs compared with the lithogenic diet. Moreover, biliary phospholipid outputs were reduced significantly ($P < 0.01$) by the β-muricholic acid feeding, but not by the UDCA feeding. No significant differences were found in biliary bile salt outputs among the three groups of mice. Abbreviations: LD, lithogenic diet; UDCA, ursodeoxycholic acid; and β-MCA, β-muricholic acid.

cholic acid to the lithogenic diet also reduced activities of cholesterol 7α-hydroxylase to 8.0 ± 2.1 pmol/min/mg and 8.8 ± 2.8 pmol/min/mg, however, these did not reach statistically significant differences compared with the chow.

**Dissolution of gallstones by UDCA and β-muricholic acid**

We have found that 100% of male C57L mice formed cholesterol gallstones at week 8 of 2% cholesterol and 0.5% cholic acid feeding, with stone size being 0.71 ± 0.23 mm in diameter in our initial study and 0.68 ± 0.27 mm in the present study. Furthermore, we observed that after the diet was changed to the normal mouse chow, cholesterol gallstones were still present in 90% (18/20) of the animals, with gallstone diameters in 0.59 ± 0.17 mm. This indicates that spontaneous dissolution does not occur in week 8 when the normal rodent chow diet was substituted for the lithogenic diet. Moreover, some mucin gel, solid and liquid crystals, and tiny gallstones (0.30 ± 0.11 mm in diameter; $P < 0.01$, compared with the chow diet group) were detected in the gallbladder biles in 40% (8/20) of UDCA-fed mice. In contrast, after the 8-week administration of 0.5% β-muricholic acid, the gallbladder biles of mice were transparent, and their examination by polarizing light microscopy revealed an abundance of liquid crystals but not cholesterol monohydratre crystals or gallstones, suggesting that preexisting cholesterol gallstones were dissolved completely by β-muricholic acid. Overall, 8 weeks of β-muricholic acid and UDCA administration produced complete gallstone dissolution rates of 100% and 60% compared with the chow (10%). **Table 2** summarizes biliary lipid compositions of pooled gallbladder biles (n = 20 per group) after gallstone dissolution study, i.e., at week 8 of 0.5% UDCA and β-muricholic acid feeding. The CSI values, cholesterol/phospholipid ratios, and mole% cholesterol of pooled gallbladder biles were decreased markedly by feeding 0.5% UDCA and β-muricholic acid compared with the chow diet (Table 2). In chow-fed mice (control), the predominant bile salt species were taurocholate (50.1 ± 6.5%) and tauro-β-muricholate (43.2 ± 5.6%). Other bile salts (tauro-ω-muricholate, tauroursodeoxycholate, taurochenodeoxycholate, and taurodeoxycholate) were present in small amounts (0.6–3.5%). Feeding UDCA produced a significant ($P < 0.001$) increase in tauroursodeoxycholate (80.3 ± 2.5%) and significant ($P < 0.001$) decreases in concentration of taurocholate (1.2 ± 0.4%) and tauro-β-muricholate (10.2 ± 1.1%). Mice fed β-muricholic acid displayed a significant ($P < 0.001$) increase in tauro-β-muricholate (85.5 ± 4.5%) and a significant ($P < 0.001$) decrease in taurocholate (9.6 ± 2.6%). The hydrophobicity indexes of pooled gallbladder biles were significantly ($P < 0.001$) lower in UDCA (−0.48 ± 0.04) and β-muricholic acid (–0.67 ± 0.03) groups compared with the chow feeding (−0.35 ± 0.04). **Figure 3** plots the relative lipid compositions of pooled gallbladder biles before and after 8 weeks of gallstone dissolution on phase diagram (total lipid concentration = 10 g/dl) from mice fed chow (control) with or without 0.5% UDCA or β-muricholic acid. It is apparent that initial biliary lipid compositions from gallbladder biles containing cholesterol gallstones plotted in the central three-phase zone (see Fig. 1, top panel, and Table 1). However, in UDCA, (Fig. 3, top panel) and tauro-β-muricholate-rich (Fig. 3, middle panel) bile systems, all crystallization pathways shift to the left and the expansion of Region E overlaps physiological lipid compositions. The relative lipid compositions of pooled gallbladder bile from mice fed UDCA were located on the phase boundary *ab* between Regions C and E (Fig. 3, top panel). Moreover, the relative lipid compositions of the bile in mice fed 0.5% β-muricholic acid crossed the phase boundary *ab* and entered Region E in which these biles are composed of liquid crystals and saturated micelles (Fig. 3, middle panel). The lipid compositions of pooled gallbladder biles with preexisting cholesterol gallstones in chow-fed mice still fell in the central three-phase zone (Fig. 3, bottom panel), where the biles consist of solid cholesterol crystals, liquid crystals, and saturated micelles. With respect to the solubilizing components of bile, cholesterol dissolution from gallstones was predictable from the solubility relationships regarding absolute and relative cholesterol and phospholipid compositions as inferred from model systems (24, 25). Furthermore, feeding β-muricholic acid and UDCA strikingly decreased cholesterol/phospholipid ratio of gallbladder biles but not phospholipid/bile salt ratio compared with the chow diet (control). This suggests that the predominant physical-chemical mechanism of in vivo gallstone dissolution with β-muricholic acid and UDCA is via liquid crystalline dispersion of cholesterol.

TABLE 2. Biliary lipid compositions of pooled gallbladder biles after gallstone dissolution[a]

| | Pooled Gallbladder Biles | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Diet | Ch | PL | BS | Ch/PL | PL/BS | [TL] | CSI[b] | Corrected CSI[c] |
| | | *mole%* | | | | *g/dl* | | |
| Chow | 8.44 | 14.80 | 76.76 | 0.57 | 0.19 | 8.06 | 1.59 | 2.48 |
| 0.5% UDCA | 6.88 | 14.85 | 78.27 | 0.46 | 0.19 | 7.81 | 1.31 | 2.75 |
| 0.5% β-MCA | 5.30 | 15.18 | 79.52 | 0.35 | 0.19 | 8.01 | 1.00 | 2.40 |

[a] Values were determined from pooled gallbladder biles (n = 20 per group).

[b] The cholesterol saturation index values of pooled gallbladder biles were calculated from the critical tables (27), so they were estimates based on taurocholate.

[c] The corrected cholesterol saturation index values of pooled gallbladder biles were calculated (26) based on tauro-β-muricholate, taurocholate, and tauroursodeoxycholate mixtures in ratios of 50:50:0 (wt/wt/wt) (the chow group), 0:20:80 (wt/wt/wt) (the UDCA group), and 85:15:0 (wt/wt/wt) (the β-muricholic acid group) (see Materials and Methods).

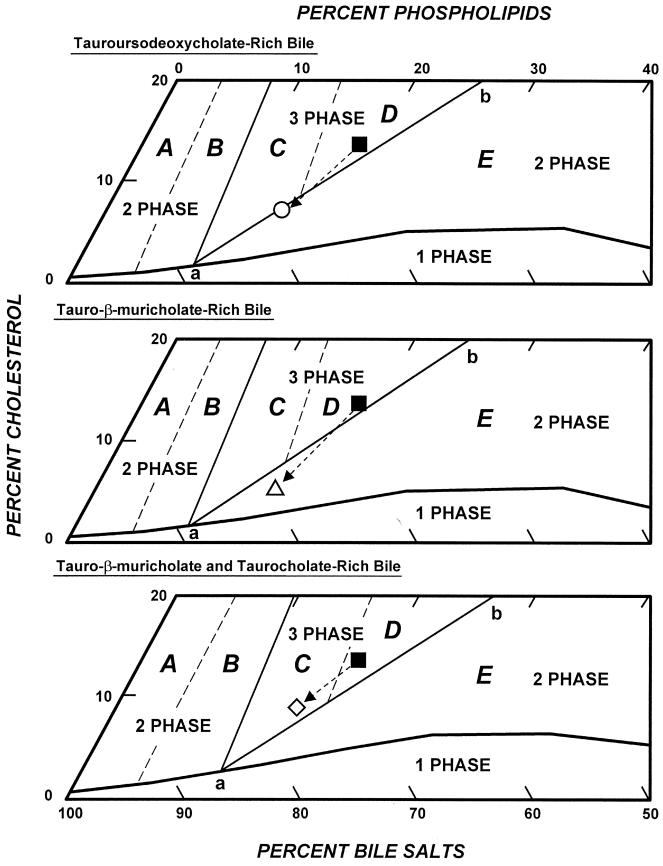


**Fig. 3.** Three condensed phase diagrams with arrows connecting data points (see Table 2) exhibits initial and final relative lipid compositions during gallstone dissolution by feeding 0.5% UDCA (top panel), 0.5% β-muricholic acid (middle panel), or chow (control) for 8 weeks (bottom panel). This system is in the same format and shows the same physical states at equilibrium, as described for Fig. 1. Of note is that three new phase diagrams were generated due to variable bile salt species proportions for mixtures of tauro-β-muricholate, taurocholate, and tauroursodeoxycholate in ratios of 0:20:80 (wt/wt/wt) (top panel), 85:15:0 (wt/wt/wt) (middle panel), and 50:50:0 (wt/wt/wt) (bottom panel). Lipid compositions of pooled gallbladder biles from mice that have formed cholesterol gallstones due to an 8-week feeding of the lithogenic diet were located in the central three-phase zone (see Fig. 1 and Table 1). Of note is that by feeding 0.5% UDCA and β-muricholic acid, relative lipid compositions of pooled gallbladder biles crossed crystallization path boundary *ab* from the central three-phase zone into Region E, explaining in part that gallstones are dissolved through liquid crystalline dispersion of cholesterol. The direction of the arrows demonstrates that mole% cholesterol decreased while gallstones were dissolved by feeding hydrophilic acids. However, relative biliary lipid compositions of pooled gallbladder biles from mice on chow were still in Region C, where at equilibrium the biles are composed of solid cholesterol crystals, liquid crystals, and saturated micelles. A square represents relative lipid compositions of pooled gallbladder biles at beginning in mice having preexisting gallstones, and a diamond represents relative lipid compositions of pooled gallbladder biles at end of gallstone dissolution study at week 8 of feeding chow (control); a circle indicates 0.5% UDCA; and a triangle 0.5% β-muricholic acid. See text for further description.

## DISCUSSION

In the present study, we investigated effects of β-muricholic acid on hepatic and biliary cholesterol metabolism and intestinal cholesterol absorption, as well as gallstone prevention and dissolution compared with UDCA in gallstone-susceptible C57L mice. The most important findings were *i*) β-muricholic acid prevented cholesterol gallstone formation through decreasing biliary cholesterol secretion, retarding phase transition of cholesterol, and inhibiting intestinal cholesterol absorption; *ii*) β-muricholic acid enhanced the dissolution rates of cholesterol gallstones via increasing a liquid crystals-containing phase; and *iii*) β-muricholic acid was more effective than UDCA in preventing or treating diet-induced or experimental cholesterol gallstone formation.

β-muricholic acid and UDCA are structurally similar with an equatorial 7β-hydroxy so that they display a similar extremely low monomer solubility and a poor micellar capacity for cholesterol (6, 15). The presence in β-muricholic acid of an additional hydroxy group in the 6β position further decreases the detergent properties of this steroid. The differences between the two bile acids for cholesterol solubilization are enhanced by the presence of phospholipids (15). Although the poor micellar properties of β-muricholic acid might lead to its poor solubilization and absorption in the intestine, it has been shown that this bile acid, like UDCA, can be well absorbed by the human intestine (30). Of note is that when administered to patients with cholesterol gallstones, UDCA constitutes only 50% of the biliary bile acid pool (8, 9), and is metabolized by intestinal microorganisms into lithocholic acid (10, 11), a hepatotoxic compound to laboratory animals (12). In contrast, in humans, β-muricholic acid fed is secreted into hepatic bile under glycine and taurine conjugated forms (30), and cannot be transformed by intestinal microflora (30, 31). This indicates that the β-muricholic acid fed is absorbed from the intestine, conjugated in the liver, and incorporated into the biliary bile salt pool in the human. These results show that β-muricholic acid can participate in the enterohepatic circulation and the presence of the 6β-hydroxy group protects it from 7-dehydroxylation in humans.

Because more hydrophobic bile salts (taurochenodeoxycholate and taurodexoycholate) replaced hydrophilic tauro-β-muricholate during the lithogenic diet feeding, all crystallization pathways are shifted to the right on the phase diagram (25), i.e., to higher phospholipid contents. This phase change facilitates solid cholesterol crystallization during lithogenesis of cholesterol-supersaturated biles in mice fed the lithogenic diet (14, 17). When hydrophilic bile acids together with the lithogenic diet were fed, tauro-β-muricholate was increased to 20–27% of bile salt pools, with a decrease in hydrophobic taurochenodeoxycholate or taurocholate, which induces all crystallization pathways shifted to lower phospholipid contents, as well as a smaller micellar zone and an expansion of Region E containing liquid crystals and saturated micelles but never solid cholesterol crystals (25). Under these conditions,

the enlarged Region E encapsulates lipid compositions of pooled gallbladder biles from mice fed β-muricholic acid and UDCA. This provides a physical-chemical explanation for the absence of solid cholesterol crystal formation in gallbladder biles of mice fed the lithogenic diet plus 0.5% β-muricholic acid (Fig. 1, bottom panel) although the biles display supersaturated with cholesterol (Table 1). Furthermore, the addition of 0.1–0.3% hyocholic acid, a very hydrophilic bile acid, to a lithogenic diet prevents cholesterol monohydrate crystals from forming in mice (32) and similarly hyodeoxycholic acid which is of approximate hydrophilicity to UDCA has been shown to prevent cholesterol gallstone formation in hamsters (33). In addition, feeding β-muricholic acid and UDCA induced bile compositions plotted principally in Region E (Fig. 3, top and middle panels), where liquid crystals that form with physiological phospholipid/bile salt ratios are highly stable and do not precipitate solid cholesterol crystals. Moreover, the capacity of β-muricholate to dissolve cholesterol is very low and the addition of phospholipid does not increase the micellar cholesterol solubility (15). In contrast, this bile salt favors the formation of vesicles. The growth of liquid crystals on the cholesterol monohydrate surface and their subsequent dispersion might occur during gallstone dissolution (15). Also, it has been shown that liquid crystalline dissolution, observed with ursodeoxycholate and its taurine and glycine conjugates, allows the transport of a great amount of cholesterol from stones (34, 35). Our results indicate, therefore, that in tauro-β-muricholate-rich bile, a liquid crystalline (mesophase formation) mechanism (36, 37), is highly like to be responsible for promoting the dissolution of cholesterol from gallstones.

Since β-muricholic acid suppresses cholesterol absorption from the small intestine significantly via tauro-β-muricholate (38), this should diminish the cholesterol content of the liver, which in turn decreases bio-availability of cholesterol for biliary cholesterol secretion (38). In contrast, the increase of cholesterol absorption observed with cholic acid feeding is followed by a large increase of biliary cholesterol, which finally induces cholesterol gallstone formation (14). Therefore, their distinct effects on cholesterol absorption may explain differences between the effects of β-muricholic acid and cholic acid on biliary cholesterol secretion. These results suggest the importance of the total input of cholesterol from the small intestine in regulating biliary cholesterol secretion in mice.

It has been suggested that hepatic HMG-CoA reductase activity is mediated through chylomicron remnant pathway (39), and dietary cholesterol curbs hepatic cholesterol synthesis by >95% in the rat (40). Other studies (41) suggest a direct effect of bile acids on hepatic HMG-CoA reductase activity. Consistent with previous studies (20, 42), these effects are less pronounced in C57L mice fed the lithogenic diet and do not reach statistically significant difference, confirming that C57L mice with susceptible *Lith* alleles resist down-regulation of HMG-CoA reductase activity by cholesterol or bile acids. Furthermore, our results showed that feeding β-muricholic acid alone or its addition to the lithogenic diet did not markedly alter the activities of hepatic cholesterol 7α-hydroxylase, suggesting that β-muricholic acid fails to inhibit cholesterol 7α-hydroxylase and a conversion of cholesterol to bile acids is not changed.

In conclusion, our studies show that β-muricholic acid is more effective than UDCA in preventing diet-induced cholesterol gallstones and in promoting the dissolution of gallstones in gallstone-susceptible C57L mice. Taken with the metabolism of β-muricholic acid in the human (30, 31), i.e., glycine and taurine conjugation by the liver, as well as the inability of intestinal microflora to 7-dehydroxylate the molecule, these characteristics give β-muricholic acid potentially interesting properties for preventing or treating cholesterol gallstones.

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