Arachidyl Amido Cholanoic Acid (Aramchol) Is a Cholesterol Solubilizer and Prevents the Formation of Cholesterol Gallstones in Inbred Mice[1]

Tuvia Gilat[a,b,*], Alicia Leikin-Frenkel[b], Ilana Goldiner[a], Hava Laufer[a], Zamir Halpern[a], and Fred M. Konikoff[a,b]

[a]Department of Gastroenterology, Sourasky Tel Aviv Medical Center, and [b]Minerva Center for Cholesterol Gallstones and Lipid Metabolism in the Liver, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

ABSTRACT: We have recently synthesized fatty acid bile acid conjugates (FABAC) that were able to reduce and retard cholesterol crystallization in model and human biles. When given orally, they prevented the formation of cholesterol crystals in the bile of hamsters. The aim of the present study was to determine whether the FABAC are cholesterol solubilizers, whether they can dissolve pre-existing crystals, whether they can prevent the formation of cholesterol gallstones, and to investigate the optimal type of bond between the fatty acid and bile acid. The presence of cholesterol crystals was determined by light microscopy, and the total crystal mass of precipitated crystals was measured by chemical means. Inbred (C57J/L) mice on a lithogenic diet were used to evaluate cholesterol crystal formation, dissolution, and gallstone formation *in vivo*. Arachidyl amido cholanoic acid (Aramchol) was the FABAC used in the present experiments. At equimolar amounts, the cholesterol-solubilizing capacity of Aramchol was higher than that of taurocholate and similar to that of phosphatidylcholine. The addition of Aramchol dissolved approximately 50% of pre-existing crystals in model bile solutions. The same phenomenon was demonstrated in human bile *ex vivo*, with a dose–response effect. All inbred mice developed cholesterol crystals in bile after 10–14 d on the lithogenic diet. Thereafter, supplementation of the diet with Aramchol progressively reduced the proportion of mice with crystals to 25% after 28 d. On the lithogenic diet, 100% of inbred mice developed cholesterol gallstones in the gallbladder by day 21. None of the mice whose diet was supplemented with 0.5 mg or 1.0 mg of Aramchol/d developed stones or crystals. FABAC are a new class of molecules that are cholesterol solubilizers and which are able to dissolve cholesterol crystals in bile. Upon oral administration, they dissolve pre-existing cholesterol crystals and prevent the formation of gallstones in gallstone-susceptible mice.

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Fatty acid bile acid conjugates (FABAC) are a new class of synthetic molecules produced with the aim of reducing cholesterol crystallization in bile (1). It has been demonstrated that phospholipids, and not bile acids, are the major cholesterol solubilizers in bile (2–5). The FABAC were designed to bring parts of the phospholipid molecule (long-chain saturated fatty acids) into bile, using the very efficient absorption and biliary secretion of bile acids. The whole FABAC molecule has, however, characteristics that are different from those of its components.

The FABAC, and in particular 3-β-arachidylamido-7-α, 12-α-dihydroxy-5-β-cholan-24-oic acid (arachidyl amido cholanoic acid, Aramchol, C_{20}-FABAC), were shown to prolong the cholesterol nucleation time markedly and reduce the total crystal mass in model bile solutions (1). In native human gallbladder bile, FABAC were even more effective, preventing crystal formation for weeks and reducing the eventual crystal mass to minute proportions. Aramchol was also shown to prevent *in vivo* biliary cholesterol crystallization in experimental animals (1). Thus, almost three decades after the introduction of chenodeoxycholic acid for gallstone dissolution (6), FABAC raise again the possibility of a medical therapy for the prevention and/or dissolution of cholesterol gallstones. Many questions in relation to the mechanism of action and metabolism of the FABAC remain open. The present study was designed to answer four main questions: (i) Do FABAC act as cholesterol solubilizers in bile? (ii) Can they dissolve formed, pre-existing cholesterol crystals *in vitro* and *in vivo*? (iii) Can they prevent the formation of cholesterol gallstones *in vivo*? (iv) Does the kind of conjugation (ester vs. amide) influence the absorption and biliary secretion of the FABAC?

The experiments were performed with Aramchol, which is a conjugate of arachidic and cholic acid (at position 3) using an amide bond. This compound in particular was found to effectively inhibit *in vitro* cholesterol crystallization in our earlier study (1).

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC), 99% pure, was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Free fatty acids (FFA), cholesterol, sodium taurocholate (TC), and cholic acid (CA), >98% pure, were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol, TC, and CA were recrystallized prior to use. All other chemicals and

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*To whom correspondence should be addressed at 10 Nehardea St., Tel Aviv 64235, Israel. E-mail: tuvgilat@netvision.net.il

Abbreviations: Aramchol, arachidyl amido cholanoic acid; CA, cholic acid; CSI, cholesterol saturation index; FABAC, fatty acid bile acid conjugates; FFA, free fatty acid; PC, phosphatidylcholine; TC, taurocholate; UDCA, ursodeoxycholic acid.

solvents were American Chemical Society or reagent grade. The glassware was acid washed, thoroughly rinsed in distilled water, and dried.

Preparation of Aramchol. Aramchol was prepared as previously described (1) by conjugating cholic acid with arachidic acid (C_{20}) in the 3-position, using an amide bond. The conjugation was in the β configuration. For the absorption studies (see below) an ester conjugate was similarly prepared. Both compounds were purified by silica gel chromatography and characterized by ^{1}H nuclear magnetic resonance and mass spectrometry.

Model biles. The model biles were prepared from a mixture of cholesterol (in chloroform), PC (in chloroform), and TC (in methanol) in various concentrations (specified in the Results section), as previously described (7). The model bile solution was incubated under argon at 37°C, and aliquots were taken for analysis at predetermined times throughout the crystallization process.

Human bile. Human gallbladder biles were obtained from gallstone patients at cholecystectomy. The bile was aspirated after needle puncture of the gallbladder prior to mobilization of the gallbladder. Informed consent was obtained according to a protocol approved by the local institutional human subjects committee. Biles were ultracentrifuged (200,000 × *g*, 45 min, 25°C) prior to testing to get rid of crystals and cell debris.

Solubilization experiments. (i) Cholesterol-solubilizing capacity. Model biles (30 mM PC, 150 mM Na-TC) were prepared with two different cholesterol concentrations (12.2 and 9.9 mM) to yield a supersaturated [cholesterol saturation index (CSI), 1.1] and an undersaturated (CSI, 0.9) bile solution, respectively. They were then incubated overnight with 10-mM ^{3}H-labeled dried cholesterol. The ^{3}H-cholesterol solubilization after 24 h of incubation at 37°C was measured. The amount of cholesterol remaining in the supernatant solution after ultracentrifugation for 5 min at 76.000 × g_{max} was the parameter for solubilization. Solubilization of cholesterol in the model bile was compared to that observed in biles that were supplemented with 5 mM Na-TC, PC, or Aramchol.

(ii) Dissolution of pre-existing cholesterol crystals in bile. Model bile (as above) or native bile from a cholesterol gallstone patient (after ultracentrifugation for 1 h at 200,000 × g_{max} at room temperature to get rid of debris or crystals) was incubated at 37°C. After crystallization, *in vitro* or *ex vivo*, had progressed and reached an apparent equilibrium (no further increase in crystal mass), incubation was continued without as well as after adding FABAC. Serial microscopic observations and determinations of total crystal mass were performed.

Evaluation of cholesterol crystal formation and growth. (i) Light microscopy. Aliquots (5 μL) of bile or model bile were examined by a light microscope (Zeiss, Jena, Germany) under polarized light and with the differential interference mode as previously described (7). Cholesterol crystals were identified by their characteristic morphology and birefringence, and counted per microscopic field at 100-fold magnification.

(ii) Measurement of crystal mass. Chemical analyses of cholesterol were performed on model bile samples after *in situ* crystallization, as previously described (8). In human biles the mass of the pelleted crystals was quantitated after ultracentrifugation.

Animal experiments. All animal experiments related to cholesterol crystallization were performed with male inbred mice (C57J/L, 4 wk old, approximately 20 g). Cholesterol crystal and gallstone formation was induced by feeding the mice a lithogenic diet containing regular chow (Koffolk, Petach Tikva, Israel), to which butterfat 15%, cholesterol 1%, cholic acid 0.5%, and corn oil 2% (w/w) were added (9).

Dissolution of preformed crystals. After 10 and 14 d on the lithogenic diet, a group of mice was sacrificed and gallbladder bile was examined to ascertain the presence of cholesterol crystals. Subsequently, in the remaining animals, Aramchol mixed with saline was administered by gavage (*via* a feeding needle) at a dose of 3 mg/animal/d. Half of the remaining animals were sacrificed after 14 d and the rest after 28 d of Aramchol feeding. The presence of cholesterol crystals and stones in the gallbladder was examined at each time interval.

Prevention of gallstone formation. Mice were sacrificed after 3 wk of consuming the lithogenic diet with or without Aramchol (0.5 or 1.0 mg/animal/d). The percentage of animals harboring gallstones (and cholesterol crystals) in their gallbladders was determined by direct inspection and microscopy of their gallbladders.

Comparison of absorption and biliary secretion of ester- and amide-bonded conjugates. Male C57Black mice received 3 mg/animal of FABAC by intragastric administration; and heart blood, portal blood, and gallbladder bile were sampled in groups of three animals sacrificed after 1, 2, and 3 h. One group (n = 9) received the regular Aramchol, with an amide (NH) bond between the bile acid and the fatty acid. Another group (n = 9) received the compound with an ester bond. In a separate experiment, animals (3 in each group) were sacrificed 24 h after the intragastric administration of the ester or amide conjugate

Analytical methods. Biliary lipids were extracted by chloroform/methanol (2:1, vol/vol) and quantitated as previously described (10–12). Aramchol concentration was determined by high-performance liquid chromatography (Kontron) employing a Phenomenex Luna reversed-phase C-18 column as previously described (1). Samples were applied dissolved in methanol. The running phase was methanol 100%, at a flow rate of 0.9 mL/min. Aramchol was detected at 206 nm.

Statistical analysis. Student's t test was used to compare the data. A P-value of <0.05 was considered significant.

RESULTS

The cholesterol-solubilizing capacity of Aramchol was studied in two model bile solutions—one undersaturated (CSI, 0.9) and the other supersaturated (CSI, 1.1) with cholesterol. The amounts of cholesterol solubilized by Aramchol in excess of the amount solubilized by the model solutions alone were 0.9 and 1.4 mM, respectively. The corresponding amounts solubilized by NaTC were 0.6 and –0.4 mM, while those by PC were

0.9 and 0.9 mM, respectively. Thus, the cholesterol-solubilizing capacity of Aramchol was higher than that of NaTC and similar to that of PC. The effect was more marked in the supersaturated model.

The ability of Aramchol to dissolve (preformed) cholesterol crystals was studied in two model biles (model A: 15 mM cholesterol, 30 mM PC, 150 mM Na-TC; model B: 18 mM cholesterol, 36 mM PC, 120 mM Na-TC). Aramchol was added at a concentration of 7 mM to model A and at 10 mM to model B, based on the maximal solubility of Aramchol in these models. As shown in Figure 1, the cholesterol crystal mass in both model biles decreased after addition of Aramchol, from 4.4 to 2.7 μmol in model A ($P = 0.28$) and from 7 to 2.9 μmol in bile B ($P = 0.0004$), respectively. Figure 2 shows the effect of Aramchol on preformed cholesterol crystals in human bile. In the native bile, the addition of Aramchol also decreased the crystal mass significantly. The effect was dose-dependent in the dose range studied (10–30 mM). Fourteen days after the addition of Aramchol (10 and 30 mM), the total crystal mass decreased from 2.4 ± 0.1 to 1.5 ± 0.1 μmol ($P < 0.02$) and to 0.1 ± 0.01 μmol ($P = 0.01$), respectively. The decreases corresponded to 38 and 94%, respectively. Three human biles were studied. In all of them, the addition of 30 mM Aramchol resulted in the disappearance of plate-like cholesterol crystals while decreasing the total crystal mass to 6–62% of control. In two-thirds of the cases, the addition of Aramchol converted the plate-like (mature) crystals to filamentous (less mature) crystal forms (13) and reduced the total crystal mass in all three.

The ability of Aramchol to dissolve preformed cholesterol crystals *in vivo* was studied in inbred mice. Two sets of experiments, with a total of 36 mice, were performed. Figure 3 shows the percentage of mice with crystals in their gallbladders as a function of time of feeding the lithogenic diet with and without Aramchol. On days 10 and 14 all sacrificed animals ($n = 7$ and 8, respectively) had crystals in their gallbladders. However, when Aramchol (at 3 mg/animal/d) was then added to the diet, the percentage of animals with crystals gradually dropped to 75% on day 28 ($n = 12$) and to 25% on

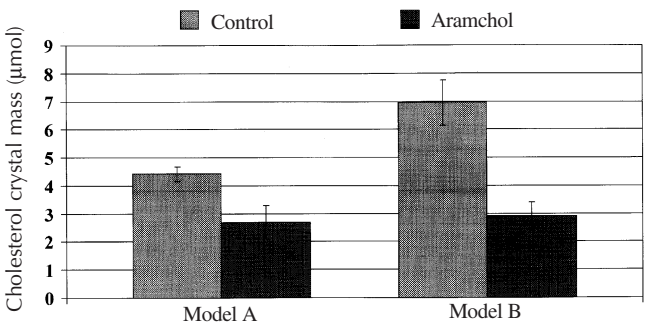

FIG. 1. Cholesterol crystal mass after 19 d of incubation of pre-existing crystals in model biles (model A: 15 mM cholesterol, 30m M egg lecithin, 150 mM Na-taurocholate; model B: 18 mM cholesterol, 36 mM egg lecithin, 120 mM Na-taurocholate) in the absence or presence of Aramchol (model A: 7 mM, model B: 10 mM; $n = 3$ for both). The decrease was 39% ($P = 0.28$) in model A and 58% ($P = 0.0004$) in model B.

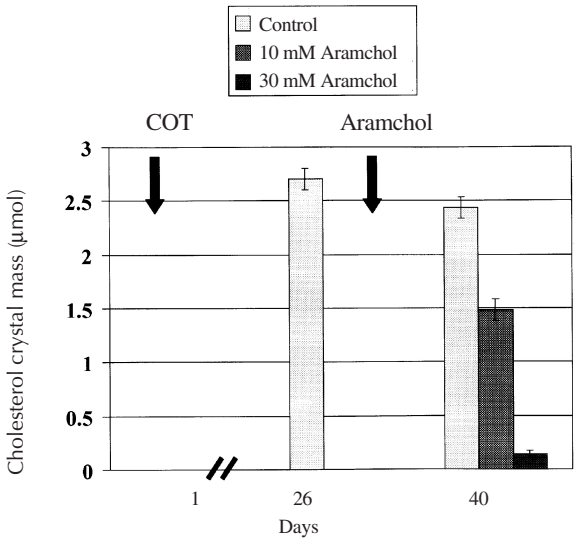

FIG. 2. Cholesterol crystal solubilization in human bile *ex vivo* by Aramchol, dose–response effect. Bile from a cholesterol gallstone patient was incubated at 37°C until apparent crystallization equilibrium (day 26). Incubation was then continued for another 14 d in the absence or presence of 10 or 30 mM Aramchol. Microscopic observations and determinations of the final crystal mass were performed. Aramchol decreased the crystal mass by 38 (grey bar: 10 mM) and 94% (black bar: 30 mM). COT, crystal observation time.

day 42 ($n = 9$). This observation contrasts with that following a continuation of the lithogenic diet without any other manipulations, which yields more crystals, and eventually stones (data not shown; Ref. 9).

The ability of Aramchol to prevent gallstone formation *in vivo* was also studied in inbred mice ($n = 17$). As seen in Figure 4, feeding of Aramchol together with the lithogenic diet completely prevented the formation of gallstones (as well as

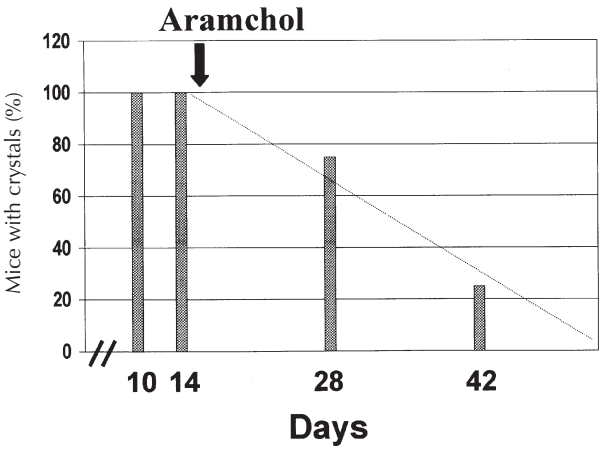

FIG. 3. Effect of fatty acid bile acid conjugate feeding on pre-formed cholesterol crystals *in vivo*. All mice ($n = 36$) were fed a lithogenic diet throughout the experiment. Seven animals were sacrificed after day 10 and another 8 after day 14: All had cholesterol crystals in their gallbladders. Thereafter, Aramchol (3 mg/d) was added to their diet. In these Aramchol-supplemented mice ($n = 21$), the proportion with crystals declined progressively to 75% after 14 d and to 25% after 28 d of Aramchol feeding.

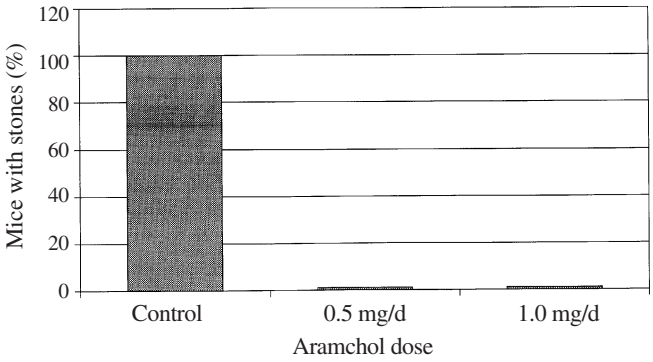

FIG. 4. Effect of Aramchol feeding on cholesterol gallstone formation *in vivo*, expressed as a percentage of inbred mice harboring cholesterol gallstones in their gallbladders after 3 wk of feeding a lithogenic diet, with or without Aramchol (0.5 or 1.0 mg/animal/d). At the end of the experiment, none of the Aramchol-fed mice ($n = 10$) had stones (or crystals) in their gallbladders; stones were found in all the controls ($n = 5$).

crystals) in the mice at both doses studied (0.5 and 1.0 mg/animal/d). All control animals developed both crystals and cholesterol gallstones by day 21, whereas none of the Aramchol- supplemented animals had either crystals or gallstones. The concentrations of Aramchol in the gallbladder bile of mice fed 0.5 mg/d varied between 0.03 and 0.17 mM (≥24 h after the last dose).

Bile and heart blood levels of amide- and ester-bonded Aramchol after a single intragastric dose of 3 mg/mouse are shown in Figure 5. In mice receiving the amide-bonded Aramchol, bile levels after 1, 2, and 3 h were 0.3 ± 0.02, 0.4 ± 0.03, and 0.7 ± 0.06 mM, respectively. In mice receiving the compound with an ester bond between the arachidic and cholic acids, bile levels remained below 0.07 mM at all times. Blood levels in the animals receiving the ester-bonded

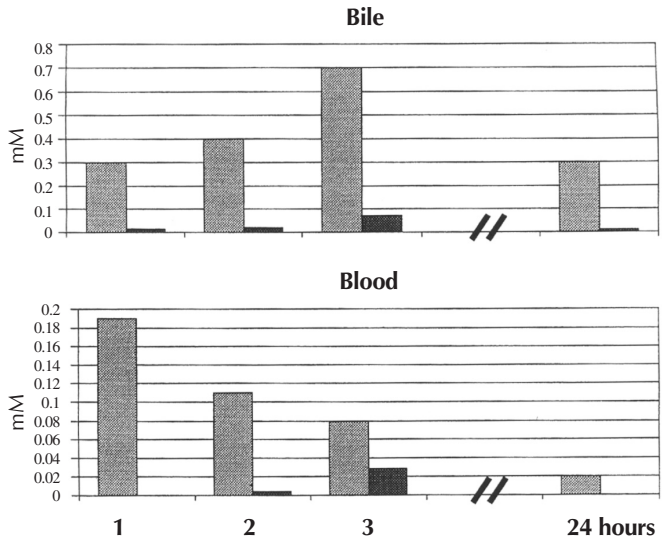

FIG. 5. Bile and heart blood levels of amide- (gray bars) and ester- (black bars) bonded Aramchol after a single intragastric administration of 3 mg/animal at 0 time. Three animals were tested at each time point with either conjugate. A separate experiment was performed to test levels at 24 h.

FABAC were below 0.03 mM at all times, whereas in the animals receiving the amide-bonded compound blood levels were mostly between 0.1 and 0.2 mM. Portal blood levels were also significantly higher with the amide-bonded Aramchol (0.1–0.3 mM) compared to the ester-bonded compound (0.001–0.1 mM). Twenty-four hours after an intragastric administration of 3 mg/animal, bile levels were 0.3 ± 0.03 mM with the amide and 0.01 ± 0.001 mM with the ester bond, while heart blood levels were 0.02 ± 0.002 and <0.0001 mM, respectively (Fig. 5).

DISCUSSION

Aramchol represents a new group of synthetic molecules (FABAC), which were recently shown to inhibit cholesterol crystallization (1). In our previous study we showed that they prolong the crystal observation time and decrease the crystal mass in model and human biles. The FABAC were absorbed after intragastric administration and prevented crystal formation in experimental animals. Their mode of action (solubilization vs. inhibition of crystallization) was not studied. The present study clearly indicates that Aramchol is a cholesterol solubilizer. *In vitro* it dissolves solid amorphous cholesterol when added to either an undersaturated or supersaturated model bile solution. Aramchol dissolves formed cholesterol crystals in model bile and also in human gallbladder bile *ex vivo*. We have now demonstrated, for the first time, that upon oral administration it can dissolve preformed cholesterol crystals *in vivo* and, most significantly, prevent the development of cholesterol gallstones in inbred mice.

Many of the structure/function relationships of the FABAC have now been elucidated. Those with longer-chain fatty acids (C_{18}–C_{22}) were the most potent in terms of cholesterol solubilization (1). FABAC containing shorter-chain fatty acids (C_6–C_{12}) were not effective, while those with C_{14}, C_{16}, and C_{24} fatty acids had a smaller effect (1). The Aramchol used in the present experiments is one of the more effective compounds.

Conjugation with cholic acid (at position 3) results in a dihydroxy compound. Conjugation with a dihydroxy bile acid (e.g., ursodeoxycholic acid) would result in a monohydroxy compound, which could be toxic. However, this is at the moment a speculative consideration, which is being further studied. Our results show that the bond between the fatty acid and the bile acid is very important. An ester bond is easily broken down by intestinal enzymes and bacteria, resulting in the separate absorption of a fatty acid and a bile acid. Thus, little of the intact ester conjugate reaches the bile (Fig. 5). Further breakdown may occur during enterohepatic cycling. Esters of fatty acids with bile acids have been found in the feces (14). These compounds have also been synthesized by Kritchevsky's group (15) in an attempt to delay the intestinal catabolism of chenodeoxycholic and ursodeoxycholic acids. The amide bond used in our experiments is a more stable bond, allowing the absorption and biliary secretion of the intact FABAC. The amide bond is, however, not exclusive. Any stable bond, permitting the absorption and biliary secretion of the intact

FABAC and providing effective cholesterol solubilization, is suitable. The bond between the fatty acid and bile acid can be *via* a bonding molecule, or it can be a direct bond (C=C).

It should be noted that the mechanism of action of the FABAC is quite different from that of chenodeoxycholic and ursodeoxycholic acids. These specific bile acids reduce the mole percentage of cholesterol in hepatic bile by an effect on biliary enzymes and/or cholesterol absorption in the gut (16). They are not cholesterol solubilizers. The direct, *ex vivo* addition of bile salts to human gallbladder bile does not prolong the nucleation time, but the addition of a cholesterol solubilizer, like phospholipids, markedly prolongs the nucleation time. This has been demonstrated by Jungst *et al.* (2). As shown in the present investigation, FABAC are cholesterol solubilizers. This was demonstrated *in vitro* where any potential effects on liver and intestines was excluded.

The cholesterol-solubilizing effect of FABAC was stronger in human bile as compared to model biles. In human bile FABAC have an effect at concentrations of 3–5 mM while in model biles concentrations of up to 30 mM are required (1). This higher effectiveness in human bile has now been confirmed many times, but the explanation is not clear at the moment.

Another major difference was observed in relation to the effect of FABAC on bile *in vivo* and *ex vivo*. To prolong the nucleation time and reduce the crystal mass in bile *ex vivo*, concentrations of 3–5 mM were required (1). To dissolve pre-existing crystals in bile *ex vivo* (using a single addition of FABAC to bile), concentrations of 10–30 mM were required. They dissolved approximately 50% or more of pre-existing crystals, usually converting the remaining crystals to earlier crystal forms (plates to filaments) (Fig. 2). After the administration of a single oral dose of 3 mg/mouse of Aramchol, biliary concentrations were 0.3–0.5 mM after 2 h and 0.1–0.6 mM after 3 h. On chronic oral administration of 3 mg/d of Aramchol, the concentrations in gallbladder bile (≥24 h after the last dose) were of the order of 0.2–0.6 mM. The dissolution of the pre-existing and continuously produced crystals was progressive (Fig. 3), with only 25% of animals having crystals after 28 d. An extrapolation of the curve predicts complete disappearance of crystals approximately 33 d after the start of Aramchol ingestion. The oral administration of 0.5 mg/d prevented the formation of crystals and gallstones in the gallbladder (Fig. 4). This was accomplished with biliary concentrations (≥24 h after the last dose) in the range of 0.03–0.17 mM.

The difference between the 3–5 mM effective in bile *ex vivo* and the 0.1–0.6 mM effective *in vivo* is marked. No definite explanation is presently available. An additional effect of the FABAC on liver, gallbladder, or intestine cannot be excluded. Studies using mass spectrometry demonstrated intact FABAC in blood and bile (Leinkin-Frenkel, A., unpublished data). We have at present no evidence for the presence of metabolites. Further research is ongoing.

The lithogenic tendency in inbred mice on a high-cholesterol diet is very strong. Under these conditions, close to 100% of the mice develop cholesterol crystals in bile within 7–10 d and gallstones within 3–8 wk (9). In human models, the process takes much longer and is less effective. During pregnancy, biliary sludge is formed in almost one-third of women but mostly during the second or third trimester, i.e., 4–9 mon (17). Cholesterol crystals are a major component of biliary sludge. In obese subjects ingesting a very low calorie diet or undergoing gastroplasty, gallstones develop in up to one-third of subjects, although usually only after several months (18). Also, patients receiving octreotide take months to develop gallstones and even then are in only some of the patients (19). The fact that FABAC almost completely prevent the formation of crystals and stones in lithogenic mice and accomplish this within 1–3 wk of ingestion suggests that their solubilizing effect is strong. Moreover, this is accomplished at doses very close to the doses (on a molar equivalence basis) used in bile salt therapy in humans (15 mg/kg/d) (16).

Unlike bile salts, FABAC are found in the systemic circulation at concentrations similar to or higher than those in the portal vein (1). This is probably due to transport *via* the lymph. Moreover, after a single oral dose they circulate in the vascular tree for over 48 h. In view of their cholesterol-solubilizing capacity, this raises the possibility of their applicability in atherosclerosis as well.

The use of chenodeoxycholic acid and ursodeoxycholic acid (UDCA) proved that cholesterol gallstones could be dissolved (16). The process was, however, too long and of low efficacy. It was further shown that for medium- and large-sized stones lithotripsy was needed. The addition of UDCA after lithotripsy did not accelerate fragment evacuation and/or dissolution (20). More importantly, UDCA therapy did not effectively prevent gallstone recurrence after successful non-surgical therapy (21,22). FABAC therapy has the potential for correcting some of these shortcomings.

In summary, the data to date suggest that FABAC may be a potential medical therapy to prevent and/or to dissolve cholesterol gallstones in humans.

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