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The cholecystokinin-1 receptor antagonist devazepide increases cholesterol cholelithogenesis in mice

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Abstract

Background—A defect in gallbladder contraction function plays a key role in the pathogenesis of gallstones. The cholecystokinin-1 receptor (CCK-1R) antagonists have been extensively investigated for their therapeutic effects on gastrointestinal and metabolic diseases in animal studies and clinical trials. However, it is still unknown whether they have a potential effect on gallstone formation.

Design—To study whether the CCK-1R antagonists enhances cholelithogenesis, we investigated cholesterol crystallization, gallstone formation, hepatic lipid secretion, gallbladder emptying function, and intestinal cholesterol absorption in male C57BL/6J mice treated by gavage with devazepide (4 mg/day/kg) or vehicle (as controls) twice per day and fed the lithogenic diet for 21 days.

Results—During 21 days of feeding, oral administration of devazepide significantly accelerated cholesterol crystallization and crystal growth to microlithiasis, with 40% of mice forming gallstones. Whereas, only agglomerated cholesterol monohydrate crystals were found in mice receiving vehicle. Compared to the vehicle group, fasting and postprandial residual gallbladder volumes in response to the high-fat meal were significantly larger in the devazepide group during cholelithogenesis, showing reduced gallbladder emptying and bile stasis. Moreover, devazepide significantly increased hepatic secretion of biliary cholesterol, but not phospholipids or bile salts. The percentage of intestinal cholesterol absorption was higher in devazepide-treated mice, increasing bioavailability of chylomicron-derived cholesterol in the liver for biliary hypersecretion into bile. These abnormalities induced supersaturated bile and rapid cholesterol crystallization.

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Conclusions—The potent CCK-1R antagonist devazepide increases susceptibility to gallstone formation by impairing gallbladder emptying function, disrupting biliary cholesterol metabolism, and enhancing intestinal cholesterol absorption in mice.

Keywords

bile salts; biliary secretion; cholesterol crystallization; gallbladder motility; gallstones; mucin

INTRODUCTION

Cholecystokinin (CCK) is a member of a family of neuroendocrine peptide hormones and is synthesized by the discrete endocrine I-cells within the mucosa in the small intestine. It stimulates gallbladder contraction, regulates pancreatic enzyme secretion and growth, promotes insulin secretion, inhibits gastric emptying and gastric acid secretion, and reduces food intake [1]. Although yet to be proved physiologically in humans, it has been found that CCK accelerates small intestinal transit and regulates glucose metabolism in animals [2, 3]. In humans, CCK is composed of varying numbers of amino acids, depending on posttranslational modification of the *CCK* gene product, preprocholecystokinin. As a result, CCK is actually a family of hormones identified by the number of amino acids, with CCK-58, CCK-33, and CCK-22 being the dominant forms in plasma and CCK-8 being the major transmitter form [4, 5]. It has been found that plasma CCK concentrations are significantly increased after ingestion of a meal, promoting the secretion of hepatic bile and stimulating the contraction of the gallbladder and the relaxation of the Sphincter of Oddi [6, 7]. All of these changes lead to the delivery of bile, including bile salts and other biliary lipids, into the small intestine for aiding in the digestion and absorption of dietary cholesterol and fatty acids.

Devazepide (L-364,718 or MK-329) is a potent non-peptide CCK antagonist selective for the CCK-1 receptor (CCK-1R) subtype and is structurally derived from the benzodiazepine family, but with quite different actions from most benzodiazepines [8-10]. It increases appetite and accelerates gastric emptying [11, 12], and may act as a potential drug for the treatment of a variety of gastrointestinal disorders [13]. It is also widely used in scientific research into the investigation of physiological and biological functions of CCK-1R [14, 15]. In addition, other potential CCK-1R antagonists, including lorglumide, loxiglumide, dexloxiglumide, proglumide, A-65186, pranazepide, tarazepide, SC-50,998, and SR-27897 (linitript), have been developed and are being investigated for their therapeutic effects on gastrointestinal diseases in animal studies and clinical trials [16].

It is widely appreciated that CCK-1R is expressed in the gallbladder smooth muscle cells and plays a key role in regulating gallbladder motor function [17]. Clinical studies have clearly demonstrated that impaired gallbladder emptying function often leads to bile stasis that accommodates time for promoting cholesterol nucleation and crystallization and for forming biliary sludge (a precursor to gallstones). Biliary sludge found by ultrasonography is one of the most common biliary features in women during pregnancy, in obese patients during rapid weight reduction, in subjects exposed to high levels of estrogen, and in patients on total parenteral nutrition (TPN) [18]. We have reported that the deletion of either the

Cck-1r or the *Cck* gene impairs gallbladder contraction function, thus leading to gallbladder stasis which, in turn, promotes cholesterol crystallization and the growth and agglomeration of solid cholesterol crystals into microlithiasis and macroscopic stones in mice [2, 19]. These studies provided the molecular mechanism underlying the therapeutic effect of CCK-8 or sincalide, the synthetic C-terminal octapeptide fragment of CCK, on the prevention of TPN-induced gallbladder disease [20, 21].

Although many therapeutic benefits of the CCK-1R antagonists have been found in animal studies and clinical trials, it is still unknown whether they cause sluggish gallbladder and bile stasis by reducing gallbladder contractility. In the present study, we investigated whether devazepide, a very commonly used CCK-1R antagonist, promotes cholesterol crystallization and gallstone formation by disrupting biliary cholesterol metabolism and impairing gallbladder contraction function in gallstone-susceptible C57BL/6J mice.

MATERIALS AND METHODS

Animals and diets

The inbred C57BL/6J mice of both genders from the Jackson Laboratory (Bar Harbor, ME) were bred to generate male mice for the studies. Mice at 8-10 weeks of age were fed normal rodent chow (Harlan Teklad F6 Rodent Diet 8664, Madison, WI) containing a trace (<0.02%) amount of cholesterol, or a lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat for 21 days. All animals were maintained in a temperature-controlled room (22±1°C) with a 12-hour day cycle (6:00 AM-6:00 PM). All procedures were in accordance with current NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University (Boston, MA).

Microscopic studies of cholesterol crystallization and gallstones

To explore whether the CCK-1R antagonists increase gallstone formation by accelerating cholesterol crystallization, mice were treated by gavage with devazepide [(3S)(2*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide] (Tocris, Ellisville, MO) at 0 or 4 mg/day/kg body weight twice per day and fed the lithogenic diet for 21 days. Devazepide was dissolved in 5% dimethyl sulfoxide (DMSO), followed by 5% Tween 80 and 90% 0.15 M NaCl. The vehicle containing 5% DMSO, 5% Tween 80, and 90% 0.15 M NaCl was used as controls.

After mice were fasted overnight and anesthetized with pentobarbital, cholecystectomy was performed. Gallbladder bile was collected before (day 0, on chow) and at frequent intervals after feeding the lithogenic diet for 3, 5, 7, 14, and 21 days for cholesterol crystallization and gallstone studies (n=5 per group for each time point). The entire gallbladder bile was examined by phase contrast and polarized light microscopy at room temperature (~22°C) for the presence of mucin gel, liquid crystals, solid cholesterol crystals, sandy stones, and gallstones [22]. Mucin gel was observed as non-birefringent amorphous strands. Arc-like and tubular crystals (assumed to be metastable transitional forms of anhydrous cholesterol being hydrated to cholesterol monohydrate crystals), solid plate-like cholesterol monohydrate crystals, as well as small, aggregated and fused liquid crystals were defined

according to previously established criteria [23]. Sandy stones were irregularly shaped, and easily disintegrable agglomerates of cholesterol monohydrate crystals surrounded by mucin gel. As visualized under the microscope, individual cholesterol monohydrate crystals projected clearly from the edges of sandy stones, and grossly they displayed a yellow color. Gallstones were hard, ball-like objects, and light yellow in color with smooth curved surfaces. Because of scattered and absorbed light, they appeared as opaque, and black in color when observed by polarizing light microscopy. The images of cholesterol monohydrate crystals and gallstones were analyzed by a Carl Zeiss Imaging System with an AxioVision Rel 4.6 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). After microscopic analysis, gallbladder bile was frozen and stored at -20°C for lipid analyses.

Cannulation of the common bile duct and collection of hepatic bile

For biliary lipid secretion study, fasted mice ($n=5$ per group) were anesthetized with pentobarbital. After cholecystectomy was performed, the common bile duct was cannulated via a PE-10 polyethylene catheter. Hepatic bile was collected by gravity during the first hour of acute fistulation for measurement of biliary lipid secretion rates and molecular composition of the bile salt pool [24]. During surgery and bile collection, mouse body temperature was maintained at $37\pm0.5^{\circ}\text{C}$ with a heating lamp and monitored with a thermometer. After determining hepatic bile volume by weighing and employing a specific density of 1, bile samples were frozen and stored at -20°C for later lipid analyses.

Gallbladder contract function

To examine the effect of the CCK-1R antagonist devazepide on gallbladder motor function, a dynamic method for measuring gallbladder emptying function was performed in mice in response to a high-fat meal after 14 days of feeding the lithogenic diet. After mice ($n=5$ per group) were fasted overnight but had free access to water, they were anesthetized with pentobarbital. During laparotomy, a PE-10 polyethylene catheter was inserted into the duodenum. The duodenal catheter was externalized through the left abdominal wall and connected to an infusion pump (Kent Scientific, Litchfield, CT). Following completion of all surgical procedures, the gallbladder was clearly exposed and its size was immediately measured with a micro-caliper. Gallbladder volume (i.e., the fasting gallbladder size) was calculated by assuming an ellipsoid shape of the organ as described elsewhere [19]. Then, mice were intraduodenally infused with corn oil (i.e., a high-fat meal) at $40\text{ }\mu\text{L/minute}$ for 5 minutes. After the duodenal infusion, postprandial gallbladder volume was measured with a micro-caliper every 10 minutes for 30 minutes. Gallbladder emptying rate was determined by a difference in gallbladder volume before and after the duodenal infusion of corn oil.

Biliary lipid analysis

Total bile salt concentration was determined by the 3α -hydroxysteroid dehydrogenase method [25]. Individual bile salt species were analyzed by HPLC [26] and hydrophobicity index of bile samples was calculated according to Heuman's method [27]. Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett [28]. Biliary cholesterol was measured using an enzymatic assay [29]. Cholesterol saturation index (CSI) of pooled gallbladder bile was calculated from critical tables [30]. Relative lipid composition of pooled gallbladder bile was plotted on condensed phased diagrams. For

graphic analysis, the phase limits of the micellar zone and the crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37°C and at a total lipid concentration of ~9 g/dL [23].

Measurement of intestinal cholesterol absorption by a fecal dual-isotope ratio method

Under chow feeding conditions, nonfasted and nonanesthetized mice (n=5 per group) were given by gavage an intragastric bolus of 150 µL of medium-chain triglyceride containing 1 µCi of [¹⁴C]cholesterol and 2 µCi of [³H]sitostanol. Mice were then transferred to individual metabolic cages with wire mesh bottoms where they continued to ingest the chow diet for the next 4 days. During this period, mouse feces were collected daily. The radioactive isotopes from 4-day pooled fecal samples were saponified, extracted, and counted according to previously established methods [31]. The ratio of the two radiolabels in the fecal extracts and in the dosing mixture was used for calculation of percent cholesterol absorption by using the following formula:

$$\% \text{ Cholesterol absorption} = \left(\frac{\left[\frac{[^{14}\text{C}]}{[^{3}\text{H}]} \right]_{\text{dosing mixture}} - \left[\frac{[^{14}\text{C}]}{[^{3}\text{H}]} \right]_{\text{feces}}}{\left[\frac{[^{14}\text{C}]}{[^{3}\text{H}]} \right]_{\text{dosing mixture}}} \right) \times 100$$

Quantitative real-time PCR assay

Total RNA was extracted from gallbladder and small intestine tissues (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reaction was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) with 1 µg of total RNA and random hexamers to generate cDNA. Primers and probes for mouse *Muc1*, *Muc2*, *Muc3*, *Muc4*, *Muc5ac*, *Muc5b*, *Abcg5*, *Abcg8*, *Npc1l1*, *Cck-1r*, *Acat2*, *Sr-b1*, and *Hmgcr* genes have been described elsewhere [32]. Real-time PCR assays for all samples were performed in triplicate. To obtain a normalized target value, the target amount was divided by the endogenous reference rodent glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as the invariant control (Part No. 4308313, Applied Biosystems, Foster City, CA).

Statistical method

All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test, Mann-Whitney U-tests, or Chi-square tests. If the *F*-value was significant, comparisons among groups of mice were further analyzed by a multiple comparison 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

Effect of devazepide on cholesterol crystallization and gallstone formation

As shown in **Figure 1**, oral administration of devazepide twice per day significantly accelerated the precipitation of solid cholesterol monohydrate crystals and the formation of gallstones in mice fed the lithogenic diet for 21 days. In the vehicle group, mucin gel and some aggregated and fused liquid crystals were detected after 3 and 7 days of feeding,

respectively. At day 14, typical cholesterol monohydrate crystals, with 79.2° and 100.8° angles, and often a notched corner were observed by polarizing light microscopy. By 21 days, many solid cholesterol monohydrate crystals agglomerated together to form larger clusters. Although the cholesterol crystallization sequence was similar between the vesicle and the devazepide groups, the crystallization and growth of solid cholesterol crystals, as well as the development of gallstones were dramatically accelerated in devazepide-treated mice. After 7 days of feeding, aggregated cholesterol monohydrate crystals surrounded by nonbirefringent amorphous mucin gel were observed. At day 14, disintegratable amorphous sandy stones were encircled by mucin gel, with individual cholesterol crystals projected from the edges. By day 21, real gallstones formed, which exhibited rounded contours and black centers from light scattering/absorption. Moreover, at 21 days on the lithogenic diet, the prevalence of gallstones was 40% in the devazepide group. The stone sizes were between 1 and 2 mm and the frequency distribution of stone number fell between 2 and 6. However, no gallstones were found in the vehicle group.

Table 1 lists the relative biliary lipid composition of pooled gallbladder bile from mice treated by gavage with devazepide (4 mg/day/kg) or vehicle (as controls) twice per day and fed the lithogenic diet for 21 days. We found that the CSI value reached supersaturated after 5 days of feeding the lithogenic diet in devazepide-treated mice, which is 9 days earlier than that in mice receiving vehicle. For the purposes of illustration, two truncated phase diagrams (**Figure 2**) for pooled gallbladder bile were created according to an average total lipid concentration of ~ 9.0 g/dL. During the 21-day period of lithogenic diet feeding, relative lipid composition of pooled gallbladder bile gradually moved upward and to the right of the phase diagrams in both the devazepide and the vehicle groups. This shift was induced by an increase in biliary cholesterol concentrations, a relative increase in biliary phospholipid concentrations, and a relative decrease in biliary bile salt concentrations. In the vehicle group, relative biliary lipid composition between day 0 and 7 days was located within the one-phase micellar zone, whereas after 14 days of feeding, lipid composition was plotted above the micellar zone. In the devazepide group, relative biliary lipid composition between 0 and 5 days was plotted within the micellar zone. We further found that relative lipid composition of pooled gallbladder bile directly entered crystallization pathway C from the micellar zone after 7 and 14 days of feeding for the devazepide and the vehicle groups, respectively. By phase analysis, the bile was predicted to be composed of three phases, namely solid cholesterol crystals, liquid crystals, and saturated micelles, exactly as what we observed by polarizing light microscopy.

Effect of devazepide on hepatic secretion of biliary lipids

Figure 3 shows that after 21 days of feeding the lithogenic diet, oral administration of devazepide twice per day leads to a significant increase in hepatic cholesterol output during the first hour of biliary secretion of interrupted enterohepatic circulation. However, hepatic output of biliary phospholipids and bile salts slightly varied and was not significantly different between the devazepide and the vehicle groups. In addition, the ratios of cholesterol to phospholipids and cholesterol to bile salts were significantly increased in devazepide-treated mice, showing that bile cholesterol saturation was high. Bile flow rates were comparable between two groups of mice. Analyses of individual bile salt species by

HPLC showed that all bile salts in hepatic bile were taurine conjugated with a similar distribution of bile salt composition between the devazepide and the vehicle groups. After 21 days of feeding, taurocholate (52.1-53.6%) became the major bile salt of biliary pool because of 0.5% cholic acid in the lithogenic diet, followed by tauro- β -muricholate (11.6-16.6%), taurodeoxycholate (11.5-13.7%) and taurochenodeoxycholate (6.1-11.5%). The concentrations of other bile salts such as tauro- ω -muricholate (4.6-7.4%) and tauroursodeoxycholate (5.4-5.9%) were low in bile. Hydrophobicity indexes of bile salts in hepatic bile were comparable (-0.06 to -0.08) between two groups of mice. These results indicate that devazepide does not influence bile salt species in bile.

Effect of devazepide on gallbladder contraction function

Figure 4 shows the changes in fasting gallbladder volumes as function of days on the lithogenic diet, which are significantly increased over time in two groups of mice during cholelithogenesis. On the chow diet (i.e., at day 0), the gallbladder wall was thin and transparent in both groups of mice. Macroscopic and light microscopic examination of gallbladder bile showed no evidence of mucin gel, solid and liquid crystals, or gallstones. When bile became supersaturated and solid plate-like cholesterol monohydrate crystal precipitated, gallbladder volumes were dramatically increased in the devazepide group, which are significantly larger than those in the vehicle group.

Because the CCK-1R antagonist blockades the stimulation of CCK to gallbladder contractility, we investigated the effect of devazepide on gallbladder emptying function in mice at 14 days on the lithogenic diet. We observed that a significant portion of the gallbladder bile was excreted in vehicle-treated mice in response to the high-fat meal. Gallbladder volumes were reduced from $38.8 \pm 14.9 \mu\text{L}$ to $22.2 \pm 10.2 \mu\text{L}$ (postprandial residual gallbladder volumes). At 30 minutes after the fatty meal, the gallbladder emptying rate was $43.4 \pm 6.7\%$. However, it was not the case in devazepide-treated mice, with the gallbladder emptying rate being $18.1 \pm 12.7\%$. As a result, only a small portion of the gallbladder bile was emptied out. Gallbladder volumes were reduced from $69.6 \pm 10.2 \mu\text{L}$ to $56.2 \pm 6.5 \mu\text{L}$ (postprandial residual gallbladder volumes). This suggests that gallbladder emptying function is impaired because the CCK/CCK-1R signaling cascade in the gallbladder is disrupted by devazepide, thus leading to gallbladder stasis.

As shown in **Figure 5**, a large amount of mucin gel was accumulated in the gallbladder lumen of devazepide-treated mice after 3 days of feeding the lithogenic diet. By 7 days, we detected that numerous classical plate-like cholesterol monohydrate crystals were embedded in mucin gel, showing the formation of biliary sludge. These findings were consistent with the concept that the mucin gel is a strong pro-crystallizing agent acting as a matrix accelerating cholesterol nucleation and crystallization in bile, as found in prairie dog experiments and in model bile studies that were supplemented with varying concentrations of purified mucin proteins [33-35].

We further observed that the concentrations of cholesterol and cholesteryl esters in the gallbladder wall were significantly higher in the devazepide group than in the vehicle group. After 21 days of feeding the lithogenic diet, oral administration of devazepide twice per day significantly reduced expression of *Cck-Ir* in the gallbladder. Moreover, devazepide-treated

mice displayed a significant increase in mRNA levels of *Muc2*, *Muc5ac*, and *Muc5b* (for producing the gel-forming mucins), but not *Muc1*, *Muc3*, and *Muc4* (for producing the epithelial mucins) compared to mice receiving vehicle. In addition, expression of *Npc1l1*, *Abcg5*, *Abcg8* (the gallbladder lipid transporters) and *Acat2* (for cholesterol esterification) was significantly increased in the devazepide group than in the vehicle group. No difference in expression of *Sr-b1* was found.

Effect of devazepide on intestinal cholesterol absorption

Because CCK-1R is expressed in the smooth muscle cells of small intestine, CCK-1R (–/–) mice display sluggish small intestinal transit and increased intestinal cholesterol absorption [2]. We examined whether the CCK-1R antagonist devazepide increase intestinal cholesterol absorption. **Figure 6** shows that the percentage of intestinal cholesterol absorption is significantly higher in the devazepide group than in the vehicle group. As expected, oral administration of devazepide twice per day resulted in a significant reduction in expression of the *Cck-1r* gene in the small intestine. After 21 days of feeding the lithogenic diet, mRNA levels of the intestinal *Abcg5*, *Abcg8*, and *Acat2* genes were significantly increased in the devazepide group compared to the vehicle group. Moreover, expression of *Npc1l1* and *Hmgcr* was significantly reduced. It is highly likely that these changes may be a secondary phenomenon due to increased amounts of the absorbed cholesterol entering the enterocyte in devazepide-treated mice.

DISCUSSION

A defect in gallbladder motor function has been strongly linked to the formation of cholesterol gallstones and impaired gallbladder contractility has been observed in humans in both the fasting and the postprandial status [36]. An abnormal gallbladder motor function has been reported in gallstone-free subjects under several conditions such as pregnant women, obese patients with rapid weight loss, and patients with TPN treatment, and this may represent a potent pathophysiologically relevant stimulus predisposing to gallstone formation [17, 37]. Thus, the role of impaired gallbladder contractility in the pathogenesis of gallstones is studied with growing interest.

Devazepide has been characterized as a new potent antagonist of CCK. Some *in vitro* and animal studies have clearly demonstrated higher affinity of devazepide for peripheral CCK-1R than for brain CCK-1R and competitive antagonization of CCK-8-stimulated gallbladder contraction, pancreatic enzyme release, and smooth muscle strip contractions [38]. Although the CCK-1R antagonists, including devazepide, have been extensively investigated for their therapeutic benefits on gastrointestinal and metabolic diseases in clinical trials [39–41], their side effects on the pathogenesis of cholesterol crystallization and gallstone formation are still unknown.

In the present study, the most important findings are: (i) devazepide has a significant effect on impairing gallbladder contractile function, thus leading to enlarged fasting and postprandial residual gallbladder volumes that predispose to gallbladder stasis and biliary sludge. (ii) Devazepide increases intestinal cholesterol absorption that may be an important source of excess cholesterol for hepatic hypersecretion into bile, thereby causing cholesterol-

supersaturated bile and enhancing cholelithogenesis. (iii) Under devazepide treatment, both elevated CSI values in bile and gallbladder stasis accelerate cholesterol crystallization and promote solid cholesterol crystal growth and agglomeration into microlithiasis and macroscopic stones. These results indicate that the potential CCK-1R antagonists, including devazepide, could have a worrisome and unwelcome lithogenic effect, leading to the formation of cholesterol gallstones.

It has been well established that the biological function of CCK is mediated by its binding to two specific receptors, CCK-1R and CCK-2R. The CCK-1R is expressed mainly in the gallbladder, small intestine, pancreas, and stomach, as well as in the brain. The CCK-1R is highly specific for sulfated CCK-8 and for the nonpeptide antagonist devazepide. Binding of mature CCK-8 to CCK-1R exerts its physiological functions; however, the CCK-8-mediated actions are inhibited by the CCK-1R antagonists through disrupting the CCK-1R signaling pathway. Obviously, this unique mechanism provides a molecular basis for elucidating the lithogenic effect of devazepide on the impairment of gallbladder contractility. In the present study, our findings show that devazepide causes gallbladder stasis by reducing gallbladder emptying, which provides a longer residence time of excess cholesterol in the gallbladder lumen for its uptake and absorption by the cholesterol transporters that are located on the apical surface of gallbladder epithelial cells, as well as for cholesterol crystallization and solid cholesterol crystal growth. These abnormalities greatly promote the agglomeration of solid cholesterol crystals into microlithiasis and macroscopic stones.

More importantly, we found for the first time that devazepide enhances intestinal cholesterol absorption in mice. This may also be the case for other CCK-1R antagonists. It has been appreciated that high intestinal cholesterol absorption efficiency is an important risk factor for gallstone formation [42]. We have reported previously that the disrupted deletion of the *Cck-1r* gene induces a significant increase in intestinal cholesterol absorption by retarding small intestinal transit in mice [2]. Thus, hepatic cholesterol secretion is significantly increased in CCK-1R ($-/-$) mice, leading to supersaturated bile and rapid cholesterol crystallization. High gallstone prevalent rate in devazepide-treated mice may be explained by increased availability of chylomicron-derived cholesterol in the liver for biliary hypersecretion into bile. Under the lithogenic diet feeding conditions, sluggish small intestinal transit rate, because of the suppression of *Cck-1r* expression by devazepide, may provide a longer time for micelle-carried cholesterol to the apical membrane of enterocytes, in where more cholesterol molecules are taken up for their intestinal absorption by the sterol influx transporter Niemann-Pick C1 like 1 (NPC1L1) protein. Although elevated expression levels of the intestinal ABCG5/G8 may be a secondary reaction to the increased absorption of cholesterol that enters the enterocytes [43], the net effect between influx and efflux of intraluminal cholesterol molecules crossing the brush border membrane of the enterocytes is positive so that intestinal cholesterol absorption efficiency is increased in devazepide-treated mice. In the present study, our results suggest that the intestine-derived cholesterol may be a key source for hepatic hypersecretion in mice treated with devazepide, thus leading to supersaturated bile.

We have found that ezetimibe prevents gallstone formation by effectively inhibiting intestinal cholesterol absorption so that hepatic cholesterol secretion is reduced in gallstone-

susceptible mice [44]. Furthermore, under ezetimibe treatment, gallbladder motility function is preserved by desaturating bile in mice even in challenge to the lithogenic diet for 56 days [45]. These studies suggest that ezetimibe may be used to prevent the undesirable side effects of devazepide on reducing gallbladder contraction function and promoting gallstone formation.

It has been proposed that in the supersaturated bile state, bile stasis enhances gallbladder cholesterol absorption and its accumulation in the gallbladder wall. The gallbladder epithelial cells have been found to actively modify lipid composition in bile by absorbing and secreting biliary lipids because many cholesterol transporters reside on the apical membrane of these epithelial cells [17]. In the normal physiological state, there may be a balance between influx and efflux of intraluminal cholesterol molecules crossing the apical membrane of gallbladder epithelial cells in mice with unsaturated gallbladder bile. Thus, no excess cholesterol is accumulated in the gallbladder tissues. However, when bile is supersaturated with cholesterol, gallbladder cholesterol absorption may be enhanced because gallbladder stasis provides a longer resident time of bile for the uptake and absorption of intraluminal cholesterol molecules by these sterol transporters. These abnormalities lead to the accumulation of excess cholesterol in the gallbladder wall because gallbladder epithelial cells cannot assemble lipoproteins for lipid transport into plasma. In the lithogenic state, the absorbed cholesterol has to be converted to cholesteryl esters for storage in the mucosa and lamina propria. As a result, the accumulation of excess cholesterol and cholesteryl esters in the gallbladder smooth muscle cells could stiffen sarcolemmal membranes and decouple the G-protein-mediated signal transduction [46-49]. This would further paralyze gallbladder contractility and worsen the impaired gallbladder emptying function that has been induced by devazepide through the disruption of the CCK-1R signaling cascade.

In the present study, we investigated the effect of devazepide on gallstone formation in male mice. Clinical studies have found that women are more susceptible to gallbladder hypomotility and the formation of biliary sludge and cholesterol gallstones compared to men. It is highly likely that high levels of estrogen may impair gallbladder contractility by disrupting CCK-1R signaling cascade through the ER α pathway in the gallbladder [50]. Thus, devazepide may be a risk factor for gallstones when it is used in women, especially under conditions of oral contraceptive steroids and conjugated estrogens, hormone replacement therapy, and pregnancy.

In addition, cholesterol gallstones often occur in obese patients during rapid weight loss, which is caused by gallbladder hypomotility and supersaturation of bile with cholesterol because of enhanced transport of cholesterol to the liver for biliary hypersecretion [51-53]. Although no consensus exists concerning the prevention of gallstones in obese patients during rapid weight loss by a very low calorie diet, pharmacological interventions or bariatric surgery, ursodeoxycholic acid (UDCA), fat-enriched diets, and CCK-8 for promoting efficient gallbladder emptying have been recommended [54]. Notably, special attention should be paid to obese patients in whom the use of devazepide and other CCK-1R antagonists should be avoided during rapid weight loss.

More recently, Shahid and colleagues reported that endogenous elevation of plasma CCK does not prevent gallstones in mice overexpressing pancreatic secretory trypsin inhibitor-I (PSTI-1) although under the condition of PSTI-I overexpression, CCK release is chronically stimulated [55]. It is likely that persistently high CCK secretion may desensitize or downregulate the gallbladder CCK1-R expression, thus impairing gallbladder contraction. This study indicated that the regulation of gallbladder contraction function is complex, which could be determined by plasma CCK levels, as well as CCK-1 sensitivity and its signaling cascade in the gallbladder. These observations are consistent with the current results demonstrating that disrupting CCK-1R signaling pathway impairs gallbladder emptying, thereby promoting the formation of biliary sludge and gallstone formation.

Although it is unclear whether increased accumulation of cholesteryl esters in the gallbladder wall is a stimulus triggering the hypersecretion of mucin gel by the gallbladder mucin-producing cells, we found that large amounts of mucin gel are indeed accumulated in the gallbladder lumen of devazepide-treated mice. Our results suggest that the accumulation of cholesteryl esters may be a risk factor for stimulating the synthesis and secretion of mucin gel. In the lithogenic bile, many solid cholesterol crystals form and precipitate in the gallbladder lumen. These solid cholesterol crystals may interact with the apical membrane of gallbladder mucosal cells, analogous to the “cytotoxic” effects of monosodium urate monohydrate and calcium pyrophosphate dihydrate crystals in the joints of gout and pseudo-gout patients [56]. These may further impair the function of gallbladder epithelial cells and cause inflammation in the gallbladder wall. More importantly, mucin gel is a powerful pro-crystallizing agent for accelerating cholesterol crystallization and crystal growth to gallstones.

The current study has a high translational value. Our results suggest that during CCK-1R antagonist treatment, gallbladder and gastrointestinal motility function should be carefully monitored and the gallbladder should be routinely examined by ultrasonography for the presence of biliary sludge (a precursor to gallstones). In addition, the duration and dosage of the CCK-1R antagonists should be reduced to prevent their undesirable side effects on gallstone formation. If a chronic use of the CCK-1R antagonists is needed in patients with some special conditions, adequate preventive measures should be taken by giving ezetimibe and/or UDCA to desaturate bile and ultimately preserve gallbladder motor function.

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Abbreviations

ABC	ATP-binding cassette (transporter)
CCK	cholecystokinin
CSI	cholesterol saturation index
HI	hydrophobicity indexes

HPLC	high-performance liquid chromatography
KO	knockout
WT	wild-type

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Figure 1.

(A) Representative photomicrographs of solid plate-like cholesterol monohydrate crystals and gallstone formation in gallbladder bile of mice treated with devazepide or vehicle and fed the lithogenic diet for 21 days. All magnifications are $\times 800$ except for the right two panels that are $\times 400$ (top) and $\times 200$ (bottom), as observed by polarizing light microscopy. (B) Schematic presentation of the solid and liquid crystal habits in the cholesterol crystallization sequence from gallbladder bile as functions of days on the lithogenic diet. The vertical axes represent arbitrary numbers of solid and liquid crystals per high power

microscopic field, all normalized to the same maximum. The arrow indicates the first appearance of real gallstones. Abbreviations: ChM, cholesterol monohydrate crystals; LC, liquid crystals, including small, aggregated and fused (multilamellar) varieties; SS, sandy stones; GS, gallstones.

Figure 2.

(A) Effect of devazepide on cholesterol saturation index in pooled gallbladder bile as functions of time on the lithogenic diet. (B) The relative lipid composition of pooled gallbladder bile from mice treated with devazepide or vehicle at each time point plotted on condensed phase diagrams for average total lipid concentrations (~9.0 g/dL) of the bile samples (from Table 1). The one-phase micellar zone at bottom is enclosed by a solid curved line. Above the micellar zone, two solid lines divide the two-phase zones from a central three-phase zone. Based upon the solid and liquid crystallization sequences present in bile,

the left two-phase and central three-phase regions are divided by dashed lines into regions A to E. With passage of time, relative lipid composition of gallbladder bile shifts upward and to the right, as well as enters crystallization pathway C directly from the one-phase micellar zone in both groups of mice. Notably, relative lipid composition of gallbladder bile enters crystallization pathway C much earlier in the devazepide group than in the vehicle group. See text for further description.

Figure 3.

Effect of devazepide on hepatic output (μmol/h/kg B.W.) of (A) biliary cholesterol, (B) phospholipids, (C) bile salts, the ratios of (D) Ch/PL and (E) Ch/BS, and (F) bile flow rate during the first hour of biliary secretion in mice after 21 days of treatment with devazepide or vehicle and feeding the lithogenic diet. Abbreviations: Ch, cholesterol; PL, phospholipids; BS, bile salts.

Figure 4.

(A) Effect of devazepide on fasting gallbladder volumes as functions of days on the lithogenic diet and (B) the percentage of gallbladder emptying during the 30-minute period after infusion of corn oil in mice treated with devazepide or vehicle at 14 days on the lithogenic diet.

Figure 5.

Effect of devazepide on (A) the formation of biliary sludge, magnifications x800; (B) lipid accumulation in the gallbladder wall; and (C) expression of the mucin genes and the genes encoding the gallbladder lipid transporters. Abbreviations: *Abcg5*, ATP-binding cassette transporter G5; *Acat2*, acyl-CoA:cholesterol acyltransferase, isoform 2; *Cck-1r*, cholecystokinin-1 receptor; *Muc1*, mucin gene 1; *Npc1l1*, Niemann-Pick C1-like 1; *Sr-b1*, scavenger receptor class B, member 1. See text for further description.

Figure 6.

Effect of devazepide on (A) intestinal cholesterol absorption, as determined by the fecal dual-isotope ratio method, and (B) gene expression of intestinal sterol transporters.

Abbreviation: *Hmgcr*, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

Table 1
Biliary lipid composition of gallbladder bile during cholesterol crystallization and gallstone formation

Day	Mole% Ch	Mole% PL	Mole% BS	Ch/PL	Ch/BS	[TL] (g/dL)	CSI
Vehicle							
0	2.02	12.24	85.75	0.165	0.024	8.42	0.44
3	2.63	12.81	84.56	0.206	0.031	8.41	0.56
5	3.26	14.58	82.16	0.224	0.040	8.51	0.64
7	5.10	15.56	79.34	0.328	0.064	9.26	0.92
14	6.20	16.62	77.18	0.373	0.080	9.07	1.07
21	7.02	17.56	75.42	0.400	0.093	9.49	1.15
Devazepide							
0	2.66	12.59	84.75	0.212	0.031	8.18	0.58
3	3.34	13.70	82.96	0.244	0.040	8.28	0.68
5	5.43	15.49	79.07	0.351	0.069	8.74	1.00
7	6.27	16.80	76.93	0.373	0.081	9.39	1.06
14	7.01	17.66	75.33	0.397	0.093	9.11	1.15
21	7.81	17.98	74.20	0.434	0.105	9.54	1.25

Values were measured from pooled gallbladder bile (n=5 per group). The CSI values of pooled gallbladder bile were calculated from the critical tables.

Abbreviations: Ch, cholesterol; PL, phospholipids; BS, bile salts; [TL], total lipid concentration; CSI, cholesterol saturation index.