Proteasome inhibition protects against diet-induced gallstone formation through modulation of cholesterol and bile acid homeostasis

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Abstract. Gallstone disease is one of the most prevalent and costly gastrointestinal disorders worldwide. Gallstones are formed in the biliary system by cholesterol secretions in bile, which result from excess cholesterol, a deficiency in bile salts or a combination of the two. The present study examined the effects of proteasome inhibition on gallstone formation using the proteasome inhibitors bortezomib (BT) and carfilzomib (CF). C57BL/6J mice were fed a lithogenic diet to generate gallstones and injected with BT or CF for 12 weeks. After 12 weeks of the lithogenic diet, 8 out of the 10 mice in the control group had developed gallstones, whereas none of the mice who received proteasome inhibitors had developed gallstones. Notably, the expression of genes associated with cholesterol synthesis (sterol regulatory element-binding protein-2 and 3-hydroxy-3-methylglutaryl-CoA reductase), cholesterol secretion [ATP-binding cassette subfamily G member 5 (*ABCG5*) and *ABCG8*] and bile acid synthesis [cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*), *Cyp7b1*, *Cyp27a1* and *Cyp8b1*] was reduced in the livers of mice injected with BT or CF. *Cyp7a1* encodes cholesterol 7α-hydroxylase, the rate-limiting enzyme in the synthesis of bile acid from cholesterol. The present study therefore measured the expression levels of transcription factors that are known to inhibit *Cyp7a1* expression, namely farnesoid X receptor (FXR), pregnane X receptor (PXR) and small heterodimer partner (SHP). Although FXR, PXR and SHP expression was predicted to increase in the presence of proteasome inhibitors, the expression levels were actually reduced; thus, it was concluded that they were not involved in the proteasome inhibition-induced regulation of *Cyp7a1*. Further investigation of the mitogen-activated protein kinase and protein kinase A (PKA) signaling pathways in human hepatoma cells revealed that proteasome inhibition-induced c-Jun N-terminal kinase (JNK) phosphorylation reduced *CYP7A1* and *CYP27A1* expression. In addition, reduced PKA phosphorylation as a result of proteasome inhibition regulated ABCG5 and ABCG8 expression. In conclusion, these findings suggest that proteasome inhibition regulates cholesterol and biliary metabolism via the JNK and PKA pathways, and is a promising therapeutic strategy to prevent gallstone disease.

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Abbreviations: ABCG, ATP-binding cassette transporter; BSEP, bile salt export pump; Cyp7a1, cholesterol 7α-hydroxylase; Cyp7b1, oxysterol 7α-hydroxylase; Cyp27a1, sterol 27-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; ERK, extracellular signal-regulated kinase; FXR, farnesoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MDR, multidrug resistance; PKA, protein kinase A; PXR, pregnane X receptor; SHP, small heterodimer partner; SREBP, sterol regulatory element-binding protein

Key words: gallstone disease, proteasome inhibition, cholesterol synthesis, bile acid synthesis, lithogenic diet, cholesterol secretion

Introduction

Gallstone disease, the most common biliary disorder affecting 10-15% of the adult population, is a significant health problem worldwide, with an incidence that has been increasing due to the rising epidemic of obesity and metabolic syndrome (1). Bile contains cholesterol, bile salts and phospholipids, and the precipitation of excessive cholesterol in bile leads to gallstone formation (2). To overcome the extremely low solubility of cholesterol in aqueous solutions, cholesterol in bile exists as mixed micelles of bile salts and phospholipids. Excess cholesterol, a lack of bile salts or phospholipids, or a combination of these factors can lead to the supersaturation of bile and the precipitation of cholesterol (2,3). Therefore, cholesterol homeostasis is required to prevent gallstone formation.

The balance between cholesterol intake/biosynthesis and biliary cholesterol secretion is important for cholesterol homeostasis (4). The master regulator of cholesterol biosynthesis,

sterol regulatory element-binding protein-2 (SREBP-2), regulates the expression of numerous genes involved in cholesterol homeostasis, including the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (4). In addition, the cytochrome P450 genes, cytochrome P450 family 7 subfamily A member 1 (*CYP7A1)*, *CYP7B1*, *CYP27A1* and *CYP8B1,* encode enzymes that catalyze various steps in cholesterol catabolism and bile acid synthesis. Transcriptional regulation of *CYP7A1*, which encodes cholesterol 7α-hydroxylase and catalyzes the initial rate-limiting step in cholesterol catabolism (5), has been relatively well-established. The farnesoid X receptor (FXR) induces expression of an atypical orphan nuclear receptor, small heterodimer partner (SHP), which then inhibits liver-related homolog-1 transactivation, thereby inhibiting *Cyp7a1* transcription (5). In addition, SHP and the pregnane X receptor (PXR) interact with hepatocyte nuclear factor 4α and block its recruitment of peroxisome proliferator-activated receptor-γ coactivator-1α to *CYP7A1* chromatin, thereby inhibiting *CYP7A1* transcription (5). Finally, the ATP-binding cassette (ABC) transporters ABCB4 [also known as multidrug resistance protein 2 (Mdr2)], ABCB11 [also known as bile salt export pump (Bsep)] and ABCG5/ABCG8 heterodimers serve essential roles in the biliary secretion of phospholipids, bile acids and cholesterol, respectively (6). Regulation of cholesterol metabolism is a therapeutic target, and statins are reported to be effective to treat gallstone disease (7) and to reduce the risk of cholecystectomy (8). However, the efficacy of statin treatment for gallstone diseases remains under debate (9). Ursodeoxycholic acid (UDCA) was Food and Drug Administration (FDA) approved for cholesterol gallstone dissolution through the increase in bile flow and the alteration of the hydrophobicity index of the bile acid pool (10). However, UDCA use in neonatal and infant cholestasis was reported to be ineffective and unsafe, and it can also cause severe toxicities, including hepatitis, pruritus, cholangitis, ascites, vanishing bile duct syndrome and liver failure, in adults (10). Therefore, novel drugs to treat gallstone disease are required to apply to the range of patients.

The proteasome, which degrades ubiquitinated proteins, has emerged as an attractive therapeutic target to treat various cancer types (11). Bortezomib (BT), the first FDA-approved proteasome inhibitor, has been used clinically to treat patients with multiple myelomas and mantle cell lymphoma (12). Moreover, BT has been under investigation to expand its therapeutic effects to various other conditions, including fatty liver (13), hepatic injury by ductal ligation (14), drug-induced liver injury (15), pancreatic cancer (16), breast cancer (17), renal cell carcinoma (18) and prostate cancer (19). In addition, other proteasome inhibitors, including carfilzomib (CF), CEP-18770, PR-047 and NPI-0052, have also been developed and tested for the treatment of a variety of diseases (20).

The present study investigated the effects and mechanism of proteasome inhibition in gallstone formation using two proteasome inhibitors, BT and CF by reducing cholesterol synthesis and biliary secretion. Furthermore, it was found that reduced transcription of *Cyp7a1* by proteasome inhibition was associated with c-Jun N-terminal protein kinase (JNK) activation and that reduced ABCG5/ABCG8 levels resulted from reduced phosphorylation of protein kinase A (PKA).

Materials and methods

Materials. The following materials were purchased: i) SP600125, 8-bromo-cAMP, Sp-cAMPS and anti-Cyp7a1 (catalog no. SAB4301212) antibody (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); ii) PD98059 and anti-p38 (catalog no. 8690), anti-phosphorylated (phospho)-p38 (Thr180/Tyr182; catalog no. 4511), anti-JNK (catalog no. 9252), anti-p-JNK (Thr183/Tyr185; catalog no. 9255), anti-extracellular signal-regulated kinase (ERK; catalog no. 4695), anti-p-ERK (Thr202/Tyr204; 4370), anti-p-PKA (Thr197; catalog no. 5661) and anti-PKA (catalog no. 5842) antibodies (all Cell Signaling Technology, Inc., Danvers, MA, USA); iii) anti-SREBP-2 (catalog no. NV100-74543) antibody (Novus Biologicals, Littleton, CO, USA); iv) anti-Cyp27a1 antibody (catalog no. GTX103718; GeneTex, San Antonio, TX, USA); v) anti-HMGCR antibody (catalog no. ab174830) (Abcam, Cambridge, MA, USA); vi) anti-BSEP (catalog no. sc-74500), anti-MDR2 (sc-58221), anti-ABCG5 (catalog no. sc-517207) and anti-ABCG8 (catalog no. sc-30111) antibodies (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA); vii) anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (catalog no. MAB374) (EMD Millipore, Billerica, MA, USA); viii) anti-mouse-horseradish peroxidase (HRP; catalog no. 115-036-003) and anti-rabbit-HRP (catalog no. 111-035-003) antibodies (both Jackson Laboratory, Ben Harbor, ME, USA); and ix) BT and CF (both Biovision, Inc., Milpitas, CA, USA).

Animals and lithogenic diet provision. Male C57BL/6J mice (n=30; 6 weeks old) were purchased from Orient Bio, Inc. (Seoul, South Korea) and were housed under special pathogen-free conditions. Experimental procedures were approved by the Animal Ethics Committee at Lee Gil Ya Cancer and Diabetes Institute of Gachon University, and all animals were treated in accordance with the Animal Care Guidelines of Lee Gil Ya Cancer and Diabetes Institute of Gachon University. Mice were housed in a controlled environment at 21-23˚C and 51-54% humidity, with a 12 h light-dark cycle, and supplied with food and water *ad libitum*. Mice were fed a lithogenic diet (D12336; Research Diets Inc., New Brunswick, NJ, USA) and were injected with BT (0.5 mg/kg/week) or with CF (0.5 mg/kg/week) for 12 weeks. Control mice were injected with an equal amount of dimethyl sulfoxide. Mice were euthanized with low flow (10-30% of total volume) of CO_2, and sera, livers and gall bladders were collected and stored at -80˚C until further analysis.

Cell culture and treatment. Hep3B cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). The Hep3B cells were treated with 50 nM BT, CF or epoxomicin (Epoxo) for 48 h along with 10 μM SP600125 (JNK inhibitor), 10 μM PD98059 (ERK inhibitor), 10 μM 8-bromo-cAMP (PKA activator) and 10 μM Sp-cAMPS (PKA activator).

Western blotting. Liver and Hep3B cells were lysed with RIPA buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet

P-40, 0.5% sodium deoxycholate and 0.1% SDS] containing protease and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA) at 4˚C for 1 h. Lysates were centrifuged at 12,000 x g, at 4˚C for 10 min, supernatants were collected and the protein concentrations of the supernatants were measured using Protein Assay Dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (50 μg) were loaded and separated on 8-13% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in phosphate-buffered saline with 0.1% Tween-20 for 1 h at 4˚C and then were incubated with primary antibodies (diluted 1:1,000) overnight at 4˚C. Membranes were then incubated with secondary antibodies (diluted 1:10,000) for 1 h at room temperature. Protein bands were detected using Enhanced Chemiluminescence Western Blotting Detection reagents (Amersham Biosciences, Little Chalfont, UK) and a ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc.).

Cholesterol, phospholipid and bile acid measurements. Cholesterol, phospholipid and bile acid levels in sera, bile and livers were measured using the total cholesterol and cholesteryl ester colorimetric/fluorometric assay kit (Biovision, Inc.), the phospholipid assay kit (Sigma-Aldrich; Merck KGaA) and the total bile acids assay kit (Biovision, Inc.), respectively, according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (qPCR). Total mRNAs from mouse livers were extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany), and cDNAs were synthesized using a Verso cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). qPCR was performed with SYBR-Green (Thunderbird; Toyobo Co., Ltd., Osaka, Japan) using a CFX Connect™ Real-Time PCR Detection system (Bio-Rad Laboratories). The thermal cycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 45 sec. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21). The primers that were used are described in Table I. GAPDH was used as the reference gene.

Statistical analysis. All of the experiments were repeated at least three independent times, and data are presented as the mean ± standard error of the mean. Statistical significance was calculated using analysis of variance followed by Tukey's post hoc test (GraphPad Prism 6.0; GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used for quantitative polymerase chain reaction.

Genes	Primer sequences
srebp-2 (mouse)	F: 5'-ACTGACCAGCACCCATACTC-3' R:5'-CAGGAGGAGAGTTGGAACCA-3'
hmgcr (mouse)	F: 5'-ATCTCCTCTCCACAAAGCTT-3' R: 5'-CATTCTCACAGCAAGCTCCC-3'
cyp7b1 (mouse)	F: 5'-GCATCATCCGAGAAGTGCAG-3' R:5'-ATGAGTGGAGGAAAGAGGGC-3'
cyp7a1 (mouse)	F: 5'-CTCCGGGCCTTCCTAAATCA-3' R: 5'-ACAGCGTTAGATATCCGGCT-3'
cyp27a1 (mouse)	F: 5'-GAGAGTGAATCAGGGGACCA-3' R: 5'-TCAGGAATGGAGGGTTTCAG-3'
cyp8b1 (mouse)	F: 5'-AGATTGCAGCGTCTCTTCCAT-3' R: 5'-CCTTGCTCCCTCAGAAACTG-3'
bsep (*abcb11*) (mouse)	F: 5'-GGGTTCTACAGGGGTTGGAA-3' R: 5'-GTGAACTTGGCCACACTCAG-3'
mdr2 (*abcb4*) (mouse)	F: 5'-TCGCAGAGAACATCGCCTAT-3' R: 5'-TCTCGATGAAGGGGTGGATG-3'
abcg5 (mouse)	F: 5'-AATTTTGGGGGAATTTCCAG-3' R: 5'-GTCCTGTGGTTGGCTCATCT-3'
abcg8 (mouse)	F: 5'-CCTGATCCGTCGTCAGATTT-3' R: 5'-CCATGGCCGTAGTAAAGGAA-3'
fxr (*nr1h4*) (mouse)	F: 5'-TGGGTACCAGGGAGAGACTG-3' R: 5'-GTGAGCGCGTTGTAGTGGTA-3'
pxr (*nr1i2*) (mouse)	F: 5'-CCCATCAACGTAGAGGAGGA-3' R: 5'-TCTGAAAAACCCCTTGCATC-3'
shp (*nr0b2*) (mouse)	F: 5'-AGCTGGGTCCCAAGGAGTAT-3' R: 5'-GGTACCAGGGCTCCAAGACT-3'
lxrα (*nr1h3*) (mouse)'	F: 5'-TACGTCTCCATCAACCACCC-3' R: 5'-CTTGCTCTGAATGGACGCTG-3'
lxrβ (*nr1h2*) (mouse)	F: 5'-CAGACGCTACAACCACGAGA-3' R: 5'-ATGAATTCCACCTGCAAGCC-3'
gapdh[a] (mouse)	F: 5'-CGACTTCAACAGCAACTCCCAC TCTTCC-3' R: 5'-TGGGTGGTCCAGGGTTTCTTA CTCCTT-3'

[a](39). SREBP-2, sterol regulatory element-binding protein-2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; Cyp7b1, oxysterol 7α-hydroxylase; Cyp7a1, cholesterol 7α-hydroxylase; Cyp27a1, sterol 27-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; BSEP, bile salt export pump; MDR2, multidrug resistance 2; ABCG, ATP-binding cassette transporter; FXR, farnesoid X receptor; PXR, pregnane X receptor; SHP, small heterodimer partner; LXR, liver X receptor, GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

Results

Proteasome inhibition reduces gallstone formation in mice. To examine the role of proteasome inhibition on gallstone formation, C57BL/6J mice were intraperitoneally injected with BT or CF (0.5 mg/kg/week) and fed a lithogenic diet for 12 weeks. After 12 weeks, 8 out of the 10 mice that were fed a lithogenic diet, exhibited gallstone formation, whereas none of the mice that were fed a lithogenic diet and were treated with BT or CF displayed gallstone formation (Fig. 1A). As expected, serum cholesterol and bile acid levels increased after 12 weeks on a lithogenic diet, but proteasome inhibition with BT or CF significantly reduced these levels (Fig. 1B and C).

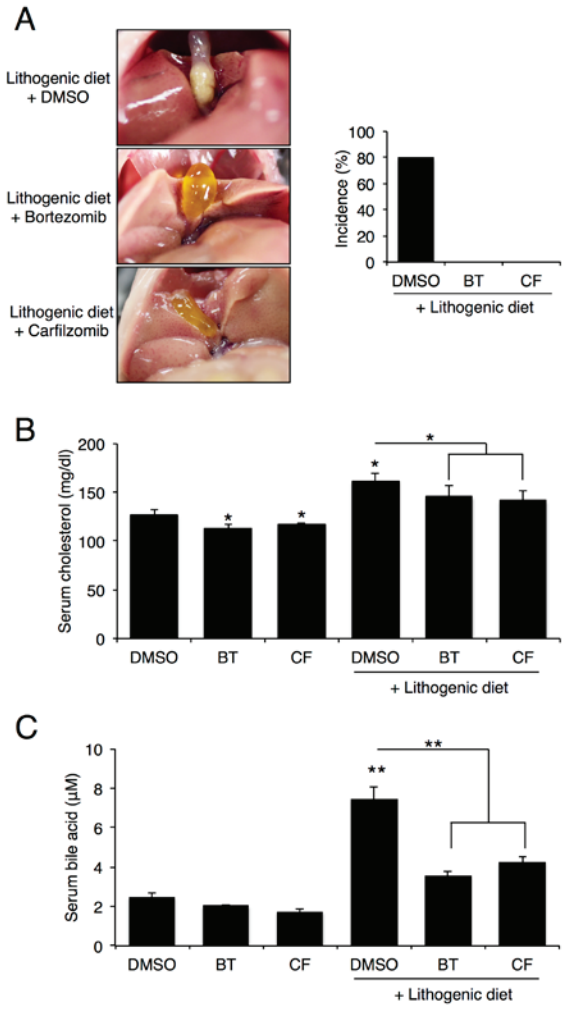

Figure 1. Proteasome inhibition reduces gallstone formation induced by a lithogenic diet. C57BL/6J mice were fed a lithogenic diet for 12 weeks and were administered intraperitoneal injections of BT (0.5 mg/kg/week) or CF (0.5 mg/kg/week). (A) Image of gallstone formation (left panel) and incidence (right panel) (n=10). (B) Serum cholesterol and (C) bile acid levels were measured with colorimetric kits (n=3). Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01. Three independent experiments were performed. DMSO, dimethyl sulfoxide; BT, bortezomib; CF, carfilzomib.

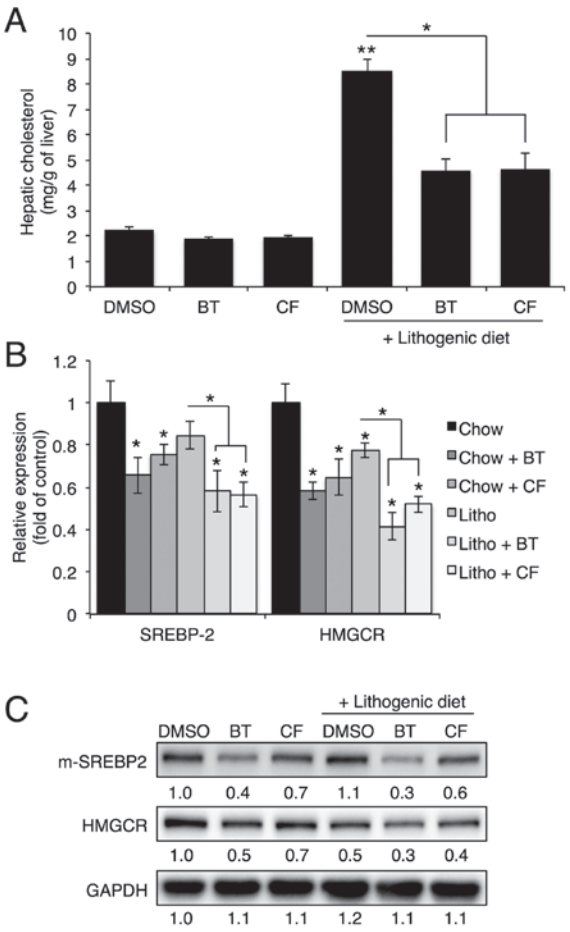

Figure 2. Proteasome inhibition reduces cholesterol synthesis by reducing SREBP-2 and HMGCR expression. (A) Total hepatic cholesterol level in mice injected with BT or CF was quantified with commercial kits (n=3). (B) mRNA levels of SREBP-2 and HMGCR in the livers of mice injected with BT or CF were measured by quantitative polymerase chain reaction (n=3). (C) Representative western blot analyses of SREBP-2 and HMGCR. Densitometric values for each band are provided below the images. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01. Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; m-SREBP-2, mature form of sterol regulatory element-binding protein-2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; Litho, lithogenic diet.

Proteasome inhibition reduces cholesterol and bile acid synthesis in the liver. To investigate the protective mechanism of proteasome inhibition on gallstone formation, the hepatic cholesterol levels and expression of genes involved in cholesterol synthesis were analyzed. In accordance with serum cholesterol levels, hepatic cholesterol levels that were elevated with a lithogenic diet were significantly reduced upon BT or CF administration (Fig. 2A). Proteasome inhibition reduced the mRNA and protein levels of *Srebp-2*, the master regulator of cholesterol synthesis, and *Hmgcr*, the rate-limiting enzyme of cholesterol synthesis (Fig. 2B and C) (4).

Next, the hepatic bile acid levels and expression of genes involved in bile acid synthesis were examined. Proteasome inhibition reduced the bile acid levels in the liver that were elevated by the lithogenic diet (Fig. 3A). Proteasome inhibition also reduced mRNA and protein expression of hepatic genes involved in bile acid synthesis, including *Cyp7a1*, *Cyp7b1*, *Cyp27a1* and *Cyp8b1* (Fig. 3B and C).

Proteasome inhibition reduces biliary cholesterol secretion. To investigate the effects of proteasome inhibition on biliary secretion, bile composition was further analyzed using commercial kits. Abcg5/Abcg8 heterodimers, Bsep and Mdr2 are crucial for the biliary secretion of cholesterol, bile acids and phospholipids, respectively (6). Notably, BT and CF treatments reduced biliary cholesterol levels, but not biliary phospholipid or bile acid levels (Fig. 4A), suggesting that proteasome inhibition alters only the biliary secretion of cholesterol. In accordance with reduced biliary cholesterol levels, *Abcg5* and *Abcg8* mRNA and protein levels were downregulated upon BT or CF injection, whereas Bsep and Mdr2 levels were not significantly affected (Fig. 4B and C). These data indicate that proteasome inhibition of Abcg5 and Abcg8 expression results in reduced biliary secretion of cholesterol and prevents gallstone formation.

Proteasome inhibition induces JNK phosphorylation, which regulates Cyp7a1 expression. Cholesterol 7α-hydroxylase,

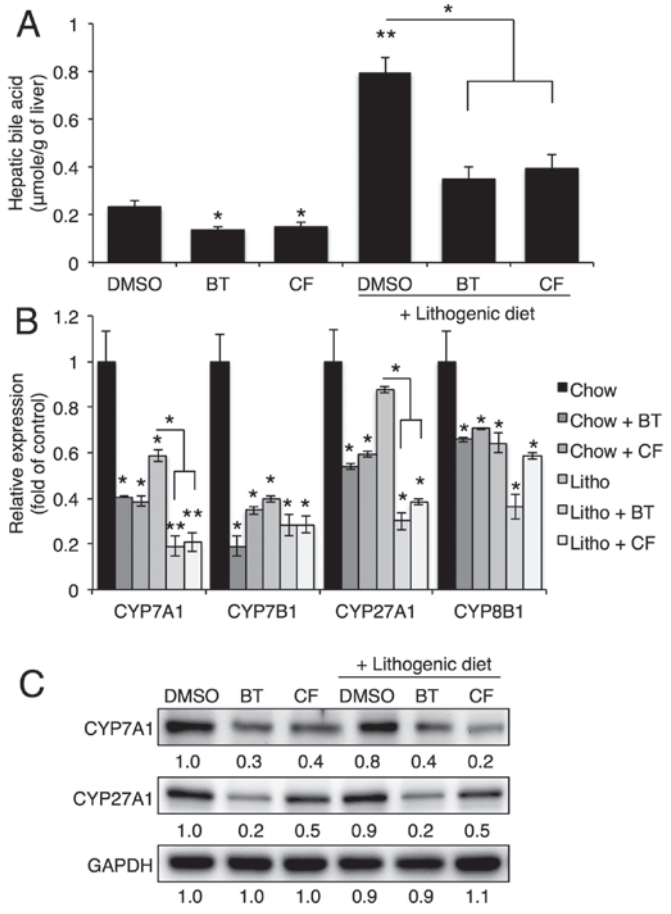

Figure 3. Proteasome inhibition reduces bile acid synthesis by reducing Cyp7a1, Cyp7b1, Cyp27a1 and Cyp8b1 expression. (A) Hepatic bile acid levels upon BT or CF injection were quantified with commercial kits (n=3). (B) mRNA levels of Cyp7a1, Cyp7b1, Cyp27a1 and Cyp8b1 in the livers of mice injected with BT or CF were measured by quantitative polymerase chain reaction (n=3). (C) Representative western blot analyses of Cyp7a1 and Cyp27a1. Densitometric values for each band are provided below the images. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01. Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; CYP7B1, oxysterol 7α-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP8B1, sterol 12α-hydroxylase; Litho, lithogenic diet.

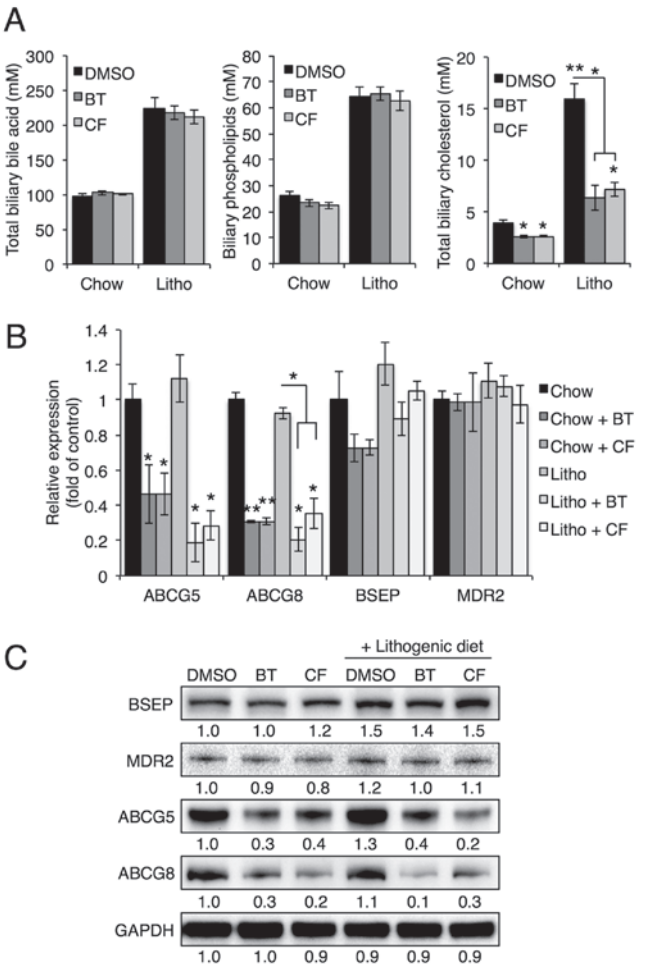

Figure 4. Proteasome inhibition reduces cholesterol secretion by reducing expression of Abcg5 and Abcg8. (A) Biliary cholesterol, bile acid and phospholipid levels were measured in mice injected with BT or CF. (B) mRNA levels of ABCG5, ABCG8, BSEP and MDR2 in the livers of mice injected with BT or CF were measured by quantitative polymerase chain reaction (n=3). (C) Representative western blot analyses of BSEP, MDR2, ABCG5 and ABCG8. Densitometric values for each band are provided below the images. Data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001. Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; Litho, lithogenic diet; BSEP, bile salt export pump; MDR2, multidrug resistance protein 2; ABCG, ATP-binding cassette transporter.

which is encoded by *Cyp7a1*, catalyzes the initial rate-limiting step in cholesterol catabolism (5), and *Cyp7a1* transcription has been shown to be inhibited by the transcription factors SHP, FXR and PXR (5). To examine whether these transcription factors are involved in the reduction of Cyp7a1 levels as a result of proteasome inhibition, SHP, FXR and PXR levels were examined upon treatment with proteasome inhibitors. As they have been shown to inhibit *Cyp7a1* transcription, SHP, FXR and PXR levels were expected to increase upon proteasome inhibition; however, SHP, FXR and PXR levels were reduced in livers treated with proteasome inhibitors (Fig. 5A). Thus it was concluded that SHP, FXR and PXR do not serve roles in the proteasome inhibition-induced reduction of Cyp7a1.

As mitogen-activated protein kinase (MAPK) has also been shown to regulate *Cyp7a1* transcription (22-24), the phosphorylation of p38, ERK and JNK was examined in livers treated with proteasome inhibitors. Hepatic phosphorylation of JNK and ERK, but not p38, was elevated upon administration of BT and CF (Fig. 5B). To determine whether proteasome inhibition-induced phosphorylation of JNK or of ERK led to the reduced expression of cholesterol and bile acid synthesis genes, the JNK inhibitor SP600125 and the ERK inhibitor PD98059 were used. In accordance with *in vivo* data, Hep3B cells treated with the proteasome inhibitors BT, CF and Epoxomicin displayed elevated ERK and JNK phosphorylation accompanied by concomitant reductions in mature form of (m)-SREBP2, HMGCR, CYP7A1 and CYP27A1 levels (Fig. 6A). Inhibition of JNK, but not ERK, reversed the downregulation of CYP7A1 and CYP27A1 that was induced by proteasome inhibition, but the proteasome inhibition-induced reduction of

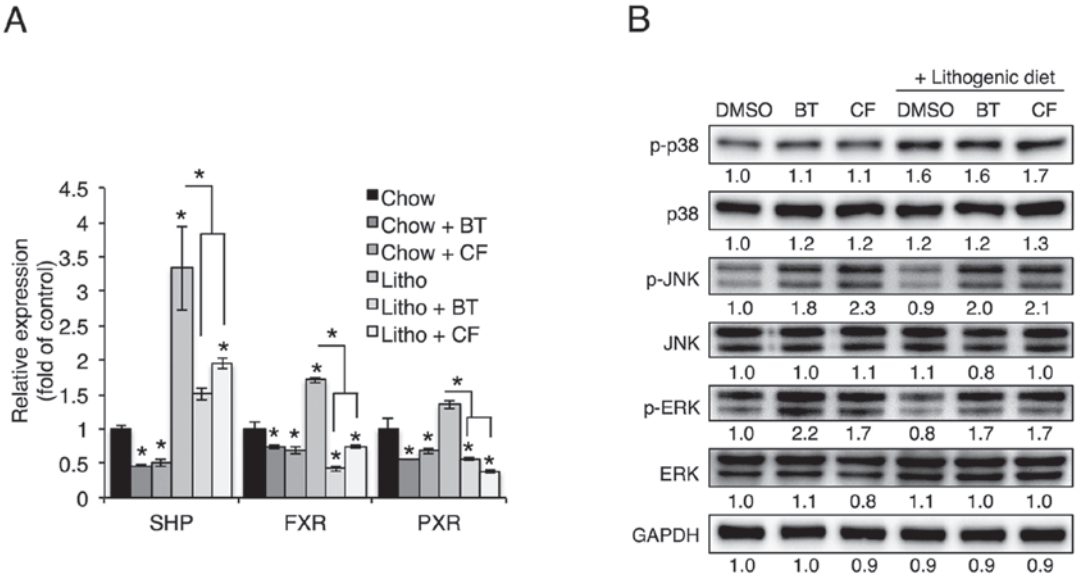

Figure 5. Proteasome inhibition increases phosphorylation of JNK and ERK. (A) mRNA levels of SHP, FXR and PXR in the livers of mice injected with BT or CF were measured by quantitative polymerase chain reaction (n=3). (B) Representative western blot analyses of proteins in the MAPK pathway. Densitometric values for each band are provided below the images. Data are expressed as the mean ± standard error of the mean. *P<0.05. Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; Litho, lithogenic diet; JNK, c-Jun N-terminal protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SHP, small heterodimer partner; FXR, farnesoid X receptor; PXR, pregnane X receptor.

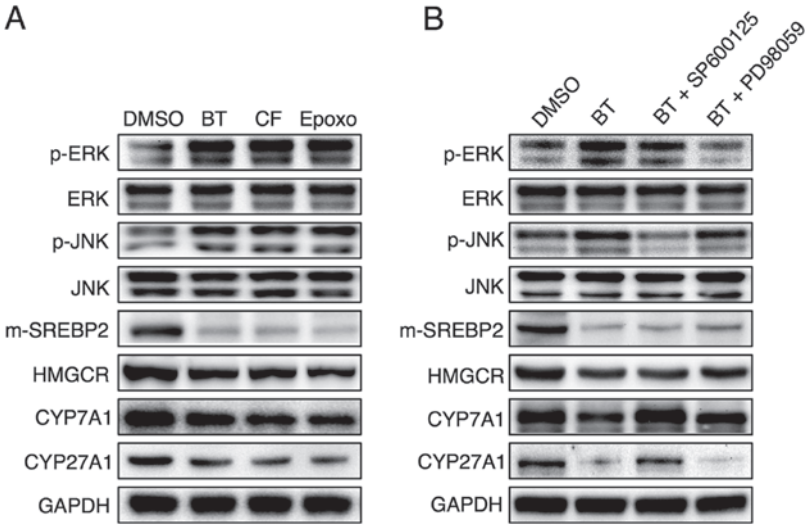

Figure 6. JNK phosphorylation as a result of proteasome inhibition serves a critical role in the downregulation of Cyp7a1 and Cyp27a1. Representative western blot analyses of (A) Hep3B cells treated with 50 nM BT, 50 nM CF or 50 nM Epoxomicin (Epoxo) and (B) Hep3B cells treated with 50 nM BT and 10 μM SP600125 (JNK inhibitor) or 10 μM PD98059 (ERK inhibitor). Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; Epoxo, epoxomicin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; JNK, c-Jun N-terminal protein kinase; ERK, extracellular signal-regulated kinase; CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; m-SREBP-2, mature form of sterol regulatory element-binding protein-2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

SREBP-2 and HMGCR was not reversed with JNK or ERK inhibitors (Fig. 6B).

Proteasome inhibition reduces ABCG5 and ABCG8 levels by reducing PKA phosphorylation. To evaluate how proteasome inhibitors regulate ABCG5 and ABCG8, the levels of liver X receptor (LXR)α and LXRβ, which are known to regulate ABCG5 and ABCG8, were examined in mouse livers in the presence of proteasome inhibitors (25). It was found that LXRα and LXRβ levels were not affected by proteasome inhibitors, suggesting that the proteasome inhibitor-induced downregulation of ABCG5 and ABCG8 was not caused by LXRα and LXRβ (Fig. 7A). Thus, the PKA signaling pathway, which has also been shown to affect ABCG5 and ABCG8 expression, was analyzed (26). Reduced PKA phosphorylation was found in Hep3B cells that were treated with proteasome inhibitors (Fig. 7B). It was also found that treatment with a PKA activator, 8-Br-cAMP or Sp-cAMPS, reversed the proteasome inhibition-induced reduction of ABCG5 and ABCG8 levels (Fig. 7C), confirming that PKA phosphorylation serves

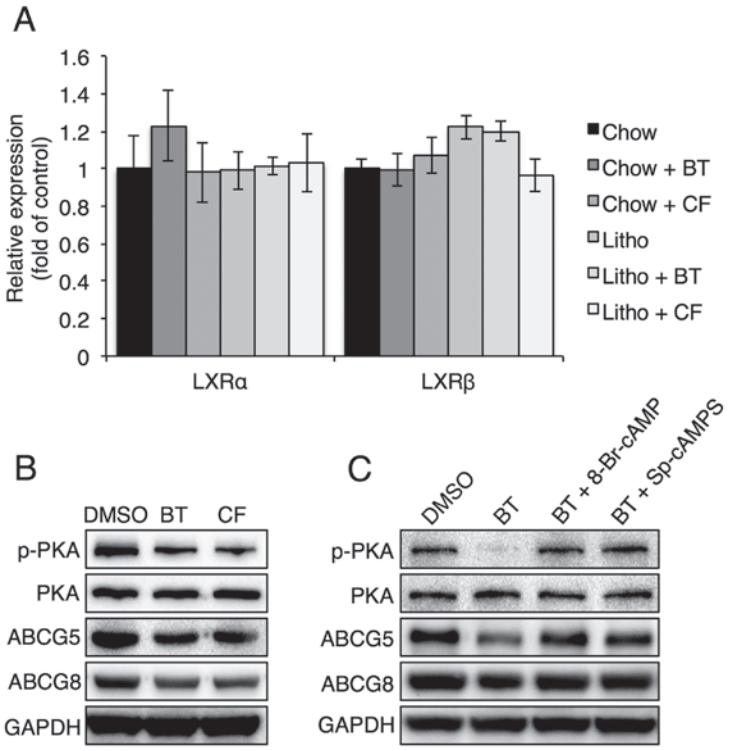

Figure 7. Reduced PKA phosphorylation as a result of proteasome inhibition serves a critical role in the downregulation of ABCG5 and ABCG8. (A) mRNA levels of LXRα and LXRβ in the livers of mice injected with BT or CF were measured by quantitative polymerase chain reaction (n=3). Representative western blot analyses of (B) Hep3B cells treated with 50 nM BT or 50 nM CF, and (C) Hep3B cells treated with 50 nM BT and 10 μM 8-Br-cAMP or Sp-cAMPS (PKA activators). Three independent experiments were performed. BT, bortezomib; CF, carfilzomib; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; Litho, lithogenic diet; ABCG, ATP-binding cassette transporter; PKA, protein kinase A; LXR, liver X receptor.

a critical role in the regulation of ABCG5 and ABCG8 levels during proteasome inhibition.

Discussion

Gallstone disease is one of the most common gastrointestinal diseases worldwide, and the cholesterol, phospholipid and bile acid composition of bile serves an important role in the pathogenesis of cholesterol gallstone formation (2). For example, cholesterol overcomes its low solubility in aqueous solutions by forming micelles with phospholipids and bile acids (2,3). Clinically, the main strategies to prevent gallstone disease have been focused on reducing cholesterol synthesis, reducing cholesterol secretion or inducing FXR, which increases bile acid and phospholipid secretion by increasing BSEP and MDR2 levels (27). In the present study, proteasome inhibition with BT or CF prevented gallstone formation by reducing cholesterol synthesis (via reductions in SREBP-2 and HMGCR) and by reducing cholesterol secretion (via reductions in ABCG5 and ABCG8). As excess cholesterol in bile can precipitate and lead to gallstone formation, reducing biliary cholesterol by proteasome inhibition is a strategy that can be utilized clinically to prevent gallstone disease. Considering that BT is currently utilized to treat hepatic conditions and certain cancer types, the protective effect of BT on gallstone formation has clinical importance.

Hepatic cholesterol levels, hepatic bile acid levels and the expression of genes involved in bile acid synthesis were significantly reduced upon proteasome inhibition. Reduced degradation of cholesterol to bile acids may lead to a higher concentration of free cholesterol that can be secreted leading to cholesterol saturation and gallstone formation (28). However, in contrast to hepatic bile acid levels, biliary bile acid levels were not affected by proteasome inhibition, thus biliary bile acid levels do not appear to affect gallstone formation. Rather, hepatic bile acid synthesis may be reduced to compensate for the reduction in hepatic cholesterol induced by proteasome inhibition.

In the present study, Cyp7a1 and Cyp27a1 protein levels were regulated by JNK phosphorylation as a result of proteasome inhibition. JNK phosphorylation has been shown to be induced by inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (29,30). However, TNF-α and IL-1β levels in the liver and serum were not altered upon proteasome inhibition (data not shown), suggesting that they are not involved in proteasome inhibition-induced phosphorylation of JNK. Although proteasome inhibition has been shown to activate MAPK (31,32), to the best of our knowledge, this is the first study to demonstrate that JNK regulates genes involved in bile acid synthesis upon proteasome inhibition. In contrast to the regulation of Cyp7a1 and Cyp27a1, JNK or ERK inhibition was unable to reverse the reduction in SREBP-2 and HMGCR levels induced by proteasome inhibition. The mechanism of the reduction in SREBP-2 and HMGCR levels by proteasome inhibition remains to be elucidated.

Proteasome inhibition also reduced the hepatic levels of the cholesterol transporters ABCG5 and ABCG8, but did not reduce the hepatic levels of the phospholipid and bile acid transporters MDR2 and BSEP, respectively. These data indicate that proteasome inhibition reduced hepatic cholesterol secretion to

bile and reduced hepatic cholesterol synthesis. Although LXR activation has been shown to increase gallstone formation by regulating ABCG5 and ABCG8 levels (25,33), BT or CF injection did not affect LXRα and LXRβ levels. PKA has also been shown to regulate ABCG5 and ABCG8 expression (26), and the present study showed that PKA phosphorylation was reduced by proteasome inhibition. In addition, it was found that PKA activation by 8-bromo-cAMP or Sp-cAMPS reversed the reduction of ABCG5 and ABCG8 upon proteasome inhibition, confirming the role of PKA in the proteasome inhibition-induced regulation of ABCG5 and ABCG8.

Although proteolytic activity of the 26S proteasome is ATP- and ubiquitin-dependent, c-AMP-induced PKA activation also stimulates the proteolytic activity of the proteasome (34). The regulatory subunit of PKA has been shown to be degraded via the proteasome (35), thus proteasome inhibition would lead to accumulation of the regulatory subunit of PKA. As the regulatory subunit of PKA functions as an inhibitor, PKA phosphorylation would be reduced (36).

BT is currently used clinically as a first-line chemotherapeutic agent for multiple myeloma, and high doses of BT induce endoplasmic reticulum stress and cell death (12,15,37). In addition, high doses of BT often lead to side effects, such as weight loss and painful neuropathy (38). It has been shown that administration of a low dose of BT (0.5 mg/kg/week) does not increase ER stress (13). Consistent with this, no signs of ER stress were observed with this low BT dose in the present study (data not shown). Although several studies have shown the protective effects of proteasome inhibition for liver diseases, including drug-induced hepatotoxicity (15) and ethanol-induced steatosis (13), to the best of our knowledge, this study is the first to show that proteasome inhibitors could be used to prevent gallstone formation.

In conclusion, in the present study, proteasome inhibitors reduced hepatic cholesterol and bile acid synthesis, and reduced biliary cholesterol secretion by reducing expression of the cholesterol transporters ABCG5 and ABCG8. This finding may lead to novel strategies for the treatment of gallstone disease.

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