Thyroid hormone receptor β1 stimulates ABCB4 to increase biliary phosphatidylcholine excretion in mice

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Abstract The ATP-binding cassette transporter ABCB4/MDR3 is critical for biliary phosphatidylcholine (PC) excretion at the canalicular membrane of hepatocytes. Defective ABCB4 gene expression and protein function result in various cholestatic liver and bile duct injuries. Thyroid hormone receptor (THR) is a major regulator of hepatic lipid metabolism; we explored its potential role in ABCB4 regulation. Thyroid hormone T3 stimulation to human hepatocyte models showed direct transcriptional activation of ABCB4 in a dose- and time-dependent manner. To determine whether THRβ1 (the main THR isoform of the liver) is involved in regulation, we tested THRβ1-specific agonists (e.g., GC-1, KB-141); these agonists resulted in greater stimulation than the native hormone. KB-141 activated hepatic ABCB4 expression in mice, which enhanced biliary PC secretion in vivo. We also identified THR response elements 6 kb upstream of the ABCB4 locus that were conserved in humans and mice. Thus, T3-via THRβ1 as a novel transcriptional activator regulates ABCB4 to increase ABCB4 protein levels at the canalicular membrane and promote PC secretion into bile. These findings may have important implications for understanding thyroid hormone function as a potential modulator of bile duct homeostasis and provide pharmacologic opportunities to improve liver function in hepatobiliary diseases caused by low ABCB4 expression.—Gautherot, J., T. Claudel, F. Cuperus, C. D. Fuchs, T. Falguières, and M. Trauner. Thyroid hormone receptor β1 stimulates ABCB4 to increase biliary phosphatidylcholine excretion in mice. J. Lipid Res. 2018. 59: 1610–1619.

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The ATP-binding cassette transporter ABCB4/MDR3 (Abcb4/Mdr2 in rodents) is expressed at the bile canalicular membrane of hepatocytes where it mediates the biliary excretion of phosphatidylcholine (PC) (1). PC emulsifies bile acids (BAs) and cholesterol to form stable mixed micelles in bile, neutralizing detergent BAs properties and preventing cholesterol crystallization in the biliary tract (2). Alcb4 knockout mice are unable to secrete PC into bile and develop bile duct injury and progressive cholestasis (3) resembling hepatobiliary diseases in humans (4). As such, mutations of ABCB4 have been shown to cause progressive familial cholestasis type 3 in children, a lethal liver disorder characterized by early onset of persistent cholestasis that progresses to cirrhosis and liver failure (5). Low phospholipid-associated cholelithiasis syndrome and intrahepatic cholestasis of pregnancy are less severe diseases and generally observed in adult patients with heterozygous ABCB4 variants (6, 7). The only therapeutic option available for these patients currently is the administration of ursodeoxycholic acid, a hydrophilic BA, which replaces more toxic endogenous BAs. However, some patients do not or only partially respond to this treatment (5). Moreover, stimulation of ABCB4 may also be beneficial in other cholangiopathies such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (8). Therefore, alternative...
therapeutic strategies, such as increasing ABCB4 expression and PC concentration in bile, may constitute an option, especially in patients with low PC concentrations in bile. So far, two nuclear receptors have been identified as ABCB4 regulators. The nuclear receptors farnesoid X receptor (FXR, NR1H4) and PPARα (NR1C1) control ABCB4 mRNA levels in both rodents and human models (9–12). FXR and PPAR response elements have already been identified in the promoter of ABCB4 (8, 12).

In our study, we hypothesized that ABCB4 expression could be regulated by thyroid hormones (THs) because these are known to exert a number of physiological roles in lipid metabolism, notably by increasing cholesterol excretion in bile (13). Triiodothyronine (T3) is the native ligand of thyroid hormones receptors (THRs). THRs present α and β subtypes with several isoforms where THRβ1 is the major expressed in the liver (14). Here, we investigated the impact of THRβ1 in the regulation of ABCB4 in immortalized human cell lines and in mice. Our data established that THRβ1 agonists increased ABCB4 expression by a direct transcriptional mechanism in a time- and dose-dependent manner. Moreover, we showed that THRβ1 agonists increased PC excretion in bile of wild-type mice via the upregulation of Abcb4. Finally, in silico analysis of ABCB4 promoter followed by gel shift assays identified three thyroid hormone receptor response elements (TRES) located in the proximal promoter of ABCB4 and conserved between human and mouse. These findings may provide novel pharmacologic opportunities to improve liver functions in patients with hepatobiliary diseases due to low ABCB4 expression.

MATERIALS AND METHODS

Cell models, culture, and treatments

HepG2 were obtained from the ATCC (LGC standards, Teddington, UK), HuH-7 were from the Japanese Collection of Research Biorepositories Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Japan). IHH were previously described (15): a clone was obtained by derivation from the original cell population, which was grown in DMEM (Gibco, ThermoFisher Scientific, Vienna, Austria). Dishes were precoated with 0.05% gelatin (Sigma-Aldrich, Vienna, Austria) for 5 min and all cells were maintained in DMEM supplemented with 10% FBS and streptomycin/penicillin in 5% CO2 at 37°C.

THR agonists and reagents

3,3′,5-Triiodo-L-thyronine (triiodothyronine/T3) (Sigma-Aldrich), GC-1 (Tocris Biosciences, Vienna, Austria), KB-2115 (Cayman Chemical, Hamburg, Germany) and KB-141 (kindly provided by Dr. Kevin J. Philips, The Methodist Hospital Research Institute, Houston, TX) were solubilized in 0.01 M NaOH to form 1 to 20 mM stock solutions. CGS-23425 (kindly provided by Dr. Ivan Tanevski, Medical University of Innsbruck, Innsbruck, Austria) was solubilized in DMSO in a 2 mM stock solution. A 5 mg/ml stock solution of the transcriptional inhibitor actinomycin D (ActD, Sigma-Aldrich) was prepared in DMSO. The translational inhibitor cycloheximide (CHX) was purchased from Sigma-Aldrich as a 100 mg/ml ready-to-use solution in DMSO.

Western blot analysis

Protein concentrations were determined with bicinchoninic acid protein assay (ThermoFisher Scientific) and cells were lysed in a loading buffer (26 mM Tris, 120 mM NaCl, 0.8 mM EDTA, 1% SDS, 0.3 M sucrose, 0.01% bromophenol blue and 0.094% β-mercaptoethanol, pH 7.4). Immunoblotting was performed with 30 μg of total proteins as previously described (16) using anti-ABCB4 antibody (P3II-26, Enzo Life Sciences, Lausanne, Switzerland) and anti-β-actin monocolonal antibody (Sigma-Aldrich) followed by peroxidase-conjugated secondary antibodies (Cell Signaling, New England Biolabs, Frankfurt, Germany). Development was performed with ECL plus detection kit (GE Healthcare, Fisher Scientific, Vienna, Austria). Blot exposure times were within the linear range of detection, and signal intensities were quantified using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij/download.html). Immunoblot quantification of ABCB4 was done using β-actin as loading control.

In silico analysis and DNA binding assay and EMSA

In silico analysis of the human ABCB4 promoter was done with both Genomatix (Genomatix, Munich, Germany) and NUBIscan (University of Basel, Switzerland) software. Alignment and analysis of the DNA loci of human and mouse Abcb4 were made by searching Homologene for ABCB4 sequences (https://www.ncbi.nlm.nih.gov/homologene/136368), followed by BLAT analysis to delineate exon/intron boundaries and walk on the chromosome sequence to generate 12 Kb of promoter (mouse genome: http://genome.ucsc.edu/cgi-bin/hgBlat?hgsql=667866713_WM1UNigaDM2tZNBrELngkW79jp&command=start). The TRES identified by Nubiscan and Genomatix were then searched and localized based on BLAT genome position across the generated sequence in mice using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Potential TRES were checked by electrophoretic mobility shift assay (EMSA) as follows. PSG5 plasmid encoding human THRβ1 (kindly provided by Dr Koshi Hashimoto from the Tokyo Medical and Dental University, Japan) was transcribed in vitro and translated using the TNT T7 Quick Coupled Transcription/Translation kit (Promega, Mannheim, Germany) following the manufacturer’s instructions. Double-stranded oligonucleotides (see sequences in Fig. 5B) were end labeled with γ-32P ATP (Hartmann Analytic, Braunschweig, Germany) using T4-polynucleotide kinase (New England Biolabs). Oligonucleotides and THRβ1 were mixed in a DNA binding buffer containing in a 20 µl final volume 10 mM Tris/HCl, pH 8.0, 40 mM KCl, 1 mM DTT, 0.05% Nonidet P40 (Sigma-Aldrich), 6% (v/v) glycerol, for 10 min at room temperature. Migration was done in 6% polyacrylamide gel on Tris borate EDTA buffer (Gibco) 0.25X (Fig. 5C).

Nuclear extract preparation

Primary human immortalized hepatocytes were grown in DMEM without FCS but with 0.5% penicillin/streptomycin (ThermoFisher Scientific), incubated for 48 h without or with T3 at 20 µM and nuclear extracts were prepared. Briefly cells were centrifuged for 5 min at 800 g, the pellet was dissolved in homogenization buffer (15 mM Tris HCl, pH 8; 15 mM NaCl; 60 mM KCl; 0.5 mM EDTA; 1 mM PMSF; and 1 mM β-mercaptoethanol). After centrifugation for 5 min at 800 g, the cell pellets were resuspended in hypotonic buffer (HB) buffer containing 0.05% Triton X-100. After another centrifugation at 1,000 g, the supernatant (cytosolic fractions) was collected and kept at −80°C. The pellets were washed with 5 ml HB buffer with Triton and centrifuged for 10 min at 1,000 g. The pellet was then washed with 5 ml HB buffer without Triton and centrifuged at 1,000 g for 10 min. Finally, pellets were suspended in 50 µl HB buffer modified with 360 mM KCl.
and incubated at 4°C for 30 min, before a final centrifugation for 5 min at 12,000 g. The supernatant (nuclear fractions) was kept at −80°C before use in gel shift. Protein concentrations were determined by bicinchoninic assay (ThermoFisher). Nuclear or cytosolic protein extracts (2 µg) were incubated for 10 min at room temperature in a total volume of 20 µl binding buffer described above before the radiolabeled probe (0.5 ng) was added. Binding reactions were further incubated for 10 min with THRβ1 antibody (sc-7838, Santa Cruz Biotechnology, Heidelberg, Germany) and resolved by 4% non-denaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA buffer at room temperature (Fig. 5D).

Animals and treatment

Fourteen C57BL/6 male mice, 8–10 weeks old, were housed under a 12:12 h light/dark cycle and permitted ad libitum consumption of water and chow diet (A04, Safe-Diet, Augy, France). Five (control) and eight (KB-141) mice were treated for 1 week, either receiving chow control or chow supplemented with KB-141 at a final concentration of 0.83 mg/kg body weight/day as described (17). After bile duct ligation, bile was collected during 30 min from cannulated gall bladders. Livers were removed and frozen in liquid nitrogen before stored at −80°C. The experimental protocols were approved by the local animal care and use committee according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the US National Academy of Science (National Institutes of Health publication 86-23, revised 1985).

RNA isolation and qRT-PCR analysis

Total RNAs were isolated with Trizol (Thermo Fisher) and reverse-transcribed into cDNA by using the M-MLV Reverse Transcriptase kit (Sigma-Aldrich) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed on a SYBR Green PCR Master Mix and with the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Vienna, Austria). qRT-PCR was performed in a 20 µl reaction mixture containing SYBR Green PCR Master Mix, primer couples, and cDNA. Reactions were performed three times in duplicate in 96-well plates. Human and mouse ABCB4, ABCB11, CYP7A1, and cDNA. Reactions were performed three times in duplicate in 96-well plates. Human and mouse ABCB4, ABCB11, CYP7A1, ABCG5, ABCG8, and 36B4 oligonucleotide primers sequences are available upon request. All expression data were normalized to 36B4 as reference gene.

Biliary phosphatidylcholine, cholesterol, and BA assays

PC was quantified as previously described (18) but adapted for bile analysis by 1:500 dilution of the bile in PBS 0.1% Triton X-100. Briefly, three enzymatic reactions followed by fluorescence measurements were done together in 96-well black plates, non cell culture-treated for fluorescence and luminescence (FluoroNunc™, Nunc, UK). Measurement of PC content was based on the amount of choline released after phospholipase D (Enzo Life Sciences) treatment, using a fluorometric assay. Fluorescence was read (ex/em 320/404 nm) with a multiplate cytofluorimeter Spectra Fluor from Tecan (MTX Lab Systems, Vienna, VA). Cholesterol was quantified with the Wako cholesterol lab assay kit (Neuss, Germany) following the manufacturer's instructions. Four microliters of bile were used for each measurement. BAs were quantified with the Diasys BAs assay kit (Holzheim, Germany) following the manufacturer's instructions; this kit can measure cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, and muricholic acid and their glyco- and tauro-conjugate. Bile samples were used at a 1:20,000 dilution for each measurement. PC, cholesterol, and BAs concentrations were determined using standard calibration curves performed in parallel of the experiments.

Statistical analysis

Data are expressed as means ± SD. Statistical analyses were carried out by Wilcoxon-Mann-Whitney tests using BiostaTVG software (INSERM/UPMC, France). P-values > 0.05 were considered statistically significant.

RESULTS

Stimulation of ABCB4 expression by THR in hepatocellular model systems in vitro

Because it has been previously described that FXR and PPARα agonists increase ABCB4 gene expression (12, 19), we compared the efficiencies of these compounds to T3 in our cellular models. PPARα agonists, particularly fibrates, have been described as slight inducers of both ABCB4 mRNA and protein expression in mice (10, 20) and humans (19, 21). We analyzed and quantified the ABCB4 protein levels in hepatocyte cancer cell models HepG2 (Fig. 1A) and HuH-7 (Fig. 1C) or in normal primary hepatocytes immortalized by the SV40 large antigen IHH (Fig. 1B), after treatment with FXR, PPARα and THR agonists at the maximally nontoxic dose [50 µM CDCA, 5 µM GW4064, 150 µM bezafibrate (BZ), 150 µM fenofibrate (FF), and 20 µM T3 determined by a toxicity assay]. At baseline, all cell lines express ABCB4 protein despite variations with HepG2 displaying the lowest amount and HuH-7 the highest (“ctrl” lanes in Fig. 1A–C and supplemental Fig. S1). Immunoblot quantification showed that GW4064 activates ABCB4 expression in the same range than T3 by two-fold. BZ significantly increased ABCB4 expression by 1.14-fold in HepG2 and HuH-7 cells. FF did not regulate ABCB4 in vitro, which confirmed a previous study performed in HepG2 cells (19). This rather moderate effect of fibrates on our cellular models could be explained by the low expression of PPARα in human liver compared with mouse liver (22).

TH stimulates ABCB4 expression in a concentration- and time-dependent manner by a direct transcriptional mechanism

We next addressed the molecular mechanisms of the regulation of ABCB4 expression by T3 in vitro. T3 increased ABCB4 gene and protein expression in a dose-dependent manner in HuH-7 cells, with an optimal effect around 20 µM (Fig. 2A). In addition, ABCB4 expression progressively increased up to 48 h upon 20 µM T3 incubation (Fig. 2B). Similar results were obtained in HepG2 and IHH cells (data not shown). Time- and dose-dependent effects suggest a direct transcriptional control of ABCB4 expression by THR. To test this hypothesis, cells were treated with the transcriptional inhibitor ActD or the translational inhibitor CHX, respectively. As shown in Fig. 2C, T3 increased ABCB4 mRNA (near 2-fold) and this effect was abolished by ActD (T3 + ActD), thus establishing that T3 regulation of ABCB4 was transcriptionally mediated. Interestingly, CHX had no effect on ABCB4 expression (T3 + CHX), therefore demonstrating that T3 effect did not require neosynthesis of a protein
and as such was direct. Because T3 signaling can be mediated by either THRα1 (NR1A1) or THRβ1 (NR1A2), we then measured their respective expressions by qRT-PCR. As shown in Fig. 2D, THRα1 was barely detectable (average Ct value 34.08, when a Ct value of 35 is considered as no expression), whereas THRβ1 expression was 2,500 times higher than THRα1 (average Ct value 29.6), in line with the well-known tissue expression pattern of these receptors (23). Taken together, our results identify T3 as a novel direct transcriptional key regulator of ABCB4 expression via THRβ1.

Selective thyromimetics of the THRβ1 isoform stimulate ABCB4 expression more efficiently than T3

Because THRβ1 is the major isoform expressed in hepatocytes, we tested the specific THRβ1 agonists GC-1 (sobetirome), CGS-2345, KB-2115 (eprotirome), and KB-141. They are structurally similar to T3 (supplemental Table S2) and were also able to stimulate ABCB4 mRNA and protein expression (Fig. 3). At 20 µM, KB-2115, CGS-2345, and mostly KB-141 showed a higher stimulating effect of ABCB4 expression than T3 (5.8-fold for KB-141 vs. 3.5-fold for T3). Collectively, these data provide novel

![Fig. 1. T3 stimulates ABCB4 expression in vitro. HepG2 (A), IHH (B), and HuH-7 (C) cells were treated with THR (20 µM T3), FXR (50 µM CDCA, 5 µM GW4064), and PPARα (150 µM BZ, 150 µM FF) agonists for 48 h. Representative immunoblots are shown in the upper panel and quantifications show ABCB4 relative expression, normalized to β-actin, compared with untreated cells in the lower panels (means ± SD of at least five independent experiments). *P < 0.05; **P < 0.01; ***P < 0.001. BF, bezafibrate; FF, fenofibrate; n.s., nonsignificant compared with nontreated control cells.](image)

![Fig. 2. Thyroid hormone T3 stimulates ABCB4 expression via a transcriptional mechanism. A: HuH-7 cells are treated with the indicated range of T3 concentration for 48 h or (B) with 20 µM T3 for the indicated time periods. Representative immunoblots are shown in the upper panel and quantifications show ABCB4 relative expression, normalized to β-actin, compared with untreated cells in the lower panels (means of at least six independent experiments ± SD). **P < 0.01; ***P < 0.001. B: Transcriptional induction of ABCB4 by T3 is shown with prior treatment of cells with actinomycin D (ActD, 5 µg/ml) or cycloheximide (CHX, 10 µg/ml) for 1.5 h, followed by 20 µM T3 treatment in a fresh medium for an additional 48 h. ABCB4 mRNA levels were normalized to the 36B4 mRNA by qRT-PCR and control set at 100%. C: THRα1 and THRβ1 relative quantification in HuH-7. mRNA levels were normalized to 36B4 mRNA by qRT-PCR and control set at 100%. Results are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., nonsignificant compared with nontreated control cells.](image)
CGS-23425, 1 µM KB-141.

following concentrations: 20 µM T3, 10 µM GC-1, 1 µM KB-2115, 2 µM THR agonist stimulation by T3 or various THR-agonists at the indicated concentrations for 48 h. ABCB4 mRNA levels were normalized to the 36B4 mRNA by qRT-PCR and control set at 1. Means ± SD are shown relative to expression levels in control conditions. *P < 0.05; **P < 0.01; n.s., nonsignificant compared with control nontreated cells. Insert: Representative immunoblot of ABCB4 protein stimulation by T3 or various THR-agonists used as the following concentrations: 20 µM T3, 10 µM GC-1, 1 µM KB-2115, 2 µM CGS-23425, 1 µM KB-141.

evidence for the involvement of THRβ1 in ABCB4 selective regulation.

TH agonist KB-141 induces Abcb4/Mdr2 expression, resulting in increased phosphatidylcholine secretion in mice

To explore whether the observed in vitro findings result in increased biliary PC excretion in vivo, we investigated the effects of KB-141 on bile flow, PC, and BA secretion and Abcb4, Abcb11, Abcg5/g8 (PC, Bas, and cholesterol transporters, respectively), and Cyp7a1 after application over 1 week. KB-141 did not affect body, liver (Table 1), nor body-weight-to-liver-weight ratio (data not shown) but increased bile flow up to 1.8-fold compared with control mice (Fig. 4A, Table 1). Concentrations of biliary cholesterol increased up to 1.8-fold, BAs up to 1.7-fold (Table 1), and, as reflected by Cyp7a1 mRNA induction (Fig. 4E) and biliary PC, up to 2.6-fold, which was the most important effect (Fig. 4B, Table 1). Accordingly, biliary output of cholesterol was increased by 3-fold (Table 1), 2.9-fold for BA excretion, and 5.4-fold for PC excretion (Fig. 4C,D, Table 1). Because PC increased in a more pronounced manner than cholesterol and BAs, the PC/Ba ratio change significantly, increasing from 0.16 ± 0.02 in controls to 0.26 ± 0.04 in KB-141-treated mice (Table 1). Biliary cholesterol secretion was also increased without significantly affecting the PC/cholesterol ratio (Table 1). In line with these data, mRNA levels of Abcb4 and Abcb11 were increased by 2.35-fold and 1.2-fold, respectively, while Abcg5 and Abcg8 expressions remained unchanged after KB-141 treatment (Fig. 4E). Taken together, these results show that THRβ1 is a major stimulator of PC secretion via Abcb4 gene upregulation in mice in vivo.

EMSA identifies new TREs in the ABCB4 promoter

In silico analysis of the human ABCB4 locus with Genomatix and NUBIscan software mapped nine potential TREs (Fig. 5A). These putative TREs are direct repeat sequences separated by four nucleotides (DR-4) (Fig. 5B). To identify which TREs interact with THRβ1, EMSA was performed (Fig. 5C). As shown here, important interaction between THRβ1 and three TREs appeared as DNA-protein complex gel shifts. These TREs are located at position −3223/−3208 (TRE3, GGTTCACgATGCTCA), −6057/−6042 (TRE6, AGTGCAgcACAGGA) and −7649/−7654 (TRE7, AAGTCAGgtAGGCCA) upstream from the transcription start site (indicated as +1 in Fig. 5A) of ABCB4. These sequences are quite similar to the DR-4 consensus TRE sequence previously described (24). In order to explore whether these sequences were bound not only by a recombinant THRβ1 obtained in vitro, but also by the endogenous THRβ1 naturally expressed in cells, nuclear and cytosolic extracts of IHH cells treated with T3 were made and tested by gel shift (Fig. 5D). Whereas cytosolic extracts did not bind any of the probes (lanes “c” in Fig. 5D), nuclear extracts of untreated cells bound weakly the probes and displayed a high molecular weight complex (Fig. 5D, lanes “n T3−”). This binding intensity increased in lanes “n T3+”, while preincubation with an antibody against THRβ1 reduced the band intensity in lanes “n+ab T3+”, therefore identifying THRβ1 as a member of this complex (our antibody cannot generate a supershift because it binds THRβ1 in the DNA binding domain). These results showed direct interactions of THRβ1 to response elements located in the ABCB4 promoter and confirmed the transcriptional mechanism of the regulation of ABCB4 expression. Interestingly, the comparison of human and mouse ABCB4 loci showed that TRE3 and 6 were conserved in the mouse genome, whereas TRE7 was not (supplemental Table S3). In addition TRE8, which was not recognized by THRβ1, was also conserved in the mouse gene (supplemental Table S3). All the other TREs tested were absent in the mouse locus. Therefore these data suggest that TREs are critical for ABCBA expression.

FXR/THR agonists show additive stimulatory effects on ABCB4 expression with a maintained repressive effect on CYP7A1

Because our data indicated that T3 has a direct transcriptional effect on the ABCB4 gene (Fig.1) and it is known that FXR agonists stimulate ABCB4 mRNA levels (25), we explored potential additive effects of FXR and THR agonists on ABCB4 expression. At the protein level, cells treated with a mix of the natural FXR agonist chenodeoxycholic acid (CDCA) “CDCA/T3” or with a mix containing the nonsteroidal FXR agonist GW4064 “GW4064/T3” increased ABCB4 stimulation more substantially than the selected compounds alone, as shown in HepG2 (Fig. 6A), IHH (Fig. 6B) and HuH7 (Fig. 6C) cells. This was significant when GW4064 was mixed with T3. At the mRNA level,
in HuH-7 cells, ABCB4 expression increased in a similar manner when CDCA or GW4064 were coadministered with T3 (Fig. 6D). Because THR agonists increase Cyp7a1 (rate-limiting enzyme of BAs synthesis) (26) while FXR agonists decrease it (25), we were also interested to explore how CYP7A1 expression would be regulated by an FXR/THR agonist mix. In IHH cells, CYP7A1 repression by FXR was maintained even in the presence of a THR agonist (Fig. 6E), suggesting that FXR repression of CYP7A1 gene is stronger than its THR activation. These results prompted us to propose a mechanism of these additive stimulatory effects in ABCB4 and CYP7A1 genes, consisting in a differential regulation depending on the costimulation (Fig. 6F).

DISCUSSION

THs exert a number of regulatory effects on lipid metabolism, including stimulation of hepatic lipase activity, induction of low density lipoprotein receptors, induction of ABCB11 and ABCG5/G8 expression, promotion of cholesterol conversion into BAs, and cholesterol secretion into the bile (13, 26–28). These key findings are clinically observed in hypothyroid patients who show a less favorable profile with an increase of plasma cholesterol levels, while hyperthyroid patients show both positive and negative effects with reduced plasma cholesterol levels (29, 30) but increased heart rates and other abnormal cardiovascular hemodynamics (31). Immune-mediated thyroid dysfunction (Hashimoto’s thyroiditis and Grave’s diseases) may be associated with immune-mediated cholestatic liver diseases such as PBC or PSC (32, 33). Notably, impaired thyroid function can aggravate cholestasis in PBC, but the molecular mechanisms remain elusive (34). Moreover, bile duct injury PBC and PSC could be modified by alterations of bile composition and biliary toxicity (8).

Because ABCB4 is an important player in lipid metabolism by translocating PC across bile canaliculi, we hypothesized that T3 may activate its expression and mediate PC secretion into the bile (Fig. 7). In this study, we demonstrate

![Table 1](image-url)
that IHH and HuH-7 cells are new potential model systems for studying ABCB4. These cells present a high endogenous expression of ABCB4 in basal conditions. By using these cell lines, we investigated a new pathway of the transcriptional regulation of ABCB4 by THs. We here show that stimulation of ABCB4 expression by the novel "THR regulatory pathway" is similarly powerful as the "FXR pathway". These results bring new potential strategies for the treatment of some ABCB4 expression defective patients (e.g., those with progressive familial cholestasis type 3 or low phospholipid associated cholelithiasis syndrome) or acquired cholangiopathies such as PBC and PSC. It may be beneficial for patients to stimulate the expression of ABCB4 and restore a better PC/BA/cholesterol ratio (Fig. 7), which prevented us from performing a successful chromatin immune precipitation with an antibody against THR-β1 antibody.

Fig. 5. EMSA showed an interaction between a predictive TRE DNA sequence in the ABCB4 promoter and in vitro translated THR-β1 protein. Predictive TRE sequences obtained with Genomatix and NUBIScan software are indicated in a graph (A) and are listed in (B). Plus (+) and minus (-) indicates coding and non-coding strands of ABCB4 gene. C: THR-β1 was in vitro translated and mixed with 32P-labeled oligonucleotides corresponding to TRE predictive sequences shown in B. Acrylamide gel electrophoresis migration shows interactions between DNA and THR-β1 as shifts (TRE3, 6, and 7 lines). D: TRE3, 6, and 7 were incubated with cytosol or nuclear fraction of IHH cells treated or not with T3. Shifts show interactions between DNA and THR-β1.

TH receptors present several isoforms encoded by THRA and THRβ genes. In the liver, THRβ1 isoform represent 80% of THRs [and the only one detected in HepG2 cells (14)], the remaining 20% are represented by THRα1 (36). THRα1 is the major THR in the heart and it is crucial for heart rate (37), whereas THRβ1 is crucial for lipid metabolism. Although we show that the native hormone T3 increases ABCB4/Abcb4 gene expression, the use of T3 for treatment of cholestasis due to a biliary phospholipid defect is of limited interest because of deleterious cardiac side effects. Previous studies focused on treatment of hypercholesterolemia have led to development of thyromimetic drugs (38). Importantly, GC-1 (also called sobetirome) and KB-2115 (also called eprotirome) have been investigated in clinical studies where they reduced plasma cholesterol levels (40, 41). As we showed in the present study, these compounds are potent stimulators of ABCB4 expression in vitro, and KB-141 increased Abcb4 expression and PC secretion in mice. T3 induces BAs and cholesterol secretion via overexpression of both the BA transporter Abcb11 (27) and the heterodimeric cholesterol transporter Abcg5/Abcg8 (26), although in our hands, Abcg5/Abcg8 expression was unchanged despite increased cholesterol excretion into the bile (Table 1), perhaps because in addition to gene control, THRβ1 agonists could also increase the activity and/or enhance the membrane localization of the dimer. Moreover, THs stimulate expression of CYP7A1 (17, 42) and thus regulate cholesterol catabolism via its conversion into BAs and its secretion into the bile. Therefore, THs increased mixed micelle stability by promoting biliary BA, cholesterol, and phospholipid secretion into the bile (2).
the identification of several polymorphisms in THRα and THRβ genes (47), across multiple populations (48), including mutations resulting in partial or complete thyroid hormone resistance (49), the liver phenotype and hence the prevalence of gallstones in such patient populations is still not reported.

Because it has been demonstrated that CDCA and the potent FXR agonist GW4064 significantly increase $ABCB4$ mRNA levels in humans and rodents models (11, 12, 41) via FXR, we explored potential synergistic effects of THR and FXR agonists. An additional stimulating effect was observed suggesting that these two pathways are separate but complementary, findings which now can be explained by the newly identified TREs in the $ABCB4$ promoter. Interestingly, we showed here that coincubation of T3 and FXR agonists resulted in a maintained repression of $CYP7A1$ expression due to FXR while THR agonists used alone showed a stimulatory effect. This would allow an increased PC secretion on the one hand, while simultaneously decreasing BAs synthesis on the other hand, the latter being considered a central mechanism in protecting against cholestatic liver injury (4). Based on our results,

Fig. 6. Additive effects of THR and FXR agonists on the stimulation of $ABCB4$ expression. HepG2 (A), IHH (B), and HuH-7 (C) cells were treated with THR and/or FXR agonists for 48 h. Representative immunoblots are shown in the upper panel and quantifications show $ABCB4$ relative expression compared with β-actin in the lower panels (means ± SD of at least five independent experiments). D: $ABCB4$mRNA levels were measured in HuH-7 cells and normalized to the 36B4 mRNA by qRT-PCR and control set at 1. E: $CYP7A1$ mRNA level was measured in IHH cells and normalized to the 36B4 mRNA by qRT-PCR and control set at 1. Means ± SD of at least four experiments per condition are shown as fold increase compared with control conditions. *P < 0.05; n.s., nonsignificant. CDCA, 50 µM; GW4064, 5 µM, T3, 20 µM. F: Schematic representation of the proposed mechanism of synergic inducibility of $ABCB4$ or $CYP7A1$ genes by THR and FXR agonists.
potential dual therapy with THR and FXR agonists could be tested in future studies. 

In addition, it was demonstrated that PPARα agonists slightly activate ABCB4 expression in humans (21), which may contribute to the benefit of fibrate therapy observed in patients with chronic cholestatic liver diseases such as PBC and PSC (8). However, the expression of PPARα is rather low in human liver compared with mouse (22), as such fibrate stimulation exerts only moderate effects and recent studies could neither demonstrate a stimulation of ABCB4 mRNA expression by fibrates in PBC and fatty liver patients (50) nor in gallstone patients (21). However, fibrates can increase the lithogenicity of bile in pediatric and adult liver disease: one gene for three diseases. Semin. Liver Dis. 21: 551–562.

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