CYP7A1 gene induction via SHP-dependent or independent mechanisms can increase the risk of drug-induced liver injury independently or in synergy with BSEP inhibition

Congrong Niu, Xiaodong Xie, Renmeng Liu, Xiaomin Liang, Yiding Hu and Yurong Lai

Drug Metabolism, Gilead Sciences Inc., Foster City, CA
Running title: CYP7A1 induction can pose a risk for DILI

Corresponding author and contact information:

Yurong Lai
Drug Metabolism, Gilead Sciences Inc., Foster City, CA 94404
Tel: 650-522-1629
Email: yurong.lai@gilead.com

Number of words in the abstract: 246
Number of words in the introduction: 714
Number of words in the discussion: 1435
Number of tables: 1
Number of figures: 5
Number of references: 41

Abbreviations:

DILI, Drug-induced liver injury; BSEP, bile salt export pump; OATP, organic anion transporting polypeptides; PXR, pregnane X receptor; AhR, Aryl hydrocarbon receptor; CYP7A1, 7 alpha-hydroxylase; MEK, mitogen-activated protein kinase kinase enzymes; SHP, small heterodimer partner.
Abstract

Drug-induced liver injury (DILI) is a frequent cause of clinical trial failures during drug development. While inhibiting BSEP is a well-documented DILI mechanism, interference with genes related to bile acid (BA) metabolism and transport can further complicate DILI development. Here, the effects of twenty-eight compounds on genes associated with BA metabolism and transport were evaluated, including those with discontinued development or use, boxed warnings, and clean labels for DILI. The study also included rifampicin and omeprazole, PXR and AhR ligands, and four mitogen-activated protein kinase kinase (MEK1/2) inhibitors. BSEP inhibitors with more severe DILI, notably pazopanib and CP-724714, significantly upregulated the expression of 7 alpha-hydroxylase (CYP7A1), independent of small heterodimer partner (SHP) expression. CYP7A1 expression was marginally induced by omeprazole. In contrast, its expression was suppressed by mometasone (10-fold), vinblastine (18-fold), hexachlorophene (2-fold), bosentan (2.1-fold), and rifampin (2-fold). All four MEK1/2 inhibitors that show clinical DILI were not potent BSEP inhibitors but significantly induced CYP7A1 expression, accompanied by a significant SHP gene suppression. SULT2A1 and BSEP were marginally upregulated, but no other genes were altered by the drugs tested. Protein levels of CYP7A1 were increased with the treatment of CYP7A1 inducers and decreased with obeticholic acid, an FXR ligand. CYP7A1 inducers significantly increased BA production in hepatocytes, indicating the overall regulatory effects of BA metabolism. This study demonstrates that CYP7A1 induction via various mechanisms can pose a risk for DILI, independently or in synergy with BSEP inhibition, and it should be evaluated early in drug discovery.
Significant Statement

Kinase inhibitors, pazopanib and CP-724714, inhibit BSEP and induce CYP7A1 expression independent of SHP expression, leading to increased bile acid (BA) production and demonstrating clinically elevated drug-induced liver toxicity. MEK1/2 inhibitors that show BSEP-independent DILI induced the CYP7A1 gene accompanied by SHP suppression. CYP7A1 induction via SHP-dependent or independent mechanisms can pose a risk for DILI, independently or in synergy with BSEP inhibition. Monitoring BA production in hepatocytes can reliably detect the total effects of BA-related gene regulation for de-risking.
Introduction

Drug-induced liver injury (DILI) is a common adverse effect that often results in regulatory action or causes drug trial failures during drug development (Watkins, 2011; Thakkar et al., 2018). For example, several marketed drugs, such as bosentan and trovafloxacin, have prescribing restrictions, and others, like troglitazone, benoxaprofen, and bromfenac, have been withdrawn from the market. Many investigational drugs, e.g., CP-724714, have been terminated during the drug development due to DILI (Senior, 2014). The causative elements behind DILI are complex and can involve various factors such as drug physicochemical properties, pharmacokinetics, mitochondrial susceptibilities, immune responses, reactive metabolites, and disruption of bile salt homeostasis (Chen et al., 2015).

The liver produces bile acids (BAs), which aid in the absorption of lipids and fat-soluble vitamins. BAs are secreted by the bile salt export pump (BSEP/ABCB11), which is indispensable for maintaining BA homeostasis. In addition, organic anion-transporting polypeptides (OATPs), sodium taurocholate co-transporting polypeptide (NTCP), organic solute transporter (OST), and multidrug resistance-associated proteins (MRPs) also play a substantial role in transporting BAs in and out of the hepatocytes (Figure 1). Inhibition of BSEP can lead to BA accumulation in hepatocytes, causing cell damage (Morgan et al., 2010; Dawson, 2011; Morgan et al., 2013). While various in vitro assays for BSEP inhibition have been developed to mitigate the DILI risk (Brouwer et al., 2013; Cheng et al., 2021), translating in vitro BSEP inhibition data directly to human DILI outcomes remains a formidable challenge, as not all potent BSEP inhibitors would cause DILI, and vice versa (Chan and Benet, 2018). Other mechanisms can also
contribute to the development of DILI (Morgan et al., 2013; Qiu et al., 2016). In addition, although preclinical species share BA transport functions with humans, they are more tolerant to hepatotoxicity related to BSEP function (Noe et al., 2001; Cheng et al., 2017). This difference in tolerance may be attributed, in part, to compensatory pathways in rodents that maintain BA homeostasis despite the absence of Bsep activity (Wang et al., 2009; Cheng et al., 2017) or form a more hydrophilic and less toxic BA pool in rodents (Perwaiz et al., 2003).

BA homeostasis is a complex process involving synthesis, metabolism, and trafficking, which are governed by feedback mechanisms that are not fully understood. Approximately a dozen enzymes are involved in the synthesis and metabolism of BAs (Figure 1) and can be divided into two major BA synthetic pathways: the classic pathway and the alternative pathway (Figure 1). Cholesterol 7 alpha-hydroxylase (CYP7A1) is the rate-limiting enzyme of cholesterol hydroxylation in the classic pathway to form 7α-hydroxycholesterol and, subsequently, cholic acid (CA) and chenodeoxycholic acid (CDCA). The alternative BA synthesis pathway begins with CYP27A1 to synthesize CA and CDCA (Figure 1). Before being secreted into bile, CA and CDCA are subsequently conjugated with glycine and taurine by BA-CoA synthetase (BACS) and BA-CoA:amino acid N-acetyltransferase (BAAT) (Figure 1). Once in the ileum, secondary BAs such as deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) are generated by gut bacterial via BA hydrolase. The secondary BAs can be further conjugated with glycine and taurine (Figure 1). About 95% of BAs are absorbed in enterocytes by apical sodium bile salt transporter (ASBT) and organic solute transporter α/β (OSTα/OSTβ) and circulated back to hepatocytes via NTCP and OATPs (Figure 1).
BAs can also undergo sulfation catalyzed by SULT2A1 or glucuronidation by UGT1A1, yielding sulfate or glucuronide conjugates in the liver (Alnouti, 2009; Huang et al., 2010).

BA metabolizing enzymes and transporters in hepatocytes are essential factors attributing to intrahepatic BA exposure and homeostasis. Recently, Saran et al. found that CYP7A1 induction by tyrosine kinase inhibitors (TKIs) can be a risk of hepatotoxicity, in addition to BSEP inhibition (Saran et al., 2022). In addition, Verzijl et al. (Verzijl et al., 2023) found that two BA-related genes, the small heterodimer partner (SHP) and Cyp7a1, are the top differentially expressed genes in liver tissues from the mice treated with PD0325901, an MEK inhibitor. The results may explain the observed DILI of MEK inhibitors in humans (Verzijl et al., 2023). This study aims to investigate the impact of BSEP inhibitor drugs and MEK1/2 inhibitors on the gene expression of BA metabolizing enzymes and transporters in human hepatocytes. The goal is to identify potential new mechanisms that may contribute to DILI as independent or synergistic risk factors in addition to BSEP inhibition.

Materials and Methods

Chemicals, Reagents, and Hepatocytes

Pazopanib, Ketoconazole, Lapatinib, Bosentan, Novobiocin, CP-724714, Bepridil, Vinblastine, Midazolam, Mometasone, Pioglitazone, Rifampicin, Benzbromarone, Fipexide, Mibefradil, Omeprazole, Nefazodone, Danazol, Sulfinpyrazone, Bithionol, Hexachlorophene, Beclomethasone, Econazole, Sitaxsentan, Obeticholic acid,
Trametinib, Cobimetinib, GDC-0623 and Mirdametinib were purchased from Sigma-Aldrich (St. Louis, MO) or obtained from Gilead sample bank. CA and CDCA were purchased from MedChemExpress LLC. (Monmouth Junction, NJ) and Alfa Aesar (Haverhill, MA), respectively. Taurochenodeoxycholic acid (TCDCA) was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), methanol, water, and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA). Glycocholic acid (GCA), glycodeoxycholic acid (GDCA), TCA, taurodeoxycholic acid (TDCA), and tauroursodeoxycholic acid (TUDCA) were purchased from EMD Millipore Corp. (Burlington, MA). Glycochenodeoxycholic acid (GCDCA) was purchased from Cayman Chemical Company (Ann Arbor, MI). Glycoursodeoxycholic acid (GUDCA) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Deoxycholic acid-d6 (D6-DCA), 2-propanol, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO).

The Matrigel™ used in the experiments was obtained from Corning (Bedford, MA). The 96-well and 24-well collagen I-coated plates were purchased from Life Technologies (Carlsbad, CA). Hepatocyte thawing medium (UCRM™) was purchased from In Vitro ADMET (Columbia, MD). Cryopreserved human hepatocytes (lots AOS, OQA, and YTW; Supplemental Table 1), hepatocyte plating medium (InVitroGRO™ CP medium), incubation medium (InVitroGRO™ HI medium), and Torpedo™ Antibiotic Mix were supplied by BioIVT (Westbury, NY). Cryopreserved human hepatocytes (lot HU8319; Supplemental Table 1), RIPA Lysis and Extraction Buffer, and the protein quantification BCA kit were obtained from Thermo Fisher. Trypsin was purchased from Promega (Madison, WI). All the oligonucleotide primer and probe sets used in the study
were purchased from Life Technologies. The CYP7A1 peptide H2N-TLENAGQK-OH and its stable isotope-labeled internal standard H2N-TLENAGQK\textsuperscript{-}-OH were synthesized and purified Biosynth (Boston, MA). High-performance liquid chromatography (HPLC) grade water and acetonitrile were obtained from EMD Chemicals, Inc. (Gibbstown, NJ) and Burdick & Jackson (Muskegon, MI), respectively. Formic acid was sourced from Sigma-Aldrich (St. Louis, MO).

**Assessment of Gene Regulations in Sandwich-Cultured Human Hepatocytes (SCHH)**

Hepatocytes were cultured in a sandwich format using a protocol adapted from the previous publication (Li et al., 2009). Briefly, cryopreserved primary human hepatocytes (details in Supplementary Table 1) were thawed at 37 °C in a plating medium (INVITROGRO CP, BioIVT) and then seeded at 65,000 cells per well in a 96-well collagen I-coated plate. The hepatocytes were allowed to attach for 4 – 6 hours. After washing once with ice-cold INVITROGRO HI media (BioIVT), the cells were overlaid with ice-cold media containing 0.25mg/ml Matrigel\textsuperscript{TM} (Corning, Tewksbury, MA). The hepatocytes were cultured at 37°C in a humidified 5% CO\textsubscript{2} incubator, and the incubation medium was replenished daily for 3 days. The cells were treated with compounds for 8 hours at concentrations ranging from 0.1 to 30 μM or up to the highest non-cytotoxic concentration.

BA-related gene regulation potentials were assessed following the previous protocols with modification (Niu et al., 2019; Niu et al., 2020). In brief, total RNA from cultured hepatocytes was isolated using an RNeasy 96 kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. RNA was eluted with 150 μL in RNase-free water. 5 μL of extracted RNA was used in all RT-qPCR reactions. All RT-qPCRs were
performed using TaqMan Fast Virus 1-step Master Mix (Life Technologies, Carlsbad, CA). The mRNA expression levels were measured as a fold-change compared to non-treated controls using the delta-delta Ct method (Livak and Schmittgen, 2001). Ct represents the cycle number at which fluorescence crosses a preset threshold. The delta Ct represents the difference between the Ct target and reference housekeeping gene, and the delta-delta Ct is the difference between the delta Ct of the treated samples and the delta Ct of the control. β-actin was selected as the housekeeping gene to normalize target gene mRNA expression. The primers and probes of target and housekeeping genes were purchased from Life Technologies and presented in Supplementary Table 2. The fold-change of a target gene was represented as a mean ± S.D. For the significance of gene regulation, a concentration-dependent increase or decrease of 2-fold and p<0.05 by t-test comparing to the untreated control was set.

**Bile Acid Production in SCHH**

A previous protocol was adapted (Qiu et al., 2016) to determine BA concentrations in the supernatant of SCHH, and the compounds were tested at a concentration of 10 µM. Briefly, the hepatocytes were seeded at 450,000 cells per well in a 24-well collagen I-coated plate (day 1) and overlaid with Matrigel™. On day 3, the HI medium was replaced with 1 mL/well fresh HI medium containing 10 µM of obeticholic acid, pazopanib, CP-724714, mirdametinib, trametinib, cobimetinib, GDC-0623 or dimethyl sulfoxide only. Each treatment was in triplicates. Then 100 µL of supernatant from each well was collected at 2, 8, 24, and 48 hours and stored at -80°C for BA analysis. The cells were washed twice with Tris-buffered saline (TBS) and stored at -80°C for CYP7A1 protein analysis.
For BA quantification, 50 μL of the culture medium at each collected time point was mixed with 200 μL of methanol containing d6-DCA (500 nM) to precipitate proteins. After centrifugation for 15 minutes at 3,500 rpm, 100 μL of supernatant was transferred to an Eppendorf Protein LoBind 96-well plate containing 100 μL of H2O. Individual BA analysis was conducted using a Sciex 7500 mass spectrometer coupling with a SHIMAZU LC system (LC-40). Chromatographic separations were performed following a previous method (Qiu et al., 2016) on a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 x 100 mm) at 60 °C with a flow rate of 550 μL/min. The mobile phase A consisted of water containing 0.1% formic acid, while the mobile phase B was acetonitrile with 0.1% formic acid. The mass spectrometer parameters included an ion source temperature = 500 °C, ion source gas 1 = 30 psi, ion source gas 2 = 70 psi, curtain gas = 40 psi, and spray voltage = 3500 V. The analysis was conducted in multiple reactions monitoring (MRM) mode, and the corresponding MRM transitions are listed in the supplementary table 3. Peak integration and quantification were achieved using Sciex OS software (v 2.2). Standard curves for each BA were constructed with a weighting factor 1/x², and concentrations in study samples were calculated from the regression line.

**CYP7A1 Protein Quantification Using Targeted Proteomics LC-MS/MS Method**

To quantify CYP7A1 protein after incubation with the inducers, the total proteins were extracted from the cell layer using the RIPA Lysis and Extraction Buffer, and the total protein concentrations were determined using BCA protein assay kits following the manufacturer's instructions. The denaturation and digestion of protein fractions were
conducted following the protocol outlined by Balogh et al., with certain modifications (Balogh et al., 2013). Aliquots of total protein (80 μg) were incubated at 95 °C for 5 minutes with 10 mM dithiothreitol in a 25 mM ammonium bicarbonate buffer containing 1% deoxycholate. Subsequently, alkylation was performed by incubating 15 mM iodoacetamide in the dark for 30 minutes at room temperature. Each sample then received trypsin at a ratio of 1:20–50 (trypsin: protein), and the digestion proceeded overnight at 37 °C. A subset of samples containing an equivalent amount of human serum albumin that underwent the same treatment was prepared for the external quantification standard curve. The digestion process was halted by adding an equal volume of water containing 0.2% formic acid and stable isotope-labeled (SIL) internal standard to each sample. For the construction of the calibration curve, the human serum albumin digestions were mixed with water containing 0.2% formic acid, synthetic unlabeled peptides (0.03–100 nM each), and the SIL internal standard.

The quantitative targeted proteomics measurement of CYP7A1 was conducted using a Sciex 7500 mass spectrometer coupled with a SHIMAZU LC system (LC-40). Peptide separation and elution were achieved using a Phenomenex Kinetex C18 (2.6 μm, 3.0 x 100 mm) column. The mass spectrometer was configured with the following settings: ion spray voltage at 4.5 kV, temperature at 500 °C, curtain gas at 40 psi, and ion source gases 1 and 2 at 40 and 50 psi, respectively. The mobile phase A was water containing formic acid (0.1% v/v) and the mobile phase B was acetonitrile with formic acid (0.1% v/v). An 11-minute linear gradient was employed for chromatographic separation, with the following profile: 0–2 minutes: 3% B; 2–6 minutes: 3–40% B; 6–7 minutes: 40–100% B; 7–8 minutes: 100% B; 8–8.5 minutes: 100–3% B; 8.5–11 minutes:
3% B. A 10 µL sample volume was injected into the LC column at a 0.4 mL/min flow rate. The ion transitions at \(430.8 \rightarrow 646.4\) and \(434.8 \rightarrow 654.4\) were used for the analyte and corresponding internal standard, respectively.

**Inhibition of BSEP in Suspended Human Hepatocytes**

The BSEP inhibition assay was conducted in Biotrex LLC (Monmouth Junction, NJ) using BSEPcyte methods. In brief, hepatocyte thawing medium (50 mL) was pre-warmed at \(37^\circ C\). Immediately taken out from a liquid N2 tank, hepatocyte vials were quickly warmed up in a \(37^\circ C\) water bath with slow rotation. The cells were then poured into the pre-warmed hepatocyte thawing medium. The tube was centrifuged at 100g at \(25^\circ C\) for 5 minutes. The supernatant was discarded, and the cell pellet was re-suspended in 8 mL of pre-warmed William’s E buffer. The hepatocyte viability was determined using the Trypan blue method, and the density was adjusted to 0.5 million cells/mL with William’s E buffer. All the assays were conducted in triplicates. Human hepatocytes at 0.25 million cells/mL were incubated with 10 µM of CA in William’s E buffer in the presence of the test compounds at \(37^\circ C\) under 5% CO\(_2\) and saturated humidity for 1 hour. At the end of the incubation, the extracellular medium was separated by centrifugation at 3310g for 15 minutes. The resulting supernatant was mixed with 3x volume of acetonitrile containing the analytical internal standard d4-GCA for bioanalysis. GCA was determined by LC-MS/MS methods, using Sciex API 3000 MS/MS system coupled with Shimadzu 10 series HPLC. The analytes were separated on a Gemini 5u C18 110A (5 µm, 3.0 mm x 50 mm) reverse phase column from Phenomenex (Torrance, CA) eluted with water containing 2 mM ammonium acetate and acetonitrile (0.1% formic acid) in a step...
linear gradient. The column chamber’s temperature was ambient. Data for GCA were acquired using MRM in the positive ion mode with an ion transition of 466.1→412.1.

**Results:**

The impact on gene expressions related to BA metabolism and transport was accessed in SCHH for twenty-eight BSEP inhibitors from various therapeutic classes (Table 1). Of these compounds, fifteen were discontinued during development or withdrawn from the market due to liver toxicity concerns, and six were found to have boxed warnings for DILI. Additionally, seven compounds without DILI warning labels were also included. Rifampicin, a pregnane X receptor (PXR) ligand, and omeprazole, an aryl hydrocarbon receptor (AhR) ligand, were also included. Furthermore, four mitogen-activated protein kinase kinase (MEK1/2) inhibitors that participate in intracellular signaling networks were also evaluated. As shown in Table 1, BSEP was not inhibited by MEK inhibitors at the highest concentration tested (40 µM), except for cobimetinib, which had an IC50 of 28.8 µM for BSEP inhibition (Table 1). The potential impact on BA metabolizing enzyme and transport genes was investigated in three donors of cryopreserved human hepatocytes. The expression of eight BA-related metabolizing and transport genes, including CYP7A1, BAAT, SULT2A1, CYP8B1, CYP27A1, BACs, and SLC10A1 and ABCB11 in hepatocytes was monitored (Figure 1).

Several BSEP inhibitors were found to induce the expression of CYP7A1. The concentration-dependent upregulation of CYP7A1 gene was observed in the hepatocytes treated with pazopanib and CP-724714 with fold changes of approximately 14.5 and 11.4, respectively, at the highest test concentrations (Figure 2). There was no association
between the induction of CYP7A1 and changes in SHP gene expression (Data not shown). Omeprazole, an AhR ligand, showed a concentration-dependent induction of CYP7A1 expression for about 2.4-fold at the concentration of 30 µM. Consistent regulation of the CYP7A1 gene was confirmed across three hepatocyte lots, as demonstrated in Supplementary Figure 1. In contrast, CYP7A1 gene expression was downregulated by rifampin, a PXR activator, for about ~2-fold (30 µM). In addition, the expression of CYP7A1 was reduced by mometasone, vinblastine, hexachlorophene, and bosentan by approximately 10-, 18-, 2.0-, and 2.1-fold, respectively, at the highest tested concentrations. CYP7A1 reduction by mometasone and vinblastine was associated with SHP induction (Supplementary Figure 2). The expression of SULT2A1, a bile-acid sulphation enzyme in the liver, was induced by rifampin, omeprazole, mometasone, hexachlorophene, nefazodone, beclomethasone, mibebradil, and danazol. These inductions were marginal (about 2-fold) at the highest tested concentrations but followed the concentration dependence. No other significant changes in the expression of BA-related genes, including CYP27A1 and CYP8B1, were observed by the treatment with these BSEP inhibitors, except for a 2.6-fold increase in ABCB11 (BSEP) by mometasone treatment (30 µM).

As depicted in Figure 3, all four MEK1/2 inhibitors significantly induced CYP7A1 gene expression, with fold changes ranging from 5.4 to 8.1. The inductions were accompanied by significant reductions in SHP expressions (12.5-20-fold) for the MEK1/2 inhibitors. In addition, SULT2A1 gene expression was induced by cobimetinib and trametinib for about 4- and 2.5-fold, respectively. Marginal increases (~2-fold) of
ABCB11, BACS, and SLC10A1 genes were also detected in the hepatocytes incubated with trametinib.

To determine whether the induction of CYP7A1 is related to its protein expression and functional activities, the protein level was measured using a targeted LC-MS/MS proteomics method. In addition, the BA production was monitored in hepatocytes treated with CYP7A1 inducers, using obeticholic acid, a known FXR ligand, as a control. Twelve BA species (supplementary table 3) were quantified in the hepatocyte culture media using an LC-MS/MS, out of which two BAs, GCA and GCDCA, were detectable and showed an increase in concentration levels over time in cell culture media (Figure 4). Treatment of CYP7A1 inducers significantly increased the protein expression, while obeticholic acid decreased the protein expression to a level under the limit of quantification (LOQ) in the hepatocytes. The total concentration of BA (the sum of GCA and GCDCA) was significantly increased at 48 hours after treatment with CYP7A1 inducers, while it was significantly decreased by obeticholic acid.

**Discussion:**

Liver injury is one of the significant safety concerns for protein kinase inhibitors that cause the discontinuation of the development or label warnings for hepatotoxicity (Vigano et al., 2023). In the current investigation, of those 8 monitored BA-associated metabolizing enzymes and transporters, CYP7A1 was the only highly modulated enzyme. Two TKIs, CP-724714 and pazopanib, significantly induced CYP7A1 expression. CP-724714 is a selective inhibitor of epidermal growth factor 2 (EGFR) tyrosine kinase that disrupts the signaling pathways for promoting the growth and survival of cancer cells.
CP-724714 was found to cause dose-limiting liver toxicities in patients, leading to early termination in the development (Munster et al., 2007). CP-724714 is a BSEP inhibitor, and the estimated IC50 of BSEP inhibition was approximately 5 μM in SCHH (Feng et al., 2009). Together with BSEP inhibition, the increased BA production by CYP7A1 induction can result in termination due to the increased risk and severity of liver injury. Another TKI, pazopanib, inhibits the vascular endothelial growth factor receptor (VEGFR) and is approved for treating advanced renal cell carcinoma. Pazopanib has the potential to cause severe or life-threatening liver damage, with fatal outcomes reported (Lee and Chan, 2016). At least 20% of patients stopped taking pazopanib due to adverse side effects, and the most frequent reason for discontinuation was elevated liver enzymes (Motzer et al., 2017). Pazopanib is a BSEP inhibitor (table 1) and significantly induced CYP7A1 expression (Figure 2), which is consistent with the findings reported by Saran et al. (Saran et al., 2022). Additionally, pazopanib is reported to induce cytochrome P450 (CYP) genes, including CYP3A4, 2C8, 2C9, 2C19, and UGTs (UGT1A1/1A4/1A9), but the induction is not through PXR activation (Moscovitz et al., 2018). Pazopanib has an elevated propensity to cause hepatotoxicity, which can result in fatality in rare cases (Keisner and Shah, 2011). In fact, pazopanib is one of the marketed TKIs that has a black box warning for hepatotoxicity (Vigano et al., 2023), and the package insert recommends monitoring hepatic function if the treatment should be interrupted, reduced or discontinued. These findings suggest that the induction of CYP7A1 may pose an additional risk to BSEP inhibition. Furthermore, CYP7A1 induction by these TKIs appeared not to be associated with an SHP suppression and was likely to interact directly through the inhibition of the MEK/ERK pathway. It is worth noting that the current experiments could not detect CYP7A1 induction by sorafenib.
(data not shown), which is inconsistent with the results reported by Saran et al. (Saran et al., 2022). Interestingly, sorafenib does not have a black box warning for hepatotoxicity, which suggests that not every TKI induces CYP7A1 gene expression, and therefore, assessing the potential of CYP7A1 induction can be critical for better safety profiles when developing TKIs.

Ligand binding to specific receptors, such as epidermal growth factors, can activate the downstream RAS/RAF/MAPK signaling pathways (Figure 5). The receptor tyrosine kinase activation triggers the phosphorylation of two MEK enzymes, MEK1 and MEK2. The downstream proteins like ERK1 and ERK2 are further activated and interact with various nuclear transcription factors (Figure 5) (Molina and Adjei, 2006). Recently, Verzijl et al. (Verzijl et al., 2023) reported the downregulation of Shp and upregulation of Cyp7a1 genes in mice treated with PD0325901, a MEK inhibitor. Here, we found that all four MEK1/2 inhibitors significantly increased CYP7A1 expression and decreased SHP expression. MEK inhibitors did not potently inhibit BSEP (Table 1) but showed clinical adverse effects of liver toxicity (Welsh and Corrie, 2015). The severity of liver injury increases when two inhibitors targeting the RAS/RAF/EMK/ERK pathway are combined (Gravbrot and Sundararajan, 2019). These results suggested that CYP7A1 induction could be a culprit for hepatotoxicity, independent of BSEP inhibition, and potentially explain the clinical observations for this class of drugs (Verzijl et al., 2023).

CYP7A1 gene expression was significantly suppressed by mometasone and vinblastine, whereas hexachlorophene and bosentan only marginally reduced its expression. As shown in Figure 5, mometasone can bind to glucocorticoid receptors (GR) and form glucocorticoid response elements (GRE). The latter can suppress nuclear
factor-kB and activator protein-1 (NF-kB/AP-1)-dependent expression of cytokines and chemokines and reduce inflammatory mediator expressions. The suppression of CYP7A1 expression was accompanied by SHP induction by mometasone (in all three lots) and vinblastine (in one of 3 lots) (Supplementary Figures 1 and 2). Of those CYP7A1 gene suppressors, bosentan is the only drug with a boxed warning of BA-related hepatotoxicity; others are clean in DILI labels. A 2-fold decrease in CYP7A1 gene expression was observed with bosentan only at 30 µM. Such a high concentration may not be clinically relevant. Although these drugs also inhibit BSEP, they suppress CYP7A1 expression and reduce BA production in the liver, potentially lowering the risk of BA-related hepatotoxicity. In rhesus monkeys, a single clinical dose of vincristine or vinblastine significantly increases plasma triacylglycerol concentrations (Sethi et al., 1983). Elevated serum cholesterol levels, including vinblastine, have been observed in chemotherapy patients (Bokemeyer et al., 1996; Yonezawa et al., 2023). In addition, treatment with mometasone led to higher plasma triacylglycerol and total cholesterol (Zimath et al., 2023). The suppression of CYP7A1 may relate to the clinical outcomes of lipid metabolisms through the mechanism of reducing cholesterol oxidation in the liver.

While BSEP is critical for hepatic BA excretion, BA concentrations in hepatocytes are maintained in multiple ways. The synthesis of BAs from cholesterol involves a complex interplay of several enzymes organized into two main pathways (Figure 1). CYP7A1, the initial and rate-limiting enzyme, begins BA synthesis. As a critical regulatory point, CYP7A1 undergoes tight regulation by BAs through the enterohepatic circulation, and its regulation involves several pathways (Chiang and Ferrell, 2020). In the liver, CDCA and CA activate FXR to induce CYP7A1 repressor SHP, which interferes
with the transcriptional activator hepatocyte nuclear factor 4 alpha (HNF4α) and liver-
related homolog 1 (LRH-1) to repress CYP7A1 promoter activity via BA-responsive 
element (BARE)(Chiang and Ferrell, 2020) (Figure 5). On the other hand, the binding to 
EGFR increased CYP7A1 expression, likely through the Ras-Raf-MEK/ERK signaling 
pathway and independent of SHP expression (Figure 5), leading to increased production 
of BAs in the liver. Activation of FXR by CDCA in the intestine can induce the release of 
fibroblast growth factor 19 (FGF19, also known as FGF15 in rodents), which travels to 
the liver and activates FGFR4/β-Klotho pathways to inhibit the transcription of CYP7A1 
(Chiang and Ferrell, 2020). The transcription of CYP7A1 genes can also be suppressed 
via the ERK/c-Jun of the mitogen-activated protein kinase (MAPK) pathway (Li et al., 
2006)(Figure 5). Additionally, TDCA/CDCA activates BA receptor TGR5 in 
macrophages and Kupffer cells, triggering inflammatory cytokine release and inhibiting 
CYP7A1 transcription via JNK signaling (Figure 5). Activating the cell signaling 
mechanism triggered by TDCA/CDCA in Kupffer cells could be an adaptive response to 
cholestatic liver injury, leading to a swift decrease in BA synthesis. In addition, nuclear 
hormone receptors (NHRs) can also interact with SHP to regulate the expression of 
CYP7A1 (Klinge et al., 2001). Interestingly, while the AhR ligand, omeprazole, 
significantly induced CYP7A1 expression for about 3-fold, the PXR activator, rifampin, 
repressed CYP7A1 expression for about 2-fold. The results suggest that the regulation 
mechanisms of CYP7A1 expression by NHRs may differ, and further investigations are 
 warranted on the interactions between CYP7A1 gene expression and the activation of 
NHRs. It is worth noting that, the quantitative correlation from in vitro to in vivo 
remains lacking in the current investigation, and the intestine-liver feedback 
mechanisms of CYP7A1 regulation were not examined in the SCHH incubation. For
example, the increased bile acid production resulting from CYP7A1 induction in the liver can trigger the FGF19 release in the intestine, which, in turn, suppresses the hepatic CYP7A1 expression. Therefore, when focusing on the regulation of the BA homeostasis, it is not possible to fully understand all the mechanisms involved by using in vitro SCHH assays alone. Further investigation is needed to determine the overall impact and identify the point of BA homeostasis disruption that leads to the development of DILI, using a system where the intestine-liver feedback mechanisms are present, which can only be studied in vivo.

The study concludes that TKI drugs, pazopanib and CP-724714, were found to induce CYP7A1 gene expression that can pose synergistic effects on DILI development and severity in addition to BSEP inhibition. While MEK1/2 inhibitors did not strongly inhibit BSEP, they were still associated with liver toxicity. The upregulation of CYP7A1 gene by MEK1/2 inhibitors through suppressing SHP can increase BA production in hepatocytes, which can be a risk factor independent from BSEP inhibition. Our findings suggest that CYP7A1 induction, which can occur through SHP-dependent or independent mechanisms, could increase the risk of DILI, either in concert with BSEP inhibition or independently.
## Data Table

### Table 1. Compounds that are included in the current analysis

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>DILI Warnings</th>
<th>DILI classification based on LTKB</th>
<th>BSEP IC50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pazopanib</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>10.3</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>12.0</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>6.5</td>
</tr>
<tr>
<td>Bosentan</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>38.1</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>8.2</td>
</tr>
<tr>
<td>Danazol</td>
<td>Boxed Warning</td>
<td>Most DILI concern</td>
<td>25.0</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Discontinued</td>
<td>NL</td>
<td>29.0</td>
</tr>
<tr>
<td>Sulfinpyrazone</td>
<td>Discontinued</td>
<td>NL</td>
<td>17.7</td>
</tr>
<tr>
<td>CP-724714</td>
<td>Discontinued</td>
<td>NL</td>
<td>6.0</td>
</tr>
<tr>
<td>Diethylenecarbamazine</td>
<td>Discontinued</td>
<td>NL</td>
<td>23.2</td>
</tr>
<tr>
<td>Bepridil</td>
<td>Discontinued</td>
<td>NL</td>
<td>35.5</td>
</tr>
<tr>
<td>Bithionol</td>
<td>Discontinued</td>
<td>NL</td>
<td>20.8</td>
</tr>
<tr>
<td>Sitaxsentan</td>
<td>Withdrawn</td>
<td>Most DILI concern</td>
<td>9</td>
</tr>
<tr>
<td>Benzbromarone</td>
<td>Withdrawn</td>
<td>Most DILI concern</td>
<td>7.8</td>
</tr>
<tr>
<td>Fipexide</td>
<td>Withdrawn</td>
<td>Most DILI concern</td>
<td>26.0</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>No mention</td>
<td>No-DILI-Concern</td>
<td>23.6</td>
</tr>
<tr>
<td>Midazolam</td>
<td>No mention</td>
<td>No-DILI-Concern</td>
<td>41.7</td>
</tr>
<tr>
<td>Econazole</td>
<td>No mention</td>
<td>NL</td>
<td>7.0</td>
</tr>
<tr>
<td>Hexachlorophene</td>
<td>No mention</td>
<td>NL</td>
<td>7.3</td>
</tr>
<tr>
<td>Beclomethasone</td>
<td>No mention</td>
<td>NL</td>
<td>2.8</td>
</tr>
<tr>
<td>Mometasone</td>
<td>No mention</td>
<td>Less-DILI concern</td>
<td>6.4</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Warnings and Precautions</td>
<td>Less-DILI concern</td>
<td>0.2</td>
</tr>
<tr>
<td>NHRs</td>
<td>Rifampicin</td>
<td>Warnings and Precautions</td>
<td>Most DILI concern</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>--------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>No mention</td>
<td>Less-DILI concern</td>
<td></td>
</tr>
<tr>
<td>MEK1/2 inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trametinib</td>
<td>Increased LFTs</td>
<td></td>
<td>NL</td>
</tr>
<tr>
<td>Cobimetinib</td>
<td>Hepatotoxicity</td>
<td></td>
<td>NL</td>
</tr>
<tr>
<td>GDC-0623</td>
<td>Investigational</td>
<td></td>
<td>NL</td>
</tr>
<tr>
<td>Mirdametinib (PD-0325901)</td>
<td>Investigational</td>
<td></td>
<td>NL</td>
</tr>
</tbody>
</table>

NL: Not listed in the databases. NHRs: nuclear hormone receptors; LTKB: Liver Toxicity Knowledge Base; LFTs: Liver function tests
Figure legends:

Figure 1. Bile acid biosynthetic pathways and homeostasis. There are two major biosynthetic pathways for bile acids: the classic and alternative pathways. In the classic pathway, cholesterol is oxidized to 7α-HC by CYP7A1, which is the rate-limiting enzyme of the classic pathway. Then, HSD3B7 converts 7α-HC to C4, which is further converted to 3α, 7α, 12α-TriHCA by CYP8B1, resulting in the synthesis of CA. Additionally, C4 can be converted to CDCA in the absence of CYP8B1. In the alternative pathway, CYP27A1 first converts cholesterol to 3-HC, which is then converted to 3α, 7α-diHCA by CYP7B1. 7α,26-diHCA can also be converted to 3α, 7α, 12α-TriHCA, which can then form CDCA. Both CA and CDCA can be conjugated with taurine or glycine by BACS or BAAT. BAs, including LCA, DCA, and CDCA, undergo sulfation catalyzed by SULT2A1 or glucuronidation by UGT1A, yielding sulfate or glucuronide conjugates.

Abbreviations: BA-Glu, bile acid glucuronide conjugates; BA-S, bile acid sulfate conjugates; CA, cholic acid; CDCA, chenodeoxycholic acid; C4, 7α-hydroxy-4-cholesten-3-one; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; CYP27A1, sterol 26-hydroxylase; HSD3B7, 3β-hydroxy-Δ5-C27-steroid dehydrogenase/isomerase; SULT2B1, sulfotransferase 2B1; UGT1A1, UDP-glucuronosyl transferase 1A1; 7α-HC, 7α-hydroxycholesterol; 3α, 7α, 12α-TriHCA, 3α, 7α, 12α-trihydroxycholestanoic acid; 3α, 7α-diHCA, 3α, 7α-dihydroxycholestanoic acid; 3-HC, 3β-hydroxy-5-cholestenoic acid.
Figure 2. Characterizing the gene expression of bile acid metabolizing enzymes and transporter in SCHH treated with BSEP inhibitors. The gene regulation studies were conducted at different concentrations of the inhibitors ranging from 0 to 30 μM, except for pazopanib, which was studied at concentrations between 0-10 μM due to its observed cytotoxicity. The gene expressions were then quantitated using real-time quantitative reverse transcription polymerase chain reaction. The mRNA expression levels were measured as a fold-change compared to non-treated controls using the delta-delta Ct method and expressed as mean ± standard deviation (SD) from three independent donors (each performed in triplicate) for each condition. Dot lines indicate the change of 2-fold of upregulation and downregulation, respectively.

Figure 3. Characterizing the gene expression of bile acid metabolizing enzymes and transporter in SCHH treated with MEK1/2 inhibitors. The gene regulation studies were conducted at different concentrations of the inhibitors ranging from 0 to 10 μM. The gene expressions were quantitated using real-time quantitative reverse transcription polymerase chain reaction. The mRNA expression levels were measured as a fold-change compared to non-treated controls using the delta-delta Ct method and expressed as mean ± standard deviation (SD) from three independent donors (each performed in triplicate) for each condition. Dot lines indicate the change of 2-fold of upregulation and downregulation, respectively.

Figure 4. The impact of CYP7A1 inducers and the FXR ligand, obeticholic acid, on protein expression of CYP7A1 and BA production in SCHH cells. The concentrations of
GCA (A) and GCDA (B) in the culture media of SCHH cells were measured at different time points (up to 48 hrs) after being treated with 10 µM CYP7A1 inducers or obeticholic acid. (C) The protein expression of CYP7A1 was determined using targeted proteomics LC-MS/MS in SCHH cells treated with 10 µM inducers or obeticholic acid for 48 hrs. (D) The total BAs in the SCHH media were measured after 48 hours of treatment with 10 µM inducers or obeticholic acid. The results were expressed as mean ± SD and statistical significance was set at P<0.05 compared to the untreated controls.

Figure 5. Schematic overview of regulatory mechanisms of CYP7A1 expression in the liver. CYP7A1 is the rating limiting enzyme for bile salt production in the liver and is a critical regulatory point that undergoes tight regulation by BAs through the enterohepatic circulation. Its regulation involves several pathways. The activation of tyrosine kinase proteins such as EGFR or VEGFR can activate the downstream Ras-Raf-MEK-ERK1/2 signaling pathways, which trigger the phosphorylation of two MEK enzymes, MEK1 and MEK2. These activated kinases then stimulate downstream proteins ERK1 and ERK2 to suppress CYP7A1 expression via various transcriptional factors and nuclear receptors such as LRH-1 and HNF4α that bind to BAREs. TGFβ1 inhibits the transcription of CYP7A1 gene through binding to BARE. Corticosteroids can bind to glucocorticoid receptors (GR) and subsequently, glucocorticoid response elements (GRE) suppress nuclear factor-κB and activator protein-1 (NF-κB/AP-1)-dependent expression of cytokines and chemokines to inhibit NF-κB-mediated inflammatory cytokine production. Proinflammatory cytokines, such as TNFα- and IL-1β, bind to cytokine receptors and activate the PKC-MAPK pathways, such as JNK, ERK, and NF-κB signaling pathways, to regulate CYP7A1 expressions. Activation of intestinal
FXR induces FGF19, which circulates to the liver and binds to hepatic FGFR4 and β-Klotho receptor, inhibiting CYP7A1 gene transcription through activating MEKs/ERK1/2 signaling pathways. Bile acids, such as CA and CDCA, are endogenous ligands of FXR. Activation of hepatic FXR induces the CYP7A1 repressor SHP expression. SHP expression is also regulated by nuclear receptors, including HNF4α, LXRα, LRH-1, RXR, and PPARγ, and transcription receptors c-Jun. Green plus, positive regulation; Red minus, negative regulation; Solid arrow, direct interaction; Dot arrow, indirect regulation.

Abbreviations: BARE, bile acid response element; EGFR, Epidermal growth factor receptor; ERK, extracellular signal-related kinase; FGF19, fibroblast growth factor19; FXR, farnesoid X receptor; FGFR4, fibroblast growth factor receptor 4; GR, glucocorticoid receptors; GRE, glucocorticoid response elements. HNF4α, hepatocyte nuclear factor 4α; JNKs, c-Jun amino-terminal kinases; IL-1β, interleukin-1β; LRH-1, liver receptor homolog-1; LXRα, liver X receptor α; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor -κB; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; SHP, heterodimer; TNF-α, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; VEGFR, vascular endothelial growth factor receptor.

Acknowledgments

The authors acknowledge Dr. Raju Subramanian at Gilead Sciences Inc. for critically reviewing the manuscript.
Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and supplementary material.

Authorship Contributions

Participated in research design: CN, XX, YL

Conducted experiments: CN, XX, RL,

Performed data analysis: CN, XX, RL, XL, YH, YL

Wrote or contributed to the writing of the manuscript: CN, XX, RL, XL, YH, YL
References:


Footnotes:

XX and CN are equal contributors.

This work received no external funding.

No author has an actual or perceived conflict of interest with the contents of this article.

Address correspondence to: Dr. Yurong Lai, Drug Metabolism, Gilead Sciences Inc., Foster City, CA 94404
Tel: 650-522-1629
Email: yurong.lai@gilead.com
Figure 2
Figure 3

- CYP7A1
- SLC10A1 (NTCP)
- BAAT
- SULT2A1
- CYP8B1
- CYP27A1
- BACS
- ABCB11 (BSEP)
- SHP
Figure 4

(A) GCA

(B) GCDCA

(C) Protein amount (fmol/μg protein)

(D) Total bile acid concentration (μM)