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Metformin Disrupts Bile Acid Efflux by Repressing Bile Salt Export Pump Expression

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Abstract

Purpose—The bile salt export pump (BSEP), a key player in hepatic bile acid clearance, has been the center of research on drug-induced cholestasis. However, such studies focus primarily on the direct inhibition of BSEP, often overlooking the potential impact of transcriptional repression. This work aims to explore the disruption of bile acid efflux caused by drug-induced BSEP repression.

Methods—BSEP activity was analyzed in human primary hepatocytes (HPH) using a traditional biliary-clearance experiment and a modified efflux assay, which includes a 72-h pretreatment prior to efflux measurement. Relative mRNA and protein expressions were examined by RT-PCR and Western blotting, respectively.

Results—Metformin concentration-dependently repressed BSEP expression in HPH. Although metformin did not directly inhibit BSEP activity, longer metformin exposure reduced BSEP transport function in HPH by down-regulating *BSEP* expression. BSEP repression by metformin was found to be AMP-activated protein kinase-independent. Additional screening of 10 reported cholestatic non-BSEP inhibitors revealed that the anti-cancer drug tamoxifen also markedly repressed *BSEP* expression and reduced BSEP activity in HPH.

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Conclusions—Repression of BSEP alone is sufficient to disrupt hepatic bile acid efflux. Metformin and tamoxifen appear to be prototypes of a class of *BSEP* repressors that may cause drug-induced cholestasis through gene repression instead of direct BSEP inhibition.

Keywords

BSEP; Cholestasis; Metformin; Tamoxifen; Gene regulation

INTRODUCTION

Drug-induced liver injury (DILI) is one of the most common causes of drug attrition from both drug development and post-marketing - a major concern for the pharmaceutical industry, regulatory agencies, and clinical practice (1,2). DILI can manifest in a variety of ways, mimicking a wide range of disorders associated with both acute and chronic liver diseases (3). One of the most prevalent manifestations of DILI is drug-induced cholestasis (1), characterized by interrupted bile flow from the liver to the intestines and accumulation of toxic levels of bile acids in the liver, particularly within hepatocytes (4). Physiologically, bile acids play an important role in lipid and fat-soluble vitamin emulsification and absorption from the small intestine. To facilitate their function, bile acids have distinct polar and non-polar regions that allow them to form micelles (5,6). These detergent-like micelles are necessary for lipid-trapping; however, this property also contributes to toxicity.

Typically, drug-induced cholestasis occurs soon after initiating a new drug, and is confirmed when discontinuation of the drug alleviates toxicity (7,8). While, often not life-threatening when diagnosed early, prolonged cholestasis can result in severe liver injury, liver transplantation, and death (2,4). With an increasing number of drugs reported to cause cholestasis after FDA approval, drug-induced cholestasis has become a major focus of investigation (9,10).

The bile salt export pump (BSEP, ABCB11) is the rate-limiting step of bile flow from the liver to the intestine (11,12). As the key determinant of hepatic bile acid elimination, BSEP is a major focus of drug-induced cholestasis research. Most current studies focus on disruption of BSEP function through direct inhibition of bile acid transport (13,14). Indeed, the recently issued FDA guidance for industry on transporter-based drug interactions focuses on transporter inhibition (15–17). Nevertheless, BSEP inhibition alone cannot fully explain BSEP-associated cholestasis. Genetic mutations in *BSEP* gene are linked to substantial attenuation or even absence of BSEP expression (e.g. progressive familial intrahepatic cholestasis type 2) (12). A group of compounds with cholestatic liability did not inhibit BSEP activity in prototypical BSEP functional assays (18). Notably, remarkable reduction of BSEP expression was observed in 8 of 12 patients with cholestatic DILI (19). Our previous study showed that several agents classified as BSEP inhibitors were also capable of repressing BSEP expression (20). Of importance, dual inhibitors and repressors of BSEP appear to be associated with severe DILI. Based on these observations, we pursued the current study wherein drug-mediated repression of BSEP expression is shown to play an important role in drug-induced cholestasis.

Metformin is one of the most commonly prescribed drugs used to treat type 2 diabetes mellitus. While generally considered a safe medication, there have been several reports of cholestasis with metformin use where discontinuation of the drug alleviated cholestatic toxicity (8,21,22). Notably, metformin does not directly inhibit BSEP (18). Accordingly, we hypothesized that instead of directly inhibiting BSEP activity, metformin represses BSEP expression, thus inducing cholestatic liver injury. Given that activation of the cellular energy sensor, AMP-activated protein kinase (AMPK), is a known mechanism through which metformin exerts many of its pharmacological actions (23), we also explored if AMPK activation is involved in the inhibitory effect of metformin on BSEP expression in HPH.

In this report, we provide experimental evidence to show that metformin exhibits potent repression of BSEP expression and disrupts BSEP-mediated bile acid efflux in HPH. Its repression of BSEP expression was independent of AMPK activation. By screening 10 cholestatic drugs that have been reported not to directly inhibit BSEP, tamoxifen was also identified as a BSEP repressor and found to disrupt BSEP efflux function in HPH. Collectively, our results indicate that a class of metformin-like compounds has the potential to repress expression of BSEP, an underappreciated mechanism that may contribute to drug-induced cholestasis.

MATERIALS AND METHODS

Reagents

Metformin, GW-4064, Compound C, D-penicillamine, nitrofurantoin, and nortriptyline were purchased from Sigma Aldrich (St. Louis, MO). Ibuprofen, sulindac, tamoxifen, trimethoprim, and verapamil were purchased from AK Scientific (Union City, CA). Carbamazepine, cimetidine, and [³H]-taurocholic acid were purchased from Perkin Elmer (Waltham, MA). PCR Primers were synthesized by Integrated DNA Technologies (Coralville, IA). High Capacity cDNA Archive Kit was obtained from Applied Biosystems (Foster City, CA). Matrigel was received from BD Biosciences (Bedford, MA). All other cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

Culture and Treatment of HPH

Human primary hepatocytes were obtained from BioIVT (Baltimore, MD). Hepatocytes (viability >90%) were seeded at 1.5×10^6 cells/well and 7.5×10^5 cells/well in 6-well and 12-well collagen-coated plates, respectively, and cultured in the sandwich format as described previously (24). Hepatocytes were treated 48 h after seeding with 0.1% DMSO or specified compounds for 24 h for mRNA analysis or 72 h for Western blotting and functional assays. Culture medium was replaced on a daily basis.

RT-PCR

Total RNA was isolated from HPH using the TRIzol® reagent and reverse transcribed using a High Capacity cDNA Archive Kit following the manufacturers' instructions. Primer sequences used for PCR amplification are as follows: GAPDH, 5'-CCCATCACCATCTTCCA GGAG-3' (forward), 5'-GTTGTCATGGATGACCTTGGC-3'

(reverse); FGF21, 5'-CTGTGGGTTTCTGTGCTGG-3' (forward), 5'-CCGGCTTCAAGGCTTTCAG-3' (reverse); BSEP 5'-ACATGCTTGCGAGGACCTTTA-3' (forward), 5'-GGAGGTTTCGTGCACCAGGTA-3' (reverse). Target gene mRNA expression was normalized against that of GAPDH. Real-time PCR assays were performed in 96-well optical plates on an StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix as described previously (25). Data are represented as mean $\pm$ SD of three individual experiments.

Western Blotting

Antibodies against BSEP (F-6) and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphor-AMPK α (Thr172) (40H9) and AMPK α were purchased from Cell Signaling Technology (Danvers, MA). Crude protein lysates from treated HPH were harvested using RIPA buffer, and protein concentrations were determined using a bicinchoninic acid (BCA) based assay from Thermo Fisher Scientific. Equal amounts of protein lysates were separated on SDS-polyacrylamide gels (4–12% bis-tris) before transferring on to polyvinylidene difluoride membranes. The membranes were subsequently probed with specific antibodies against BSEP (1:250 dilution), phosphor-AMPK (1:1000 dilution), AMPK (1:1000 dilution), or β -actin (1:30,000 dilution) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. β -actin was used as loading control. Membranes were developed with chemiluminescent reagents and the relative protein expression was estimated by densitometry analysis using NIH ImageJ (Bethesda, MD).

Biliary Efflux Assay

Human primary hepatocytes were seeded on 12-well plates as described above and cultured for 96 h after Matrigel overlay for BSEP functional assays. In a modification to the transport assay described previously (26), transport of the tritiated BSEP substrate taurocholic acid ([³H]-TCA) was monitored to assess BSEP function in the presence of selected drugs. Briefly, cells were incubated with standard or calcium (Ca²⁺)-free Hanks' balanced salt solution (ST HBSS or CF HBSS) for 10 min, before HBSS (standard and Ca²⁺-free) containing [³H]-TCA was applied to cells for a 10-min incubation. During the inhibition assay, biliary efflux was monitored in the presence of metformin or tamoxifen at different concentrations for 10 min. Conversely, the effect of BSEP repression on its function was monitored by pretreating HPH for 72 h with metformin or tamoxifen and followed by monitoring [³H]-TCA biliary efflux for 10 min in HBSS (standard or Ca²⁺-free). The assay was terminated by aspirating the [³H]-TCA-containing HBSS and adding ice-cold standard HBSS. Cell lysates harvested in 0.5% Triton X-100 were analyzed by scintillation counting and normalized to protein concentration. The amount of bile acids excreted from hepatocytes, as a measurement of [³H]-TCA, was calculated into a percentage and referred to as the biliary excretion index (BEI) as described previously (26).

$$BEI = \left(\frac{[{}^3H] - TCA \text{ in ST HBSS} - [{}^3H] - TCA \text{ in CF HBSS}}{[{}^3H] - TCA \text{ in ST HBSS}} \right) \times 100\%$$

Statistical Analysis

Results from real-time PCR and functional efflux assays are expressed as mean $\pm$ S.D. of triplicate determinations unless otherwise indicated. Statistical analyses used one-way analysis of variance (ANOVA) and Dunnett's posttest.

RESULTS

Metformin Represses BSEP Expression in HPH

The effects of metformin on BSEP expression were evaluated in sandwich-cultured HPH prepared from multiple liver donors. As demonstrated in Fig. 1A and B, expression of *BSEP* mRNA was markedly repressed by metformin treatment in a concentration-dependent manner. Maximal repression (approximately 75%) of *BSEP* mRNA expression was achieved with 1 mM metformin, a concentration that was frequently used in cell culture studies and is not cytotoxic in HPH (27,28). As expected, the positive control, GW-4064, robustly induced *BSEP* expression. Western blotting analysis confirmed metformin repressed BSEP protein expression in a pattern consistent with the changes in *BSEP* mRNA (Fig. 1C and D). These results reveal metformin as a potent repressor of *BSEP* expression in HPH.

Metformin Alters BSEP Function by Repressing its Expression

To investigate whether metformin alters BSEP function, two transport assays were performed in HPH. The effects of metformin on hepatic bile acid efflux were tested in a prototypical inhibition assay with 10 min drug exposure (Fig. 2A) or in a modified inhibition assay condition where a 72-h drug pretreatment precedes the bile acid efflux measurement (Fig. 2B). Our results demonstrated that under typical inhibitory conditions, metformin did not alter bile acid efflux compared to the vehicle control (0.1% DMSO) (Fig. 2C and E). As a positive control, a known cholestatic BSEP-inhibitor, troglitazone, potently inhibited bile acid transport through BSEP. Importantly, when HPH were pretreated for 72 h with metformin (0.1, 0.5, and 1 mM), [3 H]-TCA efflux was markedly decreased (Fig. 2D and F). Under typical inhibition assay conditions (10-min incubation), metformin (1 mM) did not alter the clearance of [3 H]-TCA (82.45% for metformin vs. 81.72% for vehicle control) whereas in the 72-h repression assay bile acid clearance in the presence of metformin was reduced to 19.22% compared to the control value of 81.52% (Table 1). These results indicate that metformin does not inhibit BSEP through direct substrate competition; instead, longer exposure to metformin repressed BSEP expression, thereby decreasing bile acid clearance capacity at 72 h.

Repression of BSEP Expression by Metformin Is Independent of AMPK Activation in HPH

AMPK activation is a known mediator of metformin actions. As shown in Fig. 3A and B, metformin markedly increased AMPK phosphorylation in HPH, without altering the expression of total AMPK. Hence, the role of AMPK activation in BSEP repression by metformin was further investigated by co-treatment with compound C, a widely used AMPK inhibitor. To confirm the inhibitory effect of compound C on AMPK in HPH, the mRNA expression of FGF21, whose expression is correlated with AMPK activation (29), was measured after treatment with metformin alone or metformin and compound C at different

concentrations (5, 10, 20 μ M). As expected, metformin at 1 mM significantly increased FGF21 mRNA expression, which was attenuated by compound C in a concentration-dependent manner, indicating the successful inhibition of AMPK activation by compound C in HPH (Fig. 3C and D). Notably, co-treatment with compound C did not reverse the repression of BSEP by metformin (Fig. 3E and F). These results suggest that repression of BSEP expression by metformin is independent of AMPK activation in HPH.

Drug-Induced Repression of BSEP Expression Is Not Unique to Metformin

In addition to metformin, which we used as a prototype compound to study BSEP repression, other drugs are reported to cause cholestasis without inhibiting BSEP (18). We evaluated 10 such drugs for their effects on BSEP expression in HPH. The concentrations of these drugs were selected based on literature search and all the compounds at the concentrations selected showed no HPH cytotoxicity during the treatments. As shown in Fig. 4A, tamoxifen, an estrogen receptor (ER) antagonist widely used to treat ER-positive breast cancer, potentially repressed *BSEP* gene expression in HPH at 20 μ M, a concentration at which this compound exhibited no cytotoxicity (data not shown). Concentration-dependent repression of BSEP by tamoxifen was confirmed in hepatocytes from additional liver donors at both the mRNA (Fig. 4B and C) and protein (Fig. 4D and E) levels. Like metformin, tamoxifen showed no effects on the [3 H]-TCA efflux in a prototypical inhibition assay in HPH (Fig. 5A and C), in line with its classification as a non-inhibitor of BSEP. However, in hepatocytes pre-treated with 10 μ M and 20 μ M tamoxifen for 72 h, [3 H]-TCA efflux was significantly reduced to 52.38% and 16.61%, respectively, compared with 71.75% in control cells (Fig. 5B, D and Table 2). Such alterations in biliary excretion index are most likely caused by the repression of BSEP expression. These results confirm disruption of BSEP efflux function in HPH by some cholestatic BSEP non-inhibitors occurs via gene repression rather than competitive inhibition.

DISCUSSION

Drug-induced cholestasis is a leading cause of drug attrition, prompting a growing research effort to understand the underlying mechanisms (30,31). Until recent years, the primary focus has been the ability of drugs to inhibit bile acid efflux from the liver by interaction with the bile salt export pump BSEP (13,14,32). Although this strategy has elucidated the mechanism of action for many cholestatic drugs, some chemicals with cholestatic liability do not directly inhibit BSEP activity (18), suggesting alternate mechanisms may contribute to drug-induced cholestasis. Our previous screen of 30 known BSEP inhibitors identified five potent (> 60% repression) and 10 moderate (20–60% repression) repressors of BSEP expression (20). Importantly, drugs exhibiting both potent repression of BSEP expression and inhibition of its activity were associated with the greatest DILI concern, i.e. black box warnings and withdrawal from the market (33). Using metformin as a model compound, the present study unequivocally shows that drug-induced BSEP repression alone is sufficient to affect bile acid clearance from HPH and, thus, may represent an overlooked mechanism leading to drug-induced cholestatic liver injury.

As the first-line medication for the treatment of type 2 diabetes, metformin exhibits several metabolic benefits and is generally well-tolerated with few side effects. Nonetheless, several case reports revealed the association of metformin with intrahepatic cholestasis and liver injury in type 2 diabetics (8,21,22). In mice, metformin treatment increased bile acid accumulation in both the liver and blood (34). The underlying mechanisms of these observations however remained elusive. Using membrane vesicles from Sf9 cells overexpressing human BSEP, Pedersen et al., screened 250 compounds for BSEP inhibition and identified metformin as a non-inhibitor of BSEP (35). In the current study, we found that metformin markedly repressed BSEP expression in sandwich-cultured HPH. These results suggest metformin may represent a pure repressor of human BSEP that could influence bile acid clearance without direct substrate competition.

Transport activity of membrane proteins has often been evaluated using insect membrane vesicles, immortalized cell lines ectopically expressing particular transporters, or sandwich-cultured hepatocytes that maintain functional endogenous transporters (14,26,36,37). Typical inhibition assays involve a short-term (10 to 30 min) incubation of test compounds with substrate probes in membrane vesicles or cultured cells (14,38). Utilizing this traditional approach, we showed that metformin does not directly inhibit BSEP-mediated efflux of taurocholic acid in HPH, which is consistent with earlier observations in inverted insect membrane vesicles (35). Nevertheless, this methodology fails to evaluate a drug's effects on BSEP after an extended period of exposure. In our modified experiments as depicted in Fig. 2B, the 72-h pretreatment prior to bile acid efflux measurements in HPH allowed a transport assay to coordinate effects of gene expression on transporter function, and confirmed that repression of BSEP expression alone can alter BSEP-mediated bile acid transport.

Despite the fact that AMPK activation is a known mechanism by which metformin exerts many of its pharmacological effects, in our study the repression of BSEP expression in HPH by metformin was independent of AMPK activation. To date, accumulating evidence has demonstrated the AMPK-independent effects of metformin, such as its inhibitory effects on hepatic gluconeogenesis and adipogenesis (39,40). Nevertheless, how metformin down-regulates BSEP expression in HPH needs further investigation. In contrast, previous studies in mice demonstrated that activation of AMPK by metformin enhanced BSEP expression (41), however this was paradoxically accompanied by increased cholestatic toxicity (34,42). Although the mechanisms underlying these contradictory observations are largely unknown, as increasingly recognized, species differences between mice and humans contribute to the relatively low pre-dictability of preclinical models for toxicological evaluation during drug development (43–45).

Recently, a list of drugs labeled “cholestatic BSEP non-inhibitors” were reported to induce cholestatic toxicity without direct inhibition of BSEP activity (18). We speculated a plausible explanation for the cholestatic effects of these drugs might be repression of *BSEP* gene expression. Of 10 drugs investigated in our study, tamoxifen potently repressed BSEP expression and disrupted BSEP-mediated bile acid efflux in HPH, while the rest had moderate or no effects. Notably, tamoxifen is classified as a selective estrogen receptor modulator that can act as either an agonist or antagonist in a tissue-specific manner (46). Interestingly, previous reports have demonstrated that estrogen receptor alpha (ER α)

exhibits transrepressive effects on BSEP expression, and estradiol-activated ER α contributes to intrahepatic cholestasis of pregnancy (47,48). As an ER α agonist in the liver (49), it is possible that, like estradiol, tamoxifen induces ER α -mediated transrepressive effects on BSEP, which merits further investigation (47). It is worth noting that while repression of BSEP expression alone is sufficient to reduce bile acid elimination in the liver and potentially leads to drug-induced cholestasis, it cannot be generalized as the mechanism for all cholestatic BSEP non-inhibitors. Given that the overall bile acid homeostasis in the liver is coordinated by its tightly controlled bio-synthesis and transport, the cholestatic effects of these BSEP non-inhibitors could be potentially contributed by compensatory mechanisms involving bile acid elimination in the liver, such as canalicular efflux transporters MRP2 and P-gp as well as sinusoidal efflux transporters MRP3, MRP4 and OST α -OST β (50).

CONCLUSION

In conclusion, BSEP inhibition cannot fully explain drug-induced cholestasis; hence, we directed our work at identifying alternate mechanisms. In the current study, we found that metformin and tamoxifen, two cholestatic BSEP non-inhibitors, alter BSEP function through repression of *BSEP* expression without directly inhibiting its activity, leading to decreased bile acid efflux from the liver. Our results suggest transcriptional BSEP repression represents an overlooked mechanism underlying drug-induced cholestasis.

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ABBREVIATIONS

AMPK	AMP-activated protein kinase
BEI	Biliary excretion index.
BSEP	Bile salt export pump
DILI	Drug-induced liver injury
DIC	Drug-induced cholestasis
HPH	Human primary hepatocytes
MRP	Multidrug resistance-associated protein
OST	Organic solute transporter
P-gp	P-glycoprotein

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Fig. 1. Metformin represses BSEP expression in human primary hepatocytes.

HPH prepared from multiple liver donors were treated with vehicle control (0.1% DMSO), metformin (0.1, 0.5, and 1 mM), or GW-4064 (2 μ M) as a positive control as detailed in *Materials and Methods*. mRNA expression of *BSEP* was measured using RT-PCR in HL#66 (A) and #111 (B). Data are expressed as mean $\pm$ S.D. ($n = 3$). The expression of BSEP protein (C & D) was measured using Western blotting in HL #111 & #120. Densitometry was determined using the NIH ImageJ software

Fig. 2. Metformin represses BSEP function in human primary hepatocytes.

The prototypical inhibition assay with short substrate incubation of 10 min (A) and a modified repression assay including a 72-h drug pretreatment (B) were schematically depicted. BSEP inhibition assay measured via biliary excretion of $[^3\text{H}]\text{-TCA}$ in two HPH donors, HL #90 (C) & #91 (E), upon 10-min exposure to vehicle control (0.1% DMSO), positive control (troglitazone 100 μM), or metformin (0.1, 0.5, and 1 mM). BSEP repression was measured via biliary excretion of $[^3\text{H}]\text{-TCA}$ in two human primary hepatocytes donors, HL #90 (D) & 91(F), upon 72-h pre-treatment with vehicle control (0.1% DMSO) or metformin (0.1, 0.5, and 1 mM). Solid bars represent dpm/mg protein of $[^3\text{H}]\text{-TCA}$ that accumulated in the cells + bile canaliculi, while open bars represent dpm/mg protein $[^3\text{H}]\text{-TCA}$ in the cells only

Fig. 3. Repression of BSEP by metformin in HPH is AMPK-independent.

HPH were treated with vehicle control (0.1% DMSO), metformin (0.1, 0.5, and 1 mM) or metformin (1 mM) and compound C (5, 10, and 20 μ M) as outlined in *Materials and Methods*. AMPK phosphorylation (A & B) was measured using Western blotting in HL#111 & #120. RT-PCR was used to measure the mRNA expression of *FGF21* and *BSEP* in HPH from donors HL #155 (C & E) and #157 (D & F). Data were expressed as mean $\pm$ S.D. (n = 3). Densitometry was determined using the NIH ImageJ software

Fig. 4. Cholestatic non-BSEP-inhibitor tamoxifen represses BSEP expression.

(A) mRNA expression of BSEP was measured using RT-PCR in HPH from donors HL #109, #130, & #131. Cells were treated with vehicle control DMSO (0.1%), tamoxifen (20 μ M, TAM), ibuprofen (50 μ M, IBUP), trimethoprim (50 μ M, TRIM), D-penicillamine (250 μ M, D-PEN), sulindac (10 μ M, SULI), carbamazepine (200 μ M, CARB), verapamil (100 μ M, VERA), nitrofurantoin (10 μ M, NITR), nortriptyline (10 μ M, NORT), and cimetidine (1 mM, CIME). Data are expressed as mean $\pm$ S.D. (n = 3). The top and bottom dotted lines indicate 20% and 60% reduction in the BSEP mRNA expression, respectively.

Concentration-dependent mRNA and protein expression of *BSEP* was measured in HPH treated with vehicle control (0.1% DMSO), positive control (GW-4064 2 μ M), or tamoxifen (5, 20 μ M). mRNA expression was measured using RT-PCR in HPH from donors HL#113 (B) & #127 (C). Data are expressed as mean $\pm$ S.D. (n = 3). *** P < 0.001; ** P < 0.01. Protein

expression of BSEP was measured using Western blotting in HPH from donors HL #120 (D) & #123 (E). Densitometry was determined using the NIH ImageJ software

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Fig. 5. Tamoxifen disrupts BSEP function in human primary hepatocytes.

The prototypical inhibition assay and a modified repression assay including a 72-h drug pretreatment were described in *Materials and Methods*. BSEP inhibition assay was measured via biliary excretion of [3 H]-TCA in two HPH donors, HL #158 (A) & #159 (C), upon 10-min exposure to vehicle control (0.1% DMSO), positive control (troglitazone 100 μ M), or tamoxifen (5, 10, and 20 μ M). BSEP repression assay was measured via biliary excretion of [3 H]-TCA in two human primary hepatocytes donors, HL #158 (B) & #159 (D), upon 72-h pre-treatment with vehicle control (0.1% DMSO) or tamoxifen (5, 10, and 20 μ M). Solid bars represent dpm/mg protein of [3 H]-TCA that accumulated in the cells + bile canaliculi, while open bars represent dpm/mg protein [3 H]-TCA in the cells only

Table 1

Biliary excretion index (BEI) of metformin treatments in inhibition and repression assays

	No Pre-treatment (Inhibition)	72 h Pre-treatment (Repression)
DMSO 0.1%	81.72 ± 6.75	81.52 ± 4.83
MET 0.1 mM	82.71 ± 8.53	83.50 ± 1.75
MET 0.5 mM	84.18 ± 2.14	68.95 ± 11.77
MET 1 mM	82.45 ± 1.08	19.22 ± 16.22 ***

 $P < 0.001$

Table 2
Biliary excretion index (BEI) of tamoxifen treatments in inhibition and repression assays

	No Pre-treatment (Inhibition)	72 h Pre-treatment (Repression)
DMSO 0.1%	65.99 ± 2.52	71.75 ± 5.14
Tamoxifen 5 µM	62.86 ± 3.95	58.94 ± 5.49
Tamoxifen 10 µM	65.60 ± 6.98	52.38 ± 4.75 [*]
Tamoxifen 20 µM	52.30 ± 7.43	16.61 ± 9.32 ^{***}

^{*} $P < 0.05$;

^{***} $P < 0.001$