Recent advances in the exploration of the bile salt export pump (BSEP/ABCB11) function

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Abstract

Introduction

The bile salt export pump (BSEP/ABCB11), residing in the apical membrane of hepatocyte, mediates the secretion of bile salts into the bile. A range of human diseases is associated with the malfunction of BSEP, including fatal hereditary liver disorders and mild cholestatic conditions. Manifestation of these diseases primarily depends on the mutation type; however, other factors such as hormonal changes and drug interactions can also trigger or influence the related diseases.

Areas covered

Here, we summarize the recent knowledge on BSEP by covering its transport properties, cellular localization, regulation, and major mutations/polymorphisms, as well as the hereditary and acquired diseases associated with BSEP dysfunction. We discuss the different model expression systems employed to understand the function of the BSEP variants, their drug interactions, and the contemporary therapeutic interventions.

Expert opinion

The limitations of the available model expression systems for BSEP result in controversial conclusions, and obstruct our deeper insight into BSEP deficiencies and BSEP-related drug interactions. The knowledge originating from different methodologies, such as clinical studies, molecular genetics, as well as *in vitro* and *in silico* modeling, should be integrated and harmonized. Increasing availability of robust molecular biological tools and our better understanding of the mechanism of BSEP deficiencies should make the personalized, mutation-based therapeutic interventions more attainable.

Abbreviations:

4-PBA	4-phenylbutyrate
ABC	ATP-Binding Cassette
BRIC2	benign recurrent intrahepatic cholestasis type II
BSEP/ABCB11	bile salt export pump
DBD	4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole
DILI	drug-induced liver injury
ESE	exonic splicing enhancers
ESS	exonic splicing silencer
FDA	U.S. Food and Drug Administration
FXR	farnesoid X receptor
HCC	hepatocellular carcinoma
ICP	intrahepatic cholestasis of pregnancy
MDR1	multidrug resistance protein 1 (P-gp)
NBD	nucleotide binding domain
NTCP	sodium-dependent bile salt uptake transporter (SLC10A1)
OATPs	organic anion transporters
PC	phosphatidyl choline
PFIC2	progressive familial intrahepatic cholestasis type II
P-gp	P-glycoprotein (MDR1)
QSAR	quantitative structure–activity relationship
SNP	single nucleotide polymorphism
Spgp	sister of P-glycoprotein
TMD	transmembrane domain
TMH	transmembrane helix
UDCA	ursodeoxycholic acid

1. Introduction

The bile salt export pump (BSEP) is a liver resident transporter protein, which plays an essential role in the enterohepatic circulation of the bile salts [1, 2]. BSEP belongs to the ATP-Binding Cassette (ABC) protein family, the members of which are integral membrane proteins, defined by the existence of conserved sequences, the Walker A, Walker B, and the so called ABC signature motif. ABC transporters are composed of transmembrane domains (TMDs) containing typically 5-6 transmembrane helices (TMHs) and cytoplasmic ABC units or nucleotide binding domains (NBDs), which are responsible for the binding and hydrolysis of ATP, the energy of which is utilized for the translocations of transported substrates across the membrane. ABC proteins are present in all sorts of organisms; both uptake and efflux transporters (importers and exporters) can be found in prokaryotes, whereas ABC transporters in eukaryote plasma membrane are exclusively exporters.

The 48 human ABC proteins (classified into seven subfamilies, ranging from ABCA to ABCG) possess very different transport characteristics and contribute to diverse physiological functions, including detoxification, antigen presentation, and secretory processes. A group of ABC transporters, expressed in various physiological barriers and sanctuary sites of the body, form a complex network constituting an innate defense mechanism against endo- and xenobiotics, which is called the chemoimmunity system [3]. An important component of this defense system is the hepatic transport network, in which the bile salt export pump plays a pivotal role.

In this paper we give an overview on the current knowledge on the hepatic ABC transporter, BSEP. We discuss the model systems employed to understand its functional properties, the diseases associated with its malfunction, as well as the conventional and recently introduced therapies to cure these disorders.

2. Architecture, localization, and transport function of the bile salt export pump

BSEP (or ABCB11) belongs to the B subfamily of ABC transporters. It shows high sequence and structural homology to the multidrug transporter P-glycoprotein (P-gp, MDR1, ABCB1), thus, BSEP was originally named sister of P-gp or Spgp [4]. Similar to P-glycoprotein, BSEP possesses a canonical ABC transporter membrane topology, having a domain order of TMD1-NBD1-TMD2-NBD2, as well as containing a large, heavily glycosylated extracellular loop in the first transmembrane domain, between TMH1 and TMH2. As a basic building principle of all other ABC transporters, organization of domains shows an internal dimeric arrangement; the two TMDs constitute a common structure in the membrane, as well as the two nucleotide binding pockets are composite sites jointly formed by the Walker A and Walker B motifs from one NBD with the ABC signature motif from the other NBD. It is generally accepted that intracellular coupling helices located between TMDs and NBDs convert the energy from ATP hydrolysis to conformational changes in the TMDs, thereby allowing substrate translocation. In BSEP the coupling helices have not been examined in detail, but their presence can be assumed on the basis of the high sequence homology to P-glycoprotein, in which these interacting helices have already been identified [5].

In recent years, higher resolution crystal structures of several ABC exporters have become available. These include the bacterial SAV1866 [6], the murine MDR1a/ABCB1 [7, 8], and the human ABCB10 [9] transporters. Thus far the structure of BSEP has not been resolved, although homology models have been generated based on the crystal structures of SAV1866 and mouse MDR1a [10, 11].

Unlike P-glycoprotein, which is present in a wide range of cell types, BSEP is selectively expressed in hepatocytes [4]. Although low levels of mRNA and protein expression were detected in the testis and adrenal gland of humans [12, 13], the function of BSEP in these

tissues is unknown. BSEP is specifically expressed in the apical (canalicular) membrane of hepatocytes, but a substantial amount of BSEP can also be found in subapical vesicles [14, 15]. The apical surfaces of adjacent hepatocytes form a microscopic tube-like structure, the bile canaliculus, which is sealed by tight junctions (see Fig. 1). The bile canaliculi are interconnected, forming a complex tubular network, the smallest branches of the biliary tree of the liver. A detailed biochemical study demonstrated that under basal conditions only a small portion of BSEP resides in the canalicular membrane, the larger part is located in the intracellular vesicular pool, from which it can be rapidly mobilized to the cell surface [16]. Following biosynthesis most canalicular membrane proteins, such as the transferrin receptor, are targeted from the Golgi apparatus to the basolateral surface first, then transcytosed to the canalicular membrane compartment. However, newly synthetized BSEP is delivered directly to the intracellular pool and then to the canalicular surface [16]. It has also been demonstrated that BSEP undergoes continuous cycling between the canalicular membrane and the rab11a-positive recycling endosomal pool [17].

As most ABC transporters, BSEP is an active transporter powered by ATP binding and hydrolysis. Contrary to P-glycoprotein, which transports a wide variety of compounds, BSEP exports a restricted number of molecules, predominantly monovalent taurine- and glycine-conjugated bile salts [18]. It has been proposed that unconjugated bile salts are also BSEP substrates; however, these molecules are probably transported only at very low rate (see in [19]). Non-bile acid compounds, such as pravastatin and fexofenadine, have been reported to be transported by the human BSEP [20, 21]. In addition, it has been suggested that vinblastine and calcein-AM are substrates of the mouse BSEP (mBSEP) [22]. However, these results should be taken with caution, because these compounds are good MDR1 substrates, and MDR1 can easily be up-regulated in many cellular model systems. Although slight species differences in the substrate recognition of the human, rat, canine, and feline BSEP have been

reported [23, 24], the major transport function of this transporter seems to be evolutionary conserved [19].

3. Physiological role and regulation of BSEP

A large fraction of bile constituents are continuously cycled in the enterohepatic circulation. About 95% of the bile salts travel through this cycle. A critical step in this process is the hepatic secretion of the bile, which is conducted by the transporters residing in the canalicular membrane of hepatocytes [2]. BSEP expels bile salts to the bile canaliculi, whereas MDR3/ABCB4 flips phosphatidyl choline (PC) to the outer leaflet of the membrane, resulting in net efflux of PC. The ABCG5/G8 heterodimer exports cholesterol to mixed micelles and PC vesicles. Other canalicular transporters including ATP8B1/FIC1, MRP2/ABCC2, MDR1/ABCB1, ABCG2, and the chloride-bicarbonate anion exchanger 2 (AE2) also contribute to the composition of bile by retaining aminophospholipids and exporting divalent bile salts, anionic conjugates, organic cations, glutathione, and bicarbonate to the bile (reviewed in [2, 25]). It has been proposed that ABCG2 is capable of bile acid transport [26], however, its physiological role, especially in the liver, is debated as discussed in detail in Ref. [2]. The basolateral (sinusoidal) transporters for bile acids include various solute carriers, such as NTCP, OATP1B1, OATP1B3, and OSTα/β [27, 28]; export pumps, such as MRP3/ABCC3, MRP4/ABCC3 [29, 30]. NTCP and the OATPs responsible for the uptake of bile acids from the sinusoidal space into the hepatocytes, whereas MRP3, MRP4 and OSTα/β mediate a reverse transport, when it becomes necessary (see Fig. 1).

Export of bile salts driven by BSEP was shown to be the rate limiting step in bile secretion [1]. Thus, the expression and function of BSEP is a critical determinant of the enterohepatic circulation of the bile salts. The expression of *BSEP* is under the control of the farnesoid X receptor (FXR), a bile acid sensor, which forms heterodimer with RXRα upon activation, and consequently binds to FXR response element in the promoter of *BSEP* [31].

This FXR-mediated transcriptional activation can be further modified by other nuclear receptor co-activating proteins including ASCOM, CARM1, and SRC2 [32]. Moreover, FXR-independent transcriptional regulation of *BSEP* involves LRH1, NRF2, and PXR [32].

In addition to the transcriptional control, the cell surface expression of BSEP can be rapidly modulated by mobilization of the intrahepatic, BSEP-containing vesicles, or inversely by controlling the internalization of the transporter from the canalicular membrane. This redistribution between the endosomal pool and the cell surface provides a fine-tuned regulatory mechanism, reacting promptly to the demand for the bile secretory capacity. The taurine-conjugated bile salt, taurocholate was shown to act not only as a bile constituent but also as a signaling molecule, which can trigger the delivery of BSEP to the canalicular membrane [16]. Recently, we have demonstrated that taurocholate-stimulated canalicular trafficking of BSEP is controlled by a signaling pathway that involves the EPAC/LKB1/AMPK kinase cascade [33]. In addition to taurocholate, cAMP also promotes BSEP trafficking to the canalicular surface by an alternative, PKA-dependent mechanism [16, 33]. PI3K has been shown to participate in the taurocholate-mediated redistribution of BSEP but not in the cAMP-stimulated response [34]. Another bile salt, tauroursodeoxycholate has been demonstrated to stimulate delivery of BSEP from the Golgi to the cell surface through the activation of p38 MAP kinase [35]. The presence and the half-life time of BSEP on the cell surface are determined not only by the delivery rate, but also by anchoring and internalization mechanisms. Numerous regulatory proteins such as cortactin, HAX-1, EPS15, AP-2, and IP3-receptor II have been suggested to participate in this process [36].

Canalicular ABC transporters including MDR1, MRP2, ABCG5, and BSEP are localized in bile salt-resistant, cholesterol- and sphingomyelin-rich microdomains of the hepatocyte canalicular membrane [37, 38]. As previously shown for MDR1, MRP2, and ABCG2 [39-42], the activity of BSEP is modulated by the cholesterol content of the membrane [43, 44].

However, cholesterol increases only the transport capacity (V_{max}) of BSEP, without affecting the affinity for the substrates (K_m) [45]. This differs from what was observed with MRP2, where both V_{max} and K_m are modified by cholesterol. The exact mechanism how cholesterol modulates BSEP activity is unknown. Using a heterologous *in vitro* system, the phosphorylation of BSEP by PKCα has also been demonstrated [46], however, its physiological relevance remains to be clarified.

Transporters in hepatocytes, including both canalicular and basolateral transport proteins, constitute a complex, coordinated system. Expression of the enzymes and transporters involved in the bile salt synthesis, uptake, and secretion is concertedly regulated. In addition, the function of transporters exporting other bile constituents is also essential for proper BSEP function. Even drug transporters can influence the function of BSEP by controlling of the distribution of compounds interacting with the transporter either on the intracellular or the intraluminal side. The latter is exemplified by the trans-inhibition of BSEP by estradiol-17β-glucuronide or progesterone metabolites, which are exported by MRP2 to the canalicular lumen [47, 48].

4. Human diseases associated with BSEP mutations

A large number of mutations and polymorphisms in the *BSEP* gene have been identified, which either cause hereditary liver diseases, or represent susceptibility factor for the development of various conditions affecting mainly the liver [11, 36, 49-53]. An up-to-date database summarizes the available information on the mutant variants of ABC transporters including BSEP (see http://abcmutations.hegelab.org/) [54]. All regions of BSEP can be affected, but the hot spots for mutations are the NBDs and the cytoplasmic loops. *BSEP* polymorphisms are ethnic dependent and usually rare, with the exception of the V444A and M677V variants, which exhibit allele frequencies ranging 50-93% and 0-15%, respectively, depending on the ethnic background [51].

Hereditary liver diseases caused by loss of function mutations in BSEP include the progressive familial intrahepatic cholestasis type II (PFIC2) and the benign recurrent intrahepatic cholestasis type II (BRIC2). In these syndromes impaired bile salt secretion caused by the lack or insufficient amount of functioning BSEP in the canalicular membrane leads to intrahepatic cholestasis characterized by diminished bile production, reduced bile flow, and intrahepatic accumulation of toxic bile salts, concomitant with high plasma levels of bile salts and bilirubin. PFIC2 is an early onset disease with rapid progression frequently leading to end-stage liver complications before adulthood, whereas BRIC2 is characterized by recurrent episodes of cholestasis and consequent pruritus without substantial liver damage in most cases. PFIC2 patients are also susceptible to development of pediatric hepatocellular carcinoma (HCC) and cholangiocarcinoma [55, 56]. Several mutations in *BSEP* have been shown to be responsible for PFIC2 and/or BRIC2 (see Table 1). These include the relatively frequent E297G and D482G variants, which are present in 58% of European PFIC2 patients [49].

The intrahepatic cholestasis of pregnancy (ICP) is a transient form of cholestatic liver diseases manifesting usually in the third trimester of pregnancy, and characterized by elevated serum bile acid levels, variable pruritus, fetal distress and increased risk for preterm delivery. Numerous polymorphisms and mutations in*BSEP*, including the common V444A variant, have been identified as susceptibility factor for ICP [11, 51, 57-61]; however, the mechanism how these variants contribute to the progression of ICP is unclear. High levels of estrogens and progesterone have been proposed to be responsible for the trans-inhibition of BSEP in the later stage of pregnancy [48], but transcriptional repression of BSEP expression by 17β-estradiol and epiallopregnanolone sulfate has recently been demonstrated, providing an alternative model for the development of ICP [62].

Substantial interindividual variability has been observed in the BSEP expression at both mRNA and protein levels even in healthy individuals [51, 63]. The frequent V444A variant was associated with low expression levels. Patients with BSEP-deficiencies usually exhibit reduced canalicular expression of BSEP. Staining with anti-BSEP antibodies of liver tissue samples combined with genotyping is a powerful method to evaluate the disorder [64, 65]. Several studies reported recurrence of intrahepatic cholestasis due to the autoantibodies raised against BSEP after liver transplantation. The antibody originated from one of these cases recognizes an extracellular epitope of the human BSEP, moreover, suppresses its function [66], providing an ideal tool for assessment of BSEP at the protein level. Inversely, the presence of autoimmune antibodies can be detected by canalicular antibody deposits in liver tissue samples subjected to the patients' serum, which specificity can be subsequently verified by Western blot analysis [11].

Besides estrogens and progesterone metabolites, numerous drugs, including rifampicin, troglitazone, nefazodone, glibenclamide, cyclosporin A, and bosentan, have been shown to block BSEP function, thus, a cholestatic form of drug-induced liver injury (DILI) can develop upon treatment with these agents [53, 67-69]. The efforts to predict drug interaction with BSEP will be discussed later; nevertheless, the contribution of genetic factors to drug-induced cholestasis is also unambiguous. The frequent V444A variant and numerous rare mutations have been identified as risk factors for cholestatic DILI [52, 60, 61]. In addition to ICP and DILI, numerous *BSEP* variants are associated with several other liver diseases including primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), primary intrahepatic stones, gallstone disease, and neonatal cholestasis [70-72]. Despite the substantial amount of genomic data accumulated in the last decade, the etiological role of these BSEP mutations and SNPs in the acquired cholestatic liver diseases remains to be established.

Although the association of *BSEP* mutations and polymorphisms with the above discussed liver diseases is clearly documented, the mechanisms how these mutations/SNPs lead to the syndromes are diverse and controversial. Mutations in the *BSEP* gene can result in defective bile salt secretion in several ways, which include reduced mRNA stability, protein misfolding and instability, loss of transport activity, as well as impaired trafficking to the canalicular surface. Nevertheless, a close correlation between the cell surface expression of BSEP and the clinical manifestation of PFIC2/BRIC2 has been demonstrated [73]. Even though the two most frequent disease-causing *BSEP* mutations (E297G and D482G) were extensively studied, the results are controversial. The discrepancies are likely stemmed from the differences in the applied experimental arrangements, including the different origin of BSEP in terms of species (human, rat, or mouse), and diversity of heterologous expression systems used for trafficking and/or transport studies. Table 1 summarizes the identified defects caused by various *BSEP* mutations and SNPs, specifying the major features of the used model systems.

5. Assessments of expression and function of BSEP

Reliable assay systems detecting BSEP expression, localization and function are crucial for dependable diagnosis and understanding the mechanisms of BSEP-related diseased conditions. The various detection methods assessing BSEP expression at the protein level with the help of anti-BSEP antibodies were discussed in the previous Section. Another approach to investigate the basis of reduced expression of *BSEP* is the so-called minigene system, which determines aberrations at the RNA level [50]. This method revealed abnormal pre-mRNA splicing in a considerable number of mutations and SNPs in the *BSEP* gene.

Different *in vitro* model systems are used to evaluate BSEP expression and function; however, the results have to be interpreted carefully because of the complexity of bile salt circulation, drug metabolism, and transport systems. Some models aim at investigating certain

specific properties of the transporter, whereas others try to build a more complex net of transporters in a cellular context for measuring vectorial transport of bile salts. Variance in the applied cellular models may affect the intracellular fate of the studied BSEP variant. The robust Sf9 insect cell expression system commonly used for transport studies is relatively tolerant of protein misfolding, whereas mammalian cells, especially polarized epithelial cells are more sensitive to folding problems, thus, are also suitable for studying trafficking defects. However, it should be noted that the trafficking machinery of the commonly used model cells, such as MDCK, HEK293, and HepG2, can considerably differ from that of the hepatocytes, the authentic cell type. This is exemplified by the fact that the stimulatory effect of taurocholate on BSEP trafficking can be observed *in vivo* and in isolated hepatocytes [16, 33], but not in Wif-B9 liver model cells [17].

Species differences add an extra complication, since dissimilarities between the properties of human and non-human BSEP may result in discrepancies in *in vitro* model systems. Common laboratory model animals, such as rats and mice, exhibit significant differences in bile composition, bile salt levels, and regulation as compared to humans. The ratio of glycine to taurine conjugates in humans is 3:1 and the prevalent form is chenodeoxycholate. The ratio is the reverse in rodents, and the bile composition is dominated by more hydrophilic bile salts [74], making rodents less susceptible to cholestatic conditions. Human and rodent BSEP expressed in heterologous systems exhibit different affinity for various bile salts. The highest affinity substrate of the human BSEP is taurocholate, whereas the preferred substrate of rodent BSEP is taurochenodeoxycholate [14, 18]. It should be noted that affinities for monovalent bile acid conjugates are very close, and the determined affinity orders vary to some extent with the experimental conditions (see more details in [19]). Feline and canine BSEP proteins, sharing 91.2% homology with human BSEP, prefer taurine-conjugated bile acid over glycocholic acid, and have lower affinity for unconjugated cholic

acid [24]. Although the affinities of BSEP for different substrates are slightly different, the substrate spectrum in general is fairly conserved [19]. Therefore, both human and rodent BSEP are used in *in vitro* assay systems to study transporter-drug interactions. Human BSEP is regularly employed in the vesicular transport measurements; whereas mouse or rat BSEP is preferred in the ATPase assay, since rodent versions of BSEP possess higher ATPase activities than the human counterpart [75]. Since the ATP activity of either BSEP variant is very low as compared to that of the multidrug transporters, the results obtained from these assays should be interpreted cautiously, because of the high risk for false positive and negative hits for inhibitors and stimulators, respectively.

Contrary to the human clinical experience, *BSEP* knockout mice have no marked liver disease only when challenged with high cholate diet [76]. These animals still secrete 30% of normal bile salts into bile, predominantly tauromuricholate and tetrahydroxylated bile salts, and exhibit elevated P-gp expression, which may help to prevent disease development [77, 78]. An interesting model option is mBSEP overexpression in the liver of mice [79]. These animals display enhanced biliary lipid (phospholipid and cholesterol) secretion paralleled with an increased bile salt output.

Vectorial transport of labelled bile salts, measured in rat or human sandwich-cultured hepatocytes represents a more complex model system. It should be noted that similar to the case of BSEP, other canalicular efflux and basolateral uptake transporters involved in the bile salt transport also show species differences [80, 81]. Modeling the differences in the transport and drug metabolic pathways between the human and animal hepatocytes may help to increase the reliability of animal-derived hepatocyte models in drug interaction tests.

The causes of drug-induced liver toxicity are diverse, and only a minor portion of DILI is linked to BSEP. As mentioned earlier several agents including even high-profile drugs have been shown to induce liver toxicity through BSEP inhibition. A comprehensive study found

that 38 out of 200 common drugs inhibited taurocholate transport with an IC_{50} of less than 100 μM [82]. Several *in vitro* assay systems are available to evaluate potential drug interactions with BSEP. Insect cell membrane vesicles containing recombinant BSEP provide a rapid and cost-effective approach. K_i values determined by this method showed reasonably good correlation with the results of vectorial transport assays and *in vivo* data [19]. The sensitivity of the Sf9 cell-based assay can be increased by cholesterol loading of the insect cell membranes, which are normally intrinsically poor in cholesterol [43, 45]. Cholesterol treatment increases the V_{max} but not the K_m values for various bile salts, with the exception of glycochenodeoxycholate. Using this assay system for screening large compound libraries, several chemically different compounds were identified as possible BSEP inhibitors. Positive hits can be subsequently checked in more complex *in vitro* systems, such as the vectorial assays of cell monolayers using primary hepatocyte cultures or engineered model cells (MDCK or LLC-PK1) expressing BSEP and a bile salt uptake transporter, e.g., NTCP [83].

Primary hepatocyte cultures represent the most complex *in vitro* systems having the full set of bile salt transport systems together with the entire drug metabolic machinery, although in this case not only direct drug inhibitions but also the effects of drug metabolites are measured. In vesicular assays the drugs reach the transporter primarily from the cytoplasmic side, since inverted membrane vesicles are used. In contrast, in vectorial systems drugs can be applied either from the apical or the basolateral surface, which allows deciphering the sidedness of the drug interaction. This is how trans-inhibition of BSEP by estradiol-17β-glucuronide or progesterone metabolites has been revealed [47, 48]. Nevertheless, most inhibitory drugs are thought to act from the cytoplasmic side of BSEP. It should be noted that the sidedness cannot be unambiguously determine in many cases, since several therapeutic agents are lipophilic, thereby easily penetrate the cell membrane, and can reach even transmembrane drug binding sites, as shown previously for P-gp. In addition, vectorial

transport systems allow combined administration of bile acids and tested drugs, mimicking the drug-induced cholestasis. For example ritonavir, cyclosporin A, and simvastatin have been shown to become toxic only in the presence of bile acids, suggesting certain cooperativity [84]. These results should, however, be interpreted cautiously, because the high levels of bile acids far from physiological concentrations (200-fold of normal level), can alter membrane lipid structure by themselves.

Another important issue is the type of labeled substrates employed in the *in vitro* transport assays. A favored test compound is taurocholate, and most studies use radiolabeled substrates. For economical and safety reasons fluorescently labeled bile salts have also been developed. However, most fluorophores were shown interfere with the transport by substantially altering the size or hydrophobicity of the substrate. The aminofluorescein-tagged bile acids, such as chenodeoxycholylglycyl-amido-fluorescein and cholylglycyl-amido-fluorescein are substrates for both NTCP and BSEP, but not as good as taurocholate [83]. Not only the size of the fluorophore, but its position on the substrate molecule is also important. Taurocholate conjugated with 4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD) on the side chain was successfully applied in insect cell vesicular and vectorial transport assays [85, 86].

6. In silico models for predictions of cholestatic effects of drugs

Various methods have been established to predict drug interactions with BSEP, as well as to evaluate mRNA and protein stability problems. ESE (exonic splicing enhancers) and ESS (exonic splicing silencer) prediction programs are used to identify nucleotide substitutions that could alter mRNA splicing [50]. Discrepancies in the experimental methods and the *in silico* predictions have made these estimations ambiguous thus far.

More sophisticated are the structure-based models for drug interactions, but no crystal structure is available for BSEP. Homology models for ABC exporters were generated on the

basis of the few known crystal structures available, mainly that of the bacterial multidrug exporter, Sav1866 [6]. Recently, the mouse P-gp has been crystallized, and its structure has been resolved at a sufficiently good resolution. Since BSEP exhibits high sequence similarity to P-gp, its structure provides a better basis for homology modeling. It should be noted, however, that homology modeling always bears some uncertainties and especially the transmembrane regions in BSEP cannot be modeled reliably. Despite these problems, homology model was used to identify the extracellular epitope recognized by the anti-BSEP antibody mentioned above [66].

Some drug interaction prediction methods are based on ligand characteristics, such as QSAR (structure activity model for ligands), pharmacophore models, and non-linear SPECS. These methods are established and validated in high throughput BSEP in vitro drug interaction datasets, which were significantly expanded recently [10, 67, 68, 84, 87-91].

A comprehensive, membrane vesicle-based study investigated a set of 624 compounds for BSEP inhibition [10]. Based on the physicochemical properties of the identified blockers, the inhibitory effect showed strong correlation with the lipophilicity and molecular size. It should, however, be noted that the IC_{50} threshold for significant BSEP inhibition was set to 300 μM in this study, which may overestimate the number of blockers. Another study, using vesicular transport assay, identified 86 BSEP inhibitors out of 250 test compounds [87]. QSAR modeling, here too, demonstrated a strong correlation between the lipophilicity and BSEP inhibition, whereas positive charge prevented the inhibitory effect. About 60% of the FDA-approved drugs, identified as BSEP inhibitors in this study, carry the risk for DILI according to the drug labels. Correlation between the severity of DILI and the inhibitory effect of the drugs on the taurocholate transport was also demonstrated using human hepatocyte sandwich cultures [87].

Similarly, the frequency and severity of bile acid-mediated DILI in connection with BSEP inhibition are predicted by using DILIsym, a multi-scale mechanistic mathematical model (http://www.dilisym.com). A great amelioration is the incorporation of bile acid dynamics in the DILIsym [92], thus, the model includes not only the metabolic pathways, mitochondrial toxicity, key transporters, etc., but also the synthesis, uptake, recirculation, efflux, and hepatotoxicity of bile acids. This complex approach may help to reduce discrepancies originated from species differences as well as from in vitro and in vivo experimental data, thereby allowing improved prediction of BSEP-related DILI [92].

For its straightforwardness the pharmacophore modeling is commonly used for drug interaction prediction, however, the promiscuous drug binding site of ABC transporters also makes this approach challenging. Several models have been developed on congeneric series, which restricts their applicability to structurally related compounds [88]. A recent 3D pharmacophore model aims at identifying functional groups common in BSEP-inhibiting molecules along with fixed intramolecular distances between interacting motifs [93]. This model is based on the inhibitory effect of test compounds with an IC_{50} value of 50 μM or less, which was determined by human BSEP-containing membrane vesicles. The importance of hydrogen bond acceptors, anionic and loosely defined aromatic features were established as BSEP inhibitor pharmacophores, in which the exclusion volume has proven to be also important. This pharmacophore was subsequently confirmed with a set of 59 molecules.

Since latest surveys concluded that MRP4 inhibition has a significant impact in development of DILI, a new pharmacophore model has recently been developed that combines BSEP and MRP4 inhibitions [90]. Since MRP4 and BSEP have partially overlapping inhibitor profiles, modeling has to focus on both common and distinct features of these two sets of compounds. Welch et al. have built a Bayesian model, which has an increased predictive value as compared to other methods [91]. Their model underline the

importance of high molecular weight, hydrophobicity, certain rotatable bonds and the aromatic features, which is in agreement with the conclusions of previous studies [87, 93].

In summary, all the above discussed models and simulations have significant predictive potential, however, the complexity of bile acid metabolism and promiscuity of the potential drug binding site of BSEP create substantial uncertainties and require careful interpretation of predictions.

7. Pharmacological correction of BSEP deficiencies

Diseases associated with *BSEP* mutations and SNPs as well as the manifestation of these conditions are highly diverse, thus, therapeutic approaches also range from symptomatic treatment to major surgical interventions. The most severe form of BSEP deficiencies, PFIC2 is primarily treated surgically, which includes external or internal partial biliary diversion, ileal bypass, and even liver transplantation. Interestingly, the outcome of these surgical treatments to some extent depends on mutation type. For instance, the E297G and D482G mutations were associated with better prognosis after partial biliary external diversion [94]. Similarly, nonsense and splice site mutations were more frequent in cases, when PFIC phenotype recurred after liver transplantation, due to the emergence of anti-BSEP antibodies [65].

Numerous mutations and polymorphisms in the *BSEP* gene have been demonstrated to result in reduced mRNA level [50]. Transcriptional activation by nuclear receptor agonists, such as FXR ligands, provides a novel therapeutic approach to correct these deficiencies (reviewed in [32]). In addition to FXR agonists, targeting of PXR, CAR, PPARα and PPARγ, as well as the vitamin D and glucocorticoid receptors may also assist to restore the balance of bile salt metabolism in BSEP-associated liver diseases. A recent clinical report demonstrated unexpected improvement in the bile acid levels and relief of symptoms in PFIC2 patients in

response to steroid treatment [95], offering a new perspective for BSEP-deficiency therapy, although the mechanism of action remains to be investigated.

Ursodeoxycholic acid (UDCA) is a commonly used medication in various cholestatic liver diseases including PFIC2, BRIC2 and ICP. UDCA significantly reduces pruritus and improves clinical parameters; however, its beneficial effect falls short in numerous patients. Genetic factors, such as the type of *BSEP* mutations, can be responsible for the ambiguous therapy outcome, although this has not yet been demonstrated by comprehensive association analyses. Several mechanisms have been suggested to explain the effectiveness of UDCA. In the most commonly accepted view the non-toxic UDCA dilutes the cytotoxic bile salts in the endogenous bile acid reservoir, thus improving the clinical parameters. It has also been proposed that UDCA stimulates BSEP and MRP2 transcription, stabilizes the hepatocyte membrane, inhibits hepatocyte apoptosis, and promotes bile flow via cholehepatic shunting. The effect of ursodeoxycholic acids on the trafficking of BSEP is also controversial. Taurine-conjugated UDCA promotes BSEP delivery to the canalicular surface [15, 35], whereas UDCA have been shown to reduce BSEP internalization [96]. Nevertheless, the efficacy of the treatment was dependent on the BSEP mutation. A recent study demonstrated partial correction of impaired trafficking of BSEP with PFIC2-causing mutations by UDCA [97].

Using chemical chaperones or correctors has recently become a rapidly developing field in the therapy of diseases associated with mutations in ABC transporter genes [98]. Systematic screening revealed a series of small molecules that can rescue the impaired trafficking of misfolded CFTR-ΔF508, causing cystic fibrosis, the most frequent lethal inherited disease. Similar advances were achieved by *in vitro* and *in vivo* studies with ABCG2 and ABCC6 in connection with gout and pseudoxanthoma elasticum, respectively [99, 100], although clinical studies have not yet performed in these diseases. The major corrector molecule used in these experiments is the 4-phenylbutyrate (4-PBA), an FDA-approved drug

for thalassemia and urea cycle disorders. This agent was found to increase cell surface expression of the BSEP mutants, D482G and E297G *in vitro*, as well as enhance bile salt excretion *in vivo* [73, 101]. Besides 4-PBA, bile salts were shown to act as pharmacological corrector stimulating the delivery of BSEP-E297G to the cell surface [102]. Even more exciting are the recent clinical data demonstrating that high-dose 4-PBA treatment can markedly improve the clinical parameters of PFIC2 patients bearing trafficking defect mutations in BSEP such as G982R, T1210P, R1128C, or R1231Q [97, 103, 104]. For many of these cases partial correction of canalicular expression of the mutated BSEP has also been demonstrated.

8. Expert opinion

The liver-resident membrane transporter protein, the bile salt export pump (BSEP) is a key component of the enterohepatic circulation. It controls the extrusion of monovalent bile salts into the bile canaliculi, the microscopic tubular structures formed by adjacent hepatocytes (see Fig. 1), which collect and direct bile to bile ducts. Since the export of bile salts is the rate limiting step in bile secretion [1], the transporter mediating this process is of great physiological and pathophysiological importance. Many ABC transporters have overlapping substrate recognition, thus, when one of them is dysfunctional, other ones can compensate for the impaired function. However, no backup system for bile salt excretion is available; therefore, loss of function of BSEP necessarily leads to severe consequences. Although basolateral transport systems in hepatocytes can weaken this effect, deficient canalicular secretion results in accumulation of bile salts in the hepatocytes (intrahepatic cholestasis). Most bile salts are strong detergents, thus have profound cytotoxic effect. Their intrahepatic accumulation leads to hepatocellular necrosis, chronic inflammation, fibrosis, and ultimately liver failure.

Diseases caused by BSEP deficiency range from transient, mild conditions to early onset, fatal liver disorders. Manifestation of the disease greatly depends on the type of BSEP dysfunction. Several causes can lead to loss of BSEP function. Mutations in *BSEP* can result in complete loss of expression, decreased mRNA/protein stability, impaired trafficking, or diminished transport. Hormones, drugs and drug metabolites can also interfere with the transport function of BSEP. Nevertheless, the current knowledge on the mechanism for BSEP deficiencies is rather controversial (see Table 1). In numerous cases a combination of hereditary and acquired factors results in the dysfunction of the transporter. For instance, several polymorphisms in *BSEP* are suggested to be susceptibility factors for ICP or DILI.

Understanding the mechanism how mutations and polymorphisms lead to BSEP deficiency and ultimately to diseased conditions is a crucial prerequisite to develop efficient and cost-effective therapeutic interventions. Model systems, including *ex vivo*, *in vitro*, and *in silico* methods provide not only a great opportunity to decipher the disease mechanisms, but also offer screening systems for interacting drugs. The increasing availability of robust molecular biology techniques makes possible to screen for mutations in a large scale and to elucidate complex transcriptional events, giving the opportunity to understand even multifactorial conditions. However, it should be noted that extensive database analyses, clinical studies, especially case reports, as well as *in vitro* and *in silico* models still represent different worlds of perception. This phenomenon is not specific for the cholestatic liver diseases, but similar scenario has evolved in other biomedical fields, including the even more complex areas, such as the tumor biology.

A critical requirement for our better understanding of BSEP deficiencies is the refinement of the available model systems. Although numerous features of bile metabolism seem to be evolutionary conserved, even slight differences between the species may result in large deviations in consequences. This is exemplified by the fact that rodents are less susceptible to

cholestatic conditions due to the predominance of more hydrophilic bile salts as compared to humans. Results of trafficking studies should also be interpreted carefully, since targeting machinery of the employed model systems may differ from that of hepatocytes, thus, cellular routing of the mutated/polymorphic BSEP variants as well as the modulatory effect of pharmacological agents on their trafficking may also differ.

An exciting new perspective has been opened by the recent application of pharmacological correctors in PFIC2 patients. Many mutations associated with severe BSEP deficiencies cause trafficking defect, resulting in reduced plasma membrane expression of the transporter (see Table 1). If the transport capability is at least partially preserved, pharmacological chaperones may correct this type of BSEP deficiencies. Recently, encouraging clinical studies reported improvement of the clinical status of PFIC2 patients upon 4-PBA treatment [97, 103, 104]. However, progress was achieved only with a very high dose of drug, and the reported improvements were sometimes transient.

In conclusion, further efforts should be made to improve the model systems to make them more reliable and better representing the human physiological and pathological conditions. Evidently, there is a need for a change in our perception of BSEP-related disorders. We should integrate the experiences of the clinical studies, the vast information generated by contemporary molecular genetics, as well as the results of *ex vivo, in vitro* and *in silico* modeling. Only this integrative approach can make mutation-specific, personalized therapeutic interventions possible to cure BSEP-associated cholestatic liver diseases.

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Annotations

* Ref. 4. Child S. et al. 1995 – This is a historic milestone paper reporting the identification of BSEP.

* Ref. 16. Kipp H et al. 2001 – The large intracellular reservoir of BSEP, as well as its mobilization upon taurocholate and cAMP are reported in this paper.

* Ref. 18. Byrne JA et al. 2002 – The basic characteristics and major transport properties of BSEP are summarized in this classical paper.

* Ref. 25. Trauner M and Boyer JL 2003 – This is one of the most comprehensive reviews on BSEP, which covers all the important aspects of this medically important transporter.

** Ref. 49. Strautnieks SS et al. 2008 – A detailed genetic analysis of European families that are affected with PFIC2. This paper is an important milestone in understanding of the genetic background of BSEP deficiencies.

** Ref. 73. Lam P et al. 2007 – A detailed study of relevant mutations in BSEP, which demonstrates correlation between the manifestation of the disease and the cell surface expression of BSEP. This is also one of the first papers that shows the rescuing effect of 4-PBA.

** Ref. 77. Wang R et al. 2001 – A milestone paper in BSEP research, reporting the generation and phenotypic features of the BSEP knockout mouse.

* Ref. 87. Pedersen JM et al. 2013 – A comprehensive study using different model systems to identify drugs that inhibit BSEP, thus having potential risk for drug-induced cholestatic liver disease. This study also demonstrates the benefits of sandwich-cultured human hepatocytes as *in vitro* assay system.

** Ref. 101. Hayashi H, Sugiyama Y. 2007 – This is one of the first papers demonstrating that 4-PBA can promote delivery of mutant BSEP variants to the cell surface.

* Ref. 103. Gonzalez E et al. 2012 – The first clinical study demonstrating the beneficial effect of 4-PBA in a PFIC2 patient.

* Ref. 105. Wang L et al. 2002 – The first comprehensive study that characterizes the most relevant BSEP mutations associated with PFIC2, using in vitro model systems.

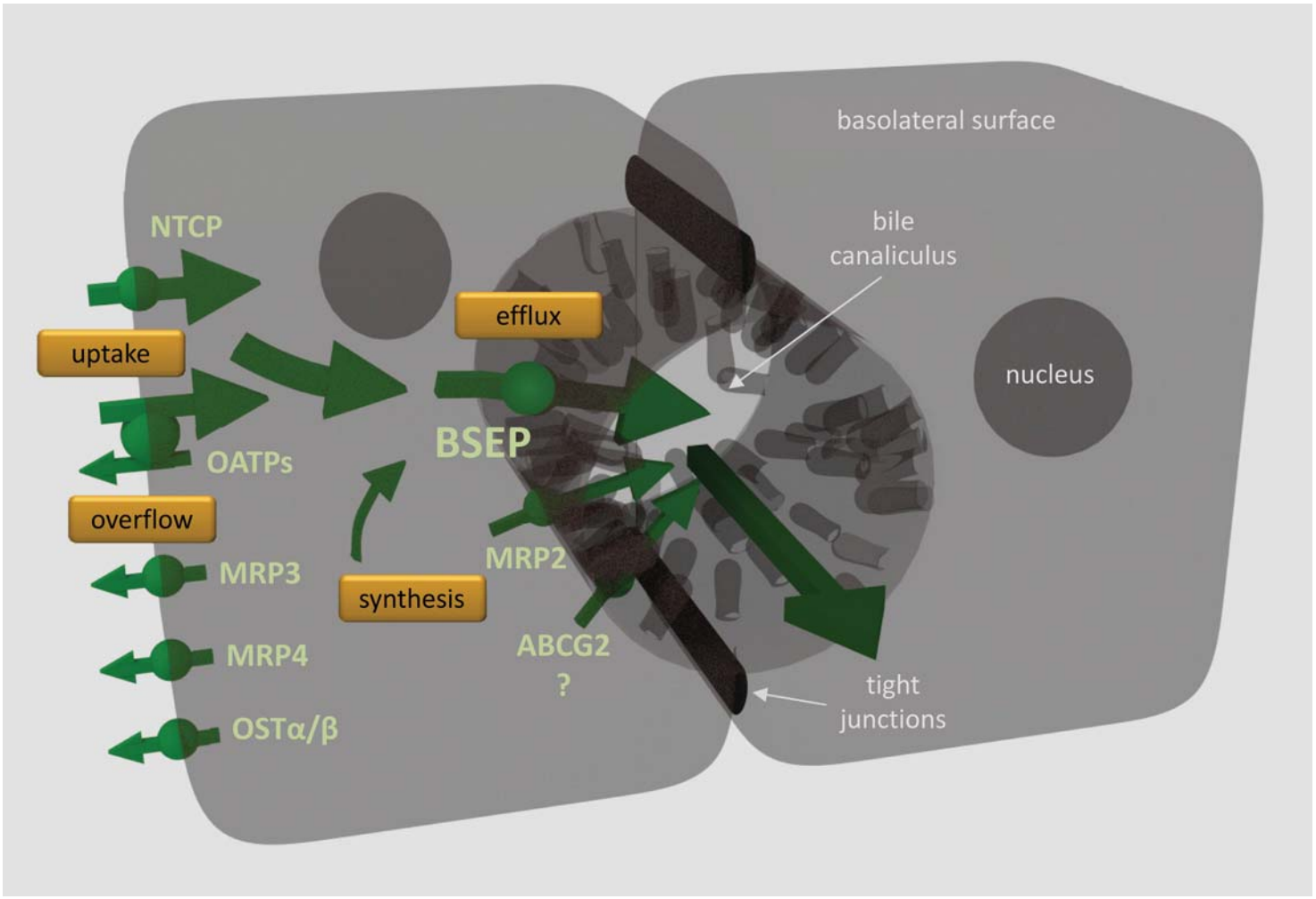
Article highlights

- A wide variety of cholestatic liver diseases is associated with the dysfunction of the bile salt export pump (BSEP), which normally mediates the secretion of monovalent bile salts into the bile. Manifestation of these disorders depends on the amount of functional BSEP on the cell surface.
- BSEP-related diseases can be either inherited or acquired. The latter is frequently associated with mild mutations or polymorphisms in BSEP. Understanding how these mutations and/or drug interactions can lead to cholestatic liver disease is a prerequisite to develop efficient, cost-effective, and possibly mutation-based, personalized therapies.
- Several human and non-human systems, including *ex vivo*, *in vitro* and *in silico* models, are employed to assess the mechanism of BSEP-related diseases and to predict the drug interactions. Most sophisticated model systems are based on the combination of various approaches.
- Recently, patients with severe BSEP deficiency were successfully treated with 4-phenylbutyrate, a pharmacological chaperone, which was able to restore at least partially the cell surface expression and the function of BSEP.

Legend for Figures and Tables

Figure 1. Routing of bile salts in hepatocytes. Bile salts, arriving from the distal ileum through the hepatic portal vein to the hepatic sinusoids, are taken up by hepatocytes via the basolateral transporters, such as NTCP and OATPs. The recycled bile salts are reconjugated and extruded together with the newly synthetized bile salts by BSEP to the lumen of bile canaliculi, the miniature tubular structures, which are assembled from the apical membranes of adjacent hepatocytes, and sealed by tight junctions. The bile salts are then collected by the bile canaliculi and transferred to the bile ducts and subsequently to the gall bladder. Alternative routes for the canalicular bile salt export include MRP2 and supposedly ABCG2. The basolateral MRP3, MRP4, and OSTα/β transporters serve as an overflow system, mediating bile salt transport to the sinusoidal space, when needed.

Table 1. BSEP mutations and polymorphisms associated with various cholestatic liver diseases. The defects in the trafficking, stability, and transport capability of BSEP are indicated as determined by different studies. The trafficking was considered abolished when no plasma membrane expression was found, whereas regarded as impaired, when the cell surface expression was reduced. The main features of the model systems used for *in vitro* assessments of the mutated BSEP are also listed. These include the species from which the transporter was originated, and the cell types used for trafficking or transport studies. n.d. – not determined


basolateral surface
NTCP
bile
canaliculus
efflux
uptake
nucleus
BSEP
OATPs
overflow
MRP2
MRP3
synthesis
ABCG2
?
MRP4
tight
junctions
OSTα/β

Mutation/ SNP	Disease	Trafficking	Protein stability	Transport	Model systems used for the studies			Reference
					BSEP from	Trafficking	Transport	
E297G*	PFIC2, BRIC2	abolished	n.d.	abolished	rat	MDCK	Sf9	[105]
		impaired	shortend $t_{1/2}$ in the membrane	normal**	human	MDCK and HEK293	HEK293	[106]
		n.d.	n.d.	reduced	human	-	Sf9	[107]
		impaired	n.d.	abolished	rat	MDCK and HEK293	Sf9	[73]
		impaired	n.d.	reduced	rat	-	MDCK	[108]
		normal	n.d.	normal	human	HepG2	HeLa-hNTCP	[51]
		n.d	elevated ubiqutination	n.d	human	MDCK	-	[109]
		impaired	rescued	n.d	human	CHO-K1	-	[50]
D482G*		normal	n.d.	reduced	rat	MDCK	Sf9	[105]
		impaired	T sensitive mRNA	normal	mouse	HepG2	SF21	[110]
		abolished	n.d.	normal	human	MDCK	HEK293	[106]
		abolished	n.d.	normal	rat	MDCK and HEK293	Sf9	[73]
		impaired	shortend $t_{1/2}$	reduced	rat	-	MDCK	[108]
		impaired	rescued	n.d.	rat	HEK293	-	[111]
		impaired	n.d.	abolished	rat	-	MDCK-rNTCP	[96 64]
		n.d.	elevated ubiqutination	n.d.	human BSEP in MDCK	-	-	[109]
G982R		abolished	n.d.	abolished	rat	MDCK	Sf9	[105]
		abolished	n.d.	n.d.	rat	HEK293	-	[111]
		impaired	n.d.	reduced	human	HepG2	HeLa-hNTCP	[51]
		n.d.	n.d.	reduced	rat	-	MDCK-rNTCP	[96 64]
		impaired	partially rescued	n.d.	rat	Can 10	-	[97]
R1153C*		abolished	n.d.	abolished	rat	MDCK	Sf9	[105]
		abolished	n.d.	abolished	rat	-	MDCK	[108]
		abolished	n.d.	n.d.	rat	HEK293	-	[111]
		impaired	n.d.	reduced	human	HepG2	HeLa-hNTCP	[51]
R1268Q*		abolished	n.d.	abolished	rat	MDCK	Sf9	[105]
		abolished	n.d.	abolished	rat	-	MDCK	[108]
		abolished	n.d.	n.d.	rat	HEK293	-	[111]
G238V		abolished	rapid proteosomal degradation	n.d.	rat	MDCK	Sf9	[105]
		impaired	rescued	n.d.	rat	HEK293	-	[111]
R1057X		normal	n.d.	abolished	rat	-	MDCK	[108]
A570T		impaired	n.d.	normal	rat	MDCK and HEK293	Sf9	[73]
		impaired	n.d.	reduced	rat	-	MDCK	[108]
		n.d.	n.d.	reduced	rat	-	MDCK-rNTCP	[96 64]
		impaired	rescued	reduced	human	CHO-K1	Hi5	[50]
R432T		n.d.	n.d.	reduced	human		Sf9	[107]
T1210P*		impaired	rescued	n.d.	rat	Can 10		[97, 103]
R1128C*		impaired	rescued	n.d.	rat	Can 10	-	[97]
		abolished	rescued	n.d.	human	CHO-K1	-	[50]
R1050C		impaired	n.d.	normal	rat	HEK293	Sf9	[73]
		impaired	n.d.	reduced	rat	MDCK	-	[108]
		impaired	rescued	n.d	human	CHO-K1	-	[50]
C336S	PFIC2***	normal	n.d.	normal	rat	MDCK	Sf9	[105]
V444A	risk for ICP	normal	n.d.	normal	human	CHO-K1	-	[50]
I206V		normal	n.d.	reduced	human	HepG2	HeLa-hNTCP	[51]
Q558H		normal	n.d.	reduced	human	HepG2	HeLa-hNTCP	[51]
N591S		impaired	n.d.	normal	rat	HEK293	Sf9	[73]
E1186K		impaired	n.d.	reduced	human	HepG2	HeLa-hNTCP	[51]
V444A	risk for DILI	n.d.	n.d.	reduced slightly	human	-	Sf9	[52]
D676Y		n.d.	n.d.	reduced	human	-	Sf9	[52]
G855R		n.d.	n.d.	reduced	human	-	Sf9	[52]

Abbreviations: BRIC2, benign recurrent intrahepatic cholestasis type II; DILI, drug-induced liver injury; ICP, intrahepatic cholestasis of pregnancy; PFIC2, progressive familial intrahepatic cholestasis type II

* also reported as susceptibility factors for ICP

** ambiguous due to low expression level

*** found in a heterozygous PFIC2 patient