Mouse Bsep ATPase Assay: A Nonradioactive Tool for Assessment of the Cholestatic Potential of Drugs

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The mouse ortholog of the human bile salt export pump (BSEP) transporter was expressed in a baculovirus-infected insect cell (Sf9) system to study the effect of membrane cholesterol content on the transporter function. The transport activity of cholesterol-loaded mouse Bsep-HAM-Sf9 vesicles was determined in a vesicular transport assay with taurochenodeoxycholate (TCDC), a known BSEP substrate. Mouse Bsep transports TCDC at a high rate that can be sensitively detected in the ATPase assay. Cholesterol upload of the Sf9 membrane potentiates both TCDC transport and TCDC-stimulated ATPase activities. Inhibitory effect of BSEP interactors on probe substrate transport was tested in both vesicular transport and ATPase assays using cholesterol-loaded membrane vesicles. A good rank order correlation was found between IC_{50} values measured in TCDC-stimulated mBsep ATPase assay and in the human BSEP vesicular transport assay utilizing taurocholate (TC) as probe substrate. This upgraded form of the mouse Bsep-HAM ATPase assay is a user friendly, sensitive, nonradioactive method for early high-throughput screening of drugs with BSEP-related cholestatic potential. It may complement the human BSEP-mediated taurocholate vesicular transport inhibition assay. (*Journal of Biomolecular Screening* 2009:10-15)

Key words: mouse, Bsep/ABCB11, cholestasis, ATPase activity, nonradioactive, taurochenodeoxycholate

INTRODUCTION

BILE ACIDS ARE OXIDATION PRODUCTS of cholesterol, synthesized by hepatocytes, and transported as bile salts by the bile salt export pump (BSEP, ABCB11), an ABC efflux transporter to the bile.[1,2] In humans there is no compensatory mechanism for the loss of this transporter.[3] Mutations of BSEP result in a genetic disease, called progressive familial intrahepatic cholestasis type 2 (PFIC2). Inhibition of BSEP by drug molecules leads to clinical cholestasis.[4] As a result, there is an increasing need for reliable, validated screening tools suited to test drug candidates for BSEP interaction potential during the drug development process.

Human BSEP protein shares high sequence identity with its rodent (rat and mouse) orthologs.[5] Gene knockout and transgenic mouse models are important in vivo systems for studying the role and regulation of the Bsep gene. In vitro expression of the mouse Bsep (mBsep) protein is essential for the analysis of functional interspecies differences and enables validation and prediction of in vivo results.[6] BSEP transporter function is often studied in vitro in membrane-based vesicular transport assays applying canalicular membrane vesicles[1] or using sandwich cultured hepatocytes as a cellular model.[6] For high-throughput applications, efflux transporters are commonly expressed in insect cell lines (e.g., Sf9) taking advantage of the robust baculovirus insect cell system,[7] and purified membranes are utilized in vesicular transport and ATPase assays.[8] After preparation, these membrane products can be stored for at least 1 year at –80 °C (without thawing) and used as biochemical "reagents" in the various assays.

Physiologically the bile salt export pump is expressed on the cholesterol-rich apical (canalicular) membrane of liver cells. Other ABC transporters, like ABCB1 (P-gp/MDR1), ABCC2 (MRP2), and ABCG2 (BCRP), expressed apically in many physiologically and pharmacologically important barriers including hepatocytes have been shown to be sensitive to the cholesterol content of the plasma membrane and are localized in cholesterol-rich microdomains.[9-13] It is known that lipid composition of insect and mammalian membranes significantly differ inasmuch as cholesterol content of Sf9 plasma membranes is approximately 5- to 10-fold lower than that of human plasma membrane.[14] In our earlier studies, the ABCG2 transporter containing Sf9 vesicles displayed an approximately 4- to 5-fold lower cholesterol level compared with vesicles prepared from human cells overexpressing the same protein. This difference markedly affects the ATPase and transport activity of the ABCG2 transporter.[11]

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In this study, we investigated the effect of membrane cholesterol level on the activity of the mBsep transporter. Cholesterol enrichment of mBsep-Sf9 vesicles resulted in a membrane product that is suitable for nonradioactive high-throughput screening for bile salt export pump interactors using the ATPase assay.

MATERIALS AND METHODS

Chemicals and biochemicals. ^{3}H-Taurochenodeoxycholate was kindly provided by Alan F. Hofmann (UCSD, San Diego, CA). The recombinant baculoviruses encoding wild-type mBsep and antibody against mBsep were kindly provided by Bruno Stieger, University Hospital (Zürich, Switzerland). Cholesterol complex of RAMEB was a product of Cyclolab (Budapest, Hungary). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise in the text.

Membrane preparation and cholesterol loading. The insect membrane vesicles were produced using Sf9 insect cells infected with recombinant baculoviruses encoding mBsep. Sf9 cells were cultured and infected with a recombinant baculovirus stock as described earlier.[7] In case of cholesterol-loaded membranes, cells were collected and treated with 10 × volume Hanks' balanced salt solution (HBSS; Gibco, Invitrogen, Carlsbad, CA) containing 1 mM cholesterol@RAMEB, for 30 min at 37 °C. After 30-min treatment, cells were washed and resuspended in HBSS. Purified membrane vesicles from cholesterol-treated and untreated baculovirus-infected Sf9 cells were prepared according to the method described by Sarkadi et al.[7] Membrane protein content was determined using the BCA method (Pierce Biotechnology, Rockford, IL).

Cholesterol treatment of membranes is indicated as "HAM" (high activity membranes) hereinafter.

SDS-PAGE and Western blotting. Proteins were separated using a 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) at 350 mA in a transfer buffer composed of 25 mM Tris, 192 mM glycine, and 15% (v/v) methanol, pH 8.3. The membrane was incubated in blocking buffer (5% nonfat dry milk powder and 0.5% bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20) for 2 h at room temperature. The membrane was then incubated with the primary antibody (rabbit anti-ratBsep monoclonal antibody recognizing mBsep as well) diluted 1:500 in blocking buffer for 2 h at room temperature. Membranes were washed 3 times with phosphate-buffered saline/0.05% Tween 20 at room temperature, followed by incubation with the secondary antibody, sheep anti-rabbit IgG-HRP, a horseradish peroxidase-conjugated species-specific whole antibody (Sigma-Aldrich) diluted 1:1000 in TPBS for 1 h at room temperature.

The membranes were subsequently washed as described above, and immunoreactive bands were visualized with ECL Western Blotting Detection System (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer, Budapest, Hungary).

Vesicular transport assay. Inside-out membrane vesicles were incubated in the presence and absence of 4 mM ATP, 50 µg of membrane protein per well at 37 °C, 5-min reaction time. The reaction mixture consisted of 2 µM taurochenodeoxycholate (TCDC), 5 mM Hepes-Tris, 0.1 M KCl, 0.01 M $Mg(NO_3)_2$, pH 7.4, with the test compounds in the concentrations indicated in the figures. The vesicles were separated by rapid filtration technique through class B glass fiber filters of a 96-well filterplate (Millipore, 1 µm). Filters were washed 5 times with 200 µl of ice-cold washing buffer (10 mM Tris-HCl, 0.1 M KNO_3), and the radioactivity retained on the filter was measured by liquid scintillation method. ATP-dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP.

ATPase assay. ATPase activity was measured in the presence and absence of sodium orthovanadate applying the protocol and reagents of the PREDEASY ATPase Kit (Solvo Biotechnology, Szeged, Hungary) according to the manufacturer's suggestions. Experiments were performed in 96-well cell culture plates (Costar 3585) in 50 µl of reaction volume. Membrane protein per well amounts, ATP concentration, and reaction time were optimized to reach the best signal-to-background ratio at 100 µM TCDC concentration and at 37 °C. Inhibitory type experiments were performed in the presence of 100 µM TCDC.

Data analysis. All experiments were run in duplicate. Data are presented as means ± standard deviations.

In case of the vesicular transport experiments, the Michaelis-Menten parameters, K_M and v_{max}, were determined by plotting the transport activity as a function of substrate concentration applying nonlinear regression (i.e., the Michaelis-Menten equation).

Relative transport values were calculated according to the following equation:

$$\text{Activity\%} = \frac{A - B}{C - B} \times 100$$

A: translocated amount of substrate in the presence of TA and ATP.

B: translocated amount of substrate in presence of TA.

C: translocated amount of substrate in the presence of solvent and ATP.

D: translocated amount of substrate in the presence of solvent.

For ATPase data, absolute vanadate sensitive activity values were calculated in units of nanomoles of liberated phosphate per milligram of protein per minute.

For the calculation of relative ATPase activities the following equation was used:

$$\text{Activity}\% = \frac{A - B}{(C - D) - (E - F)} \times 100$$

A: activity in the presence of a given concentration of the drug.

B: background vanadate insensitive activity in the presence of the drug (and 100 μM TCDC in inhibition studies).

C: maximal activation in the presence of 100 μM TCDC.

D: background, vanadate insensitive activity at maximal activation.

E: baseline activity.

F: background, vanadate insensitive activity.

To determine EC_{50} or IC_{50} values, a sigmoid dose-response curve was fitted onto the relative activity versus drug concentration plot by nonlinear regression:

$$\text{Relative activity (\%)} = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10(\text{Log}IC_{50} - c) * \text{HillSlope}).$$

PRISM 4.0 software (GraphPad Software Inc., San Diego, CA) was used for curve fitting and calculation of reaction parameters.

RESULTS

Expression of the mouse Bsep transporter in Sf9 insect cells

Presence of the mBsep protein in Sf9 membrane vesicles was shown by immunoblotting. The approximately 130-kDa band was consistent with an underglycosylated protein as observed also by other authors for this protein when expressed in insect cells.[15] Cholesterol upload of the insect membrane did not change the expression pattern of the transporter (**Fig. 1**).

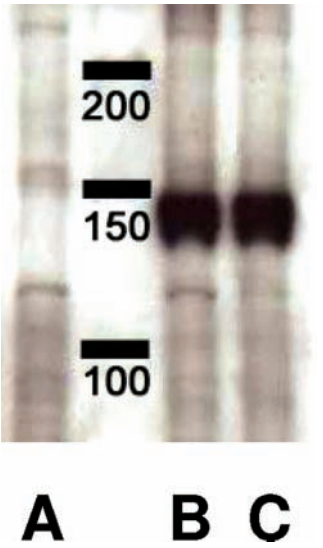

FIG. 1. Western blot image of (**A**) control Sf9 membrane (without mBsep expression), (**B**) mBsep-Sf9, and (**C**) cholesterol-loaded mBsep-HAM-Sf9 membranes. mBsep = mouse Bsep; HAM = high activity membranes; Sf9 = baculovirus-infected insect cell.

Cholesterol loading enhances mouse Bsep-mediated transport and substrate-induced ATPase activity of mBsep-Sf9 vesicles

Cholesterol loading was done as described earlier.[11] To investigate whether cholesterol loading affects transport kinetics of the transporter in mBsep-Sf9 inside-out vesicles, vesicular transport experiments were performed using ^{3}H-TCDC as a probe substrate. The accumulation of the radiolabeled substrate in the inside-out vesicles is ATP, concentration (**Fig. 2A**), and time dependent (data not shown). The transporter activities in mBsep-Sf9 and the cholesterol-loaded mBsep-HAM-Sf9 vesicles were compared. Cholesterol upload led to increased maximal velocity of the transport (967 ± 8 vs. 525 ± 5 pmol/mg/min) with a slight shift in the K_M from 8.6 ± 0.2 to 18.1 ± 0.3 μM (**Fig. 2A**).

In parallel with the TCDC transport, the ATPase activity of the transporter showed TCDC concentration dependence both in mBsep-Sf9 and in mBsep-HAM-Sf9 membranes (**Fig. 2B**). Maximal activation was observed at about 100 μM TCDC concentration. Cholesterol upload of the insect membrane resulted in a decrease of the basal vanadate sensitive activity and an increase of the maximal activation with the same EC_{50} value (16.5 μM) in both systems. No change of ATPase activity was observed in the presence of TCDC using β-gal containing Sf9 control membranes (data not shown).

Optimization of the mBsep-HAM ATPase assay parameters

ATPase assay parameters were optimized for mBsep-HAM-Sf9 to determine the parameter set with the best signal-to-background ratio. ATP concentration and protein concentration dependence were investigated at 37 °C and 100 μM TCDC. **Figure 3A** shows the TCDC-induced vanadate-sensitive ATPase activity as a function of ATP concentration. A saturation type response was detected with a v_{max} value of 21 nmol/mg of protein/min and a K_M of 0.6 ± 0.1 μM. Maximal ATPase activity was reached at about 2 mM. According to these results ATPase inhibition experiments were performed at 2 mM ATP concentration.

Time-dependence experiments were performed in the 5- to 30-min range (data not shown). After consideration of progress curve linearity as well as signal-to-background ratio, 15 min was chosen as the optimal reaction time.

ATPase activity was compared at various membrane protein concentrations in the presence and absence of sodium orthovanadate at

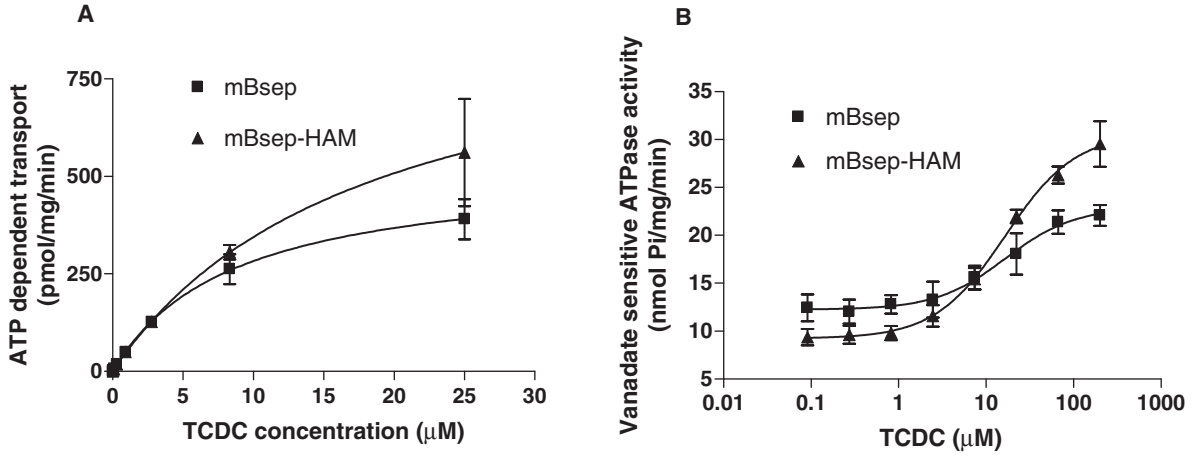

FIG. 2. (**A**) ATP-dependent transport of taurochenodeoxycholate (TCDC) into mBsep and mBsep-HAM-Sf9 inside-out vesicles. (**B**) Stimulation of the vanadate sensitive ATPase activity of mBsep-Sf9 and mBsep-HAM-Sf9 by TCDC. mBsep = mouse Bsep; HAM = high activity membranes; Sf9 = baculovirus-infected insect cell.

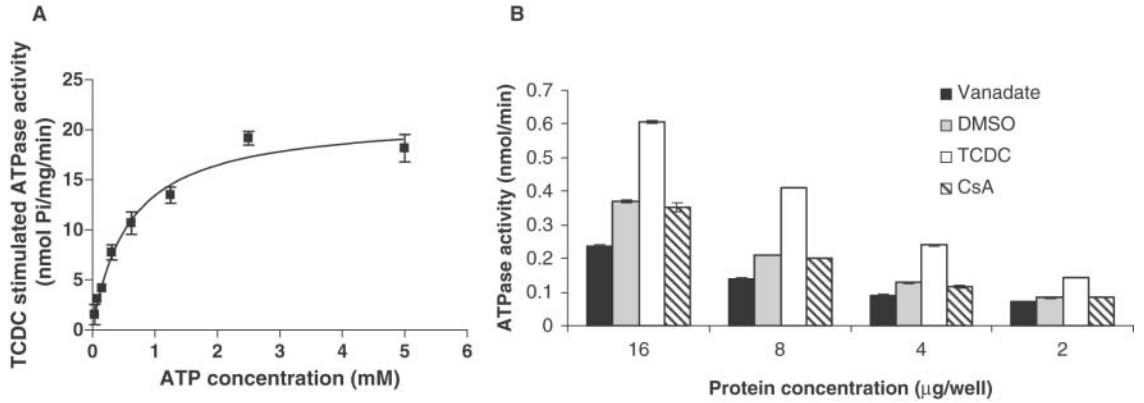

FIG. 3. (**A**) ATP concentration dependence of taurochenodeoxycholate (TCDC; 100 μM)–stimulated mBsep-HAM-Sf9 ATPase activity. (**B**) Vanadate insensitive (Vanadate), background (DMSO), drug-stimulated maximal (TCDC), and inhibited (TCDC + CsA) ATPase activities of mBsep-HAM-Sf9 membrane at different protein concentrations (μg/well). Vanadate-insensitive activity was measured in the presence of 1.2 mM sodium orthovanadate, background was determined in the presence of the solvent (2% DMSO), maximal activity was induced by 100 μM TCDC, and complete inhibition of the stimulated ATPase activity was reached by 100 μM cyclosporin A. mBsep = mouse Bsep; HAM = high activity membranes; Sf9 = baculovirus-infected insect cell.

15-min reaction time (**Fig. 3B**). Vanadate-insensitive and vanadate-sensitive background activities as well as maximal activities all increased with increasing membrane amount. The best signal-to-background ratio was observed at 8 μg of membrane protein per well concentration.

Parallel inhibition of mBsep transport and ATPase activity by cholestatic drug molecules

Various drug molecules, including ones reported to cause clinical cholestasis, were investigated using the mBsep-HAM-Sf9 membrane product. Effect on TCDC transport and TCDC-stimulated mBsep ATPase activity were measured in the vesicular transport assay and ATPase assay, respectively. Cyclosporin A, glybenclamide, rifamycin SV, ketoconazole, and troglitazone effectively inhibited the TCDC transport by mBsep and the coupled ATPase activity as well (**Fig. 4**). Rifampicin and clofazimine (not shown in the figure) did not interact significantly with the mBsep transporter. In general, IC_{50} values based on the mBsep-HAM-Sf9 ATPase data were (2 to 3 times) lower than IC_{50} values determined from the vesicular transport assay data. Human BSEP taurocholate (TC) transport experiments were performed at 2 μM total TC concentrations. Data show a nice rank order correlation with mouse Bsep ATPase and vesicular transport results because overall ranking of IC_{50} values is very similar in the 3-experiment system (Table 1).

Table 1. Correlation between TCDC-Stimulated ATPase Inhibition, TCDC Transport Inhibition of the Mouse Bsep Transporter, and Human BSEP Transporter–Mediated TC Transport Inhibition Data (IC_{50} Values)

	IC_{50} Values (μM)		
Compound	*mBSEP TCDC-Stimulated ATPase*	*mBSEP TCDC Vesicular Transport*	*hBSEP TC Vesicular Transport*
Cyclosporin A	3.5	7.2	2.0
Ketoconazole	33	>100	5.0
Troglitazone	33	97	8.0
Glybenclamide	45	120	15
Rifamycin SV	62	105	20
Rifampicin	>100	No interaction	50

TCDC = taurochenodeoxycholate; mBSEP and hBSEP = mouse and human bile salt export pump; TC = taurocholate.

DISCUSSION

The mBsep transporter is a 1321-amino-acid protein exhibiting a molecular weight of 160 kDa[15] and shows 80% to 85% similarity to human BSEP.[8] Expression of mBsep in Sf9 insect cells results in a transporter protein with proper function, but lower molecular weight of about 130 kDa (**Fig. 1**). This difference is due to the insufficient glycosylation of the protein in insect cells,[16] a known phenomenon in this expression system.[17]

A comparative study on rat Bsep transporter function in Sf9 vesicles and in isolated rat canalicular plasma membrane vesicles revealed comparable K_M values and the same rank order of transporter preferences for different bile salts. However, transport rates were significantly lower in the Sf9 system than in rat canalicular membrane vesicles (30 ± 7 vs. 65 ± 9 pmol/mg of protein/min.[16] It must be noted that these data were not corrected for the inside-out vesicle content.

Species specificity studies revealed that mouse Bsep shows significantly greater transport rate than the human and the rat ortholog when expressed in the Sf9 system.[8] The transport activity of mBsep was high enough to be detected in the ATPase assay.[8]

Earlier it was shown for the ABCG2 transporter that lack of glycosylation did not affect the transporter activity, but cholesterol level of the membrane had a serious impact on ABCG2 function[11] inasmuch as it was shown to potentiate BCRP activity.[11,12] It was hypothesized that potentiation by cholesterol may reflect a physiologically relevant regulatory role of cholesterol-rich microdomains. Indeed, it was subsequently shown that BCRP localizes to cholesterol-rich raft and caveolar membrane domains.[13]

In this study we are showing that the mBsep protein is also sensitive to the cholesterol content of its membrane environment. Transport rate of the reporter substrate (TCDC) and the corresponding ATPase activity were both increased by cholesterol upload of the Sf9 membranes. The elevated cholesterol level leads to an approximately 2-fold increase of v_{max} in the vesicular transport assay. Cholesterol loading results in a decrease of the basal ATPase activity of the transporter as well as an increased substrate-induced ATPase activity, resulting in a better signal/ background ratio and a wider dynamic range.

The mechanism of the cholesterol effect on mBsep transporter activity is yet unexplored. The canalicular membrane is a cholesterol-rich membrane, and transporters functioning in the apical membranes such as P-gp, MRP2, and BCRP have all been shown to localize to cholesterol-rich domains of the membranes.[9,10,13] Therefore, local cholesterol concentration may act as a direct regulator of mouse Bsep function.

The ATPase activity of the mBsep-HAM-Sf9 vesicles is time, temperature, and ATP dependent. After the assay parameter set has

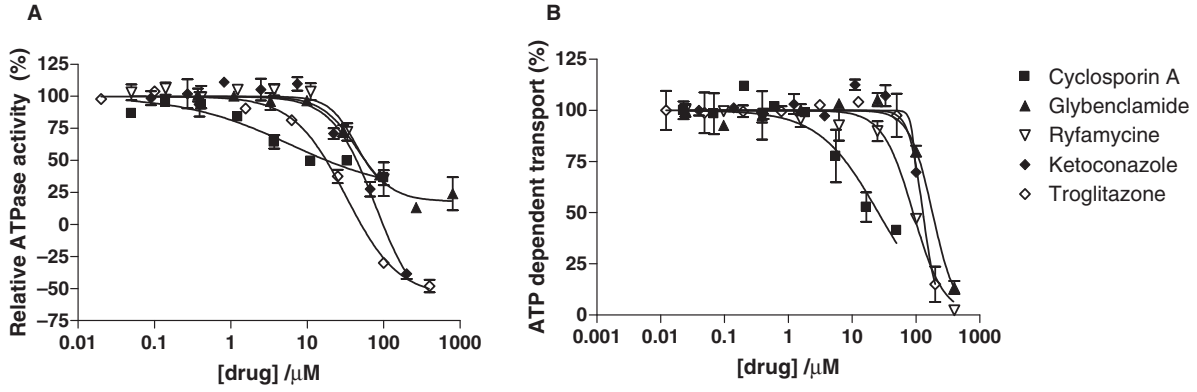

FIG. 4. (**A**) Inhibition of taurochenodeoxycholate (TCDC)–stimulated mBsep-HAM-Sf9 ATPase activity and (**B**) inhibition of mBsep-mediated TCDC transport by the selected drug molecules using mBsep-HAM-Sf9 vesicles. Inhibition curves are compared in a relative scale. mBsep = mouse Bsep; HAM = high activity membranes; Sf9 = baculovirus-infected insect cell.

been optimized (**Figs. 2, 3**), the results obtained from the mBsep-HAM-Sf9 ATPase assay were compared with those from the mBsep-HAM-Sf9 vesicles in the vesicular transport assay (**Fig. 4**). The selected drug molecules are known to cause BSEP-related cholestasis[18] and were identified as clear mBsep interactors in both assays. Furthermore, the ATPase assay proved to be more sensitive compared with vesicular transport, because slightly lower IC_{50} values were determined by this method.

A good rank order correlation was found between IC_{50} values determined in the TCDC-driven mBsep ATPase assay as well as the hBSEP vesicular transport assay using TC as a substrate.

BSEP transporter plays a key role in canalicular bile salt transport and in regulating the bile salt concentration in the hepatocytes and eventually in the systemic circulation. Identification of BSEP inhibitors, which reduce bile salt secretion thus causing cholestasis, is crucial in the early phase of drug discovery. Membrane-based rodent transporter studies are promising tools for prediction of human interactions.[5] Albeit, literature data suggest that hBSEP exhibits a wider substrate specificity than the rat analog.[18] The current transport assays, whether done in vesicles, cell monolayers (e.g., hepatocytes), or through tissue perfusion assays, all require fairly cumbersome, nongeneral analytical methods like LC/MS or the application of radiolabeled compounds. In contrast, the mBsep-HAM-Sf9 ATPase is a simple, high-throughput, sensitive, nonradioactive assay designed to detect drug-Bsep interaction. It can complement the human BSEP–mediated TC transport inhibition studies and the in vivo rodent studies.

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