Mitigating the inhibition of human Bile Salt Export Pump by drugs: opportunities provided by physicochemical property modulation, in-silico modeling and structural modification


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List of non-standard abbreviations: BSEP, human bile salt export pump; Bsep, non-human bile salt export pump; SVM, support vector machine; PLS, partial least square; ABC transporter, ATP-binding cassette transporter; DILI, drug induced liver injury; QSAR, quantitative structure-activity relationship; RF, random forest; PFIC, progressive familial intra-hepatic cholestasis; BRIC, benign recurrent intra-hepatic cholestasis.
Abstract

The human Bile Salt Export Pump (BSEP) is a membrane protein expressed on the canalicular plasma membrane domain of hepatocytes which mediates active transport of unconjugated and conjugated bile salts from liver cells into bile. BSEP activity therefore plays an important role in bile flow. In humans, genetically inherited defects in BSEP expression or activity cause cholestatic liver injury and many drugs which cause cholestatic drug induced liver injury (DILI) in humans have been shown to inhibit BSEP activity in vitro and in vivo. These findings suggest that inhibition of BSEP activity by drugs could be one of the mechanisms which initiate human DILI. To gain insight into the chemical features responsible for BSEP inhibition, we have used a recently described in vitro membrane vesicle BSEP inhibition assay to quantify transporter inhibition for a set of 624 compounds. The relationship between BSEP inhibition and molecular physicochemical properties was investigated and our results show that lipophilicity and molecular size are significantly correlated with BSEP inhibition. This data set was further used to build predictive BSEP classification models through multiple QSAR modeling approaches. The highest level of predictive accuracy was provided by a support vector machine model (Accuracy=0.87, Kappa=0.74). These analyses highlight the potential value that can be gained by combining computational methods with experimental efforts in early stages of drug discovery projects to minimize the propensity of drug candidates to inhibit BSEP.
Introduction

The pharmaceutical industry continues to face high attrition rates throughout the entire drug discovery and development process, with only 8% of all drug candidates that enter Phase I studies progressing to the market (FDA white paper 2004; Schuster et al., 2005). A major cause of compound attrition in drug development is toxicity (Kramer et al., 2007; Greaves et al., 2007). The financial burden of safety related dropouts can reach many hundreds of million dollars in late stages of drug development (DiMasi et al., 2003). Consequently, it is important to identify and mitigate potential compound related safety issues as early as possible during the drug discovery process.

One important cause of drug toxicity in humans is drug induced liver injury (DILI). This is a major cause for attrition in clinical trials, failed drug registration and withdrawal of marketed drugs (Abboud and Kaplowitz, 2007). For a few drugs, for example acetaminophen (Wallace 2004), DILI is a dose dependent and reproducible process which occurs in humans only following accidental or deliberate over-dosage and is reproducible in animals (Pirmohamed et al., 1998). However many drugs cause DILI only infrequently or very rarely in humans, which is not overtly dose dependent and is not reproducible (Zimmerman 1978). The most common patterns of clinical presentation of DILI in humans are defined as either hepatocellular (i.e. primarily affecting hepatocyte function), cholestatic (primarily affecting the biliary system) or mixed hepatocellular/cholestatic (Mumoli et al., 2006). The underlying mechanisms are complex and include both compound related properties and factors which are specific to individual susceptible patients (Thompson et al., 2011). A variety of compound related DILI risk factors have been identified or proposed. These include formation of chemically reactive metabolites, mitochondrial impairment, potent cell cytotoxicity and inhibition of the Bile Salt Export Pump (BSEP) (Greer et al., 2010; Dawson et al., 2012).

BSEP (encoded by the ABCB11 gene) is a liver specific ABC transporter which is expressed on the canalicular domain of the hepatocyte plasma membrane and which mediates the
active secretion of monovalent bile acids or salts into the bile canaliculi (Gerloff et al., 1998; Noé et al., 2002; Byrne et al., 2002). Mutations in the ABCB11 gene occur via codon deletions, insertions and various point mutations. These have been shown to cause either progressive familial intrahepatic cholestasis type 2 (PFIC2), which is a very rare but severe form of cholestatic liver disease that results in fatal liver failure unless treated by liver transplantation, or a milder form of liver injury termed benign recurrent intrahepatic cholestasis type 2 (BRIC-2) (Davit-Spraul et al., 2009). Many drugs which cause cholestatic DILI in humans have been found to inhibit the activity of human BSEP, and its rat analogue Bsep, \textit{in vitro}. In several cases inhibition of rat Bsep activity \textit{in vivo} has also been demonstrated (Kostrubsky et al., 2006; Fattinger et al., 2001). Furthermore, it has been shown recently that drugs which cause cholestatic DILI in humans exhibit markedly greater potencies and frequencies of BSEP or Bsep inhibition \textit{in vitro} than drugs which cause hepatocellular DILI or drugs that do not cause DILI (Dawson et al., 2012; Morgan et al., 2011). These findings suggest that mitigating BSEP inhibition may help reduce the likelihood of DILI for humans.

However, applying high volume screening for BSEP inhibition in a drug discovery project would be cost intensive and time consuming and potentially could delay the rate of progression of the project. A more desirable strategy would be to combine \textit{in vitro} BSEP screening with \textit{in silico} BSEP predicting models (Saito et al., 2009). In the present study we have investigated the use of various computational algorithms to build a BSEP inhibition model for a set of 624 chemically diverse compounds. The effect of these compounds on BSEP activity was quantified \textit{in vitro} using a membrane vesicle assay. An accurate classification model for BSEP inhibition has been developed, which can be used for library profiling. The relationship between some molecular physicochemical properties, such as lipophilicity and size, and propensity of BSEP inhibition were also highlighted in this analysis.
Materials & Methods

Experimental Measurement

All standard reagents and chemicals were purchased from Sigma-Aldrich Company Ltd (St. Louis, MO, USA), Merck (Hohenbrunn, Germany) or WAKO (Osaka, Japan) and were of the highest purity available. [3H]-taurocholate was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Baculoviruses expressing human BSEP (ABCB11) were provided by Bruno Stieger (Stieger et al., 2000; Noé et al., 2002). Test compounds (>95% purity) were provided by the Compound Management Group, Discovery Sciences, AstraZeneca, Alderley Park.

hBSEP (ABCB11) was expressed in Spodoptera frugiperda Sf21 insect cells from which inside-out membrane vesicles were prepared as described previously (Dawson et al., 2012) with the modification that at the end of the isolation procedure vesicles were resuspended in buffer containing 50 mM sucrose, 10 mM HEPES/Tris, pH 7.4 and protease inhibitor tablets (Roche, Basel, Switzerland). Inhibition of hBSEP mediated ATP-dependent transport of [3H]-taurocholate into vesicles was measured by a rapid filtration method as described previously (Dawson et al., 2012). Briefly, 60 μg hBSEP vesicles were incubated with test compound or dimethyl sulfoxide (DMSO) vehicle and 0.5 μM [3H]-taurocholate for 5 minutes at 37°C in buffer containing 5 mM ATP, 10 mM MgCl2, 15.0 mM HEPES/Tris pH 7.4, 142 mM KNO3, 158 mM sucrose, 12.5 mM Mg(NO3)2. For each compound, three independent experiments were performed with triplicates in each experiment for each compound test concentration (10, 30, 100, 250, 500, 1000 μM). The DMSO concentration in all reactions was 2% (v/v). The transport reaction was stopped by addition of vesicles to stop buffer containing 50 mM sucrose, 100 mM KCl, 10 mM HEPES/Tris pH 7.4, 5 mM EDTA, and 0.1 mM taurocholate. [3H]-taurocholate uptake into vesicles was measured by determining the counts per minute (CPM) using a TopCount NXT (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). CPM values determined for reactions containing AMP were subtracted from reactions containing ATP to determine the ATP-dependent uptake activity. The
percentage of uptake activity relative to DMSO vehicle control (100%) was determined for each compound test concentration. IC$_{50}$ values were calculated with non-linear regression for a sigmoidal dose-response using the following formula:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{\text{log}_{10}(\text{IC}_{50}) - X}}$$  \hspace{1cm} (1)$$

where X is the common logarithm of the concentration, Y is the response, nH is the variable Hill slope, Bottom is 0 and Top is 100.

**Data Set**

The BSEP data set for QSAR modeling comprises 624 internal compounds and external reference compounds (Supplemental Table S4). A BSEP IC$_{50}$ of 300 µM was used as a threshold value for classifying compounds, i.e. compounds with a geometric mean IC$_{50}$ value less than 300 µM were regarded as BSEP active compounds (labeled as POS class), otherwise they were regarded as BSEP inactive compounds (labeled as NEG class). An IC$_{50}$ value of 300 µM determined in the membrane vesicle model has previously been found to be a useful operational threshold to identify drugs which are associated with cholestatic or mixed hepatocellular/cholestatic liver injury in human (Dawson et al., 2012). An in-house perl script was run to randomly split the BSEP data set into a training set and a test set with the ratio of 7:3 resulting in a training set of 437 compounds (231 POS, 206 NEG) and the test set of 187 compounds (94 POS, 93 NEG).

**Physical property dependencies**

Logistic regression analysis of the relationships between molecular properties and BSEP activity were conducted using JMP8.0 software (SAS Institute Inc., Cary, NC, USA) by estimating the probability of a compound being free from BSEP inhibition as a smooth function of a single parameter (e.g., $\log_{10}$ molecular weight, calculated ClogP (BioByte Corp., Claremont, CA, USA), calculated logD and acid dissociation constants (Advanced Chemistry Development, Inc., Toronto, ON, Canada)). The statistical significance of probabilities based
on the relationship as compared with the background probabilities were evaluated using a Chi-square test statistic \( G^2 \) evaluated as in equation 2.

\[
G^2 = 2 \left( \sum - \ln p(\text{background}) - \sum - \ln p(\text{relationship}) \right)
\]  

(2)

The observed significance probability (or p-value) for the Chi-square test is the probability of obtaining, by chance alone, a Chi-square test statistic greater than the one computed. Probabilities of <0.05 were considered significant.

\( R^2 \) is the metric used to quantify how well each molecular property predicts BSEP activity, and is defined according to equation 3.

\[
R^2 = \frac{\sum - \ln p(\text{background}) - \sum - \ln p(\text{relationship})}{\sum - \ln p(\text{background})}
\]  

(3)

\( R^2 \) ranges from 0 to 1: a value of 0 would indicate no benefit in the use of a given molecular property to classify BSEP activity, whereas an \( R^2 \) of 1 would indicate perfect classification.

**Modeling methods**

Multiple approaches for building a classification model of a compound’s BSEP inhibition activity were investigated. The first and simplest approach used a basic recursive partitioning (RP) algorithm, based solely on the molecular weight and calculated lipophilicity of the structures. The partition scheme was built using JMP software. In contrast to the 1D physical properties modeling above, the scheme was derived from 437 training set compounds and evaluated using the randomly selected 187 test set compounds. Automatic parameter selection was applied at each point in the scheme to maximize the significance of the split. Partitioning was performed until \( R^2 \) (equation 3) for the test set ceased to improve, resulting in a total of four partitions, two for each parameter.

To further refine our description of the structure-activity relationships surrounding this transporter, two more elaborate sets of descriptors were calculated for all 624 molecular
structures. A set of 196 2D/3D descriptors (referred to as the AZdesc set), including descriptors for molecular size, lipophilicity, hydrogen bonding, electrostatics and topology were calculated with an in-house program. The descriptors themselves are described elsewhere (Paine et al., 2010; Bruneau 2001; Katritzky et al., 1998). A second descriptor set (referred to as the AZFP_AZtop14 set) comprises a combination of fingerprint based descriptors including the in-house developed Ghose-Crippen atom type fingerprints (Ghose and Crippen, 1987) and a set of functional groups fingerprints (Arnold et al., 2004). This collection of structural descriptors was further supplemented with a subset of the AZdesc set, comprising an additional 14 descriptors to encode molecular bulk properties like lipophilicity, size, ionic states, etc.

Partial least squares (PLS), and two non-linear machine learning methods – support vector machine (SVM) and random forest (RF) – were used to build the classification models based on the AZdesc set. For the AZFP_AZtop14 set, which was mostly focused on structural fingerprints, only the two non-linear techniques (RF and SVM) were used. All three methods are implemented in the in-house machine learning package AZOrange (Stålring et al., 2011), which is an extension of the open source package Orange (Orange official web site). Classification performance measurements based on the confusion matrix were generated to assess the quality of the classification across all six approaches (Supplemental Table S2).

**WizePairZ methodology**

Matched molecular pairs analysis (MMPA) (Leach et al., 2011) was performed on the full set of 624 compounds using the WizePairZ algorithm (Warner et al., 2010). In order to deconvolute the intrinsic effects of structural transformations from their associated effect on molecular properties, two variables were considered in the analysis: BSEP activity, expressed as -log_{10}(IC_{50}) (or pIC_{50}) and ClogP. As in our original study, the mean change in activity for each molecular pairing was plotted as a function of the change in lipophilicity,
allowing those transformations which link the anticipated trend with physicochemical properties to be easily identified.

**Results**

**Measurement of BSEP inhibition by the tested compounds**

Typical concentration response curves of test compound effects on BSEP activity are shown in Fig 1, which includes data obtained with a compound that did not inhibit BSEP (acetaminophen), a compound which exhibited potent BSEP inhibition (bosentan) and two compounds which exhibited less potent inhibition (bezafibrate and labetalol). Experiment results are the mean ± standard error of the mean (S.E.M.) from three separate test occasions. For each compound an IC$_{50}$ value was calculated and these values are summarized in the supplemental data. As can be seen from Fig. 2, 240 compounds did not exhibit BSEP inhibition. For 269 compounds, BSEP inhibition IC$_{50}$ values were observed ranging between 1000 and 10 $\mu$M, i.e. were within the range of tested compound concentrations. An additional 115 compounds exhibited very potent BSEP inhibition, which was defined as IC$_{50} < 10 \mu$M.

**Molecular property dependencies**

The influence of several molecular properties on the likelihood of BSEP inhibition with an apparent IC$_{50}$ value of <300 $\mu$M is displayed in Figure 3. The line of fit (blue) in each plot of Figure 3 is interpreted as the probability ($y$) of a data point appearing below the line (i.e. being free of BSEP inhibition for a given property value ($x$)). The scattered dots in the plots are the tested compounds and their color refers to the class (POS/NEG class) that they belong to. It seems that the likelihood of a compound inhibiting BSEP activity is highly dependent on its molecular weight and calculated lipophilicity. Any compound with a molecular weight of greater than 309 is more likely to exhibit a BSEP inhibition than not and compounds whose molecular weight below this threshold become more likely to be free from
BSEP inhibition (Figure 3d). The strong statistical significance of the relationship with molecular weight (p<0.0001) and reasonably high $R^2$ (0.22) dictate that changes in the likelihood of activity occur fairly sharply. Calculated octanol/water partition coefficients provide an even more powerful mean of classifying the compounds into BSEP inhibition actives vs. BSEP inactives ($R^2 = 0.29$). A ClogP of 2.27 marks the point at which the balance shifts from a compound being more likely to be free of BSEP inhibition to becoming a more likely BSEP inhibitor (Figure 3a).

In an attempt to identify an even more discriminatory lipophilicity metric, we investigated the impact of calculated logD on BSEP inhibition potency below the selected threshold value. While this approach demonstrated less utility than the analysis based on ClogP described above (data not shown), we noticed an interesting improvement in classification with the use of ACDlogD at pH 6.5 over the same calculation based on pH 7.4 ($R^2 = 0.19$ and 0.17 respectively). This indicates that while Biobyte’s logP algorithm provides the more effective classifier in this case, the ionization state of the active species may also play a role. This led us to investigate further the influence of pKa on BSEP inhibition (Figure 3b and 3e). The two hypotheses to be tested were: the reduced pH was causing weak acids to appear more lipophilic to the binding site thus improving their BSEP inhibition potency classification; or reduced pH caused weak bases to appear more hydrophilic, thus reducing their potency of BSEP inhibition. The data would indicate the latter to be true, as while there was no significant relationship with ACD pKa (Acid 1) (p=0.2751), a significant decrease in the likelihood of BSEP inhibition with increasing basicity was observed (p=0.0002).

To seek additional support for the argument that the formation of cations disfavors BSEP inhibition potency, we classified all 624 compounds into five ion class categories: neutrals, bases, acids, cations and zwitterions (Figure 4). Across the full dataset the distribution between BSEP inhibition potency above and below the threshold value was almost completely even, and this was mirrored across the acids, bases and neutral compounds.
However, it is notable that cations formed the least likely class of BSEP inhibitors, with only two “active” compounds and ten “inactive” compounds (17%). The final paired logistic regression analysis compared the hydrogen bond donor/acceptor characteristics of the dataset (Figure 3c and 3f). Here, the only significant relationship was with the hydrogen bond donor count, where molecules with increasing number of donors were at reduced risk of BSEP inhibition (p<0.0001). There was no significant relationship between BSEP inhibition category and the acceptor count.

**BSEP classification model**

In an effort to devise a predictive *in silico* model of BSEP inhibition, we embarked on the exploration of a number of approaches. Since molecular weight (MW) and lipophilicity are useful predictors of BSEP inhibition, as presented in the previous section, we decided to build a classification model based on a recursive partition (RP) algorithm regarding these two properties to serve as a baseline model (RP_ClogP_MW, Figure 5). At the top of the scheme, the first partition occurs at a ClogP threshold of 1.697, which provides reasonable separation between BSEP actives and inactives on the second tier. The second partition takes place to the far left of the scheme (on the more lipophilic compounds), such that those with molecular weight above and below 296.2 are separated. This third tier is where the recursion ends for 204 of these compounds, as those with molecular weight > 296.2 are deemed almost certainly active with a probability of 91% (red section in the contour plots of Figure 5b). Classification of the 78 lower weight compounds within this more lipophilic group is more ambiguous, but generally favors inactive compounds with a probability of 66% (light blue section in the contour plots of Figure 5b).

To the right of the scheme, the situation for the less lipophilic compounds is somewhat more complex. In this category where ClogP is < 1.697, compounds are most likely to be inactive, and overall, this does not change either way with the third tier partitioning at a ClogP of 0.59. Below a ClogP of 0.59, the training set compounds are almost unanimously inactive (95
compounds, 98% inactive). However, the fourth and final tier shows an interesting effect, where for the compounds of intermediate lipophilicity ($0.59 \leq \text{ClogP} < 1.697$) the classification returns to being dependant on molecular weight (mid-blue/light-red area of figure 5b). In summary, with high lipophilicity and molecular weight $\geq 296.2$, or alternatively, intermediate lipophilicity and molecular weight $\geq 360.4$, compounds are more likely to be BSEP inhibitors than not.

Although a highly predictive baseline model (RP model) could be obtained using only ClogP and MW descriptors, we sought to expand the number of descriptors included in the RP model to further refine the classifications through the identification of more subtle effects. Several BSEP classification models were built, which allowed comparison of two nonlinear machine learning algorithms with a linear algorithm and comparison of bulk property based descriptors (AZdesc) with structural descriptors (AZFP_AZtop14). The models were built using a training set of 437 compounds (231 POS, 206 NEG) and then evaluated using a test set of 187 compounds (94 POS, 93 NEG). The analysis of the test set using the different models is summarized in Table 1 and the details of individual metrics for measuring model performance are presented as supplemental data (Table S1). Of the six models evaluated, the combination of the SVM algorithm and the AZdesc set (SVM_AZdesc) showed the best performance across most of the parameters (Table 1). The probabilities of this model correctly classifying compounds predicted to be positive and negative for BSEP inhibition were 0.85 and 0.90, respectively. Thus, if a compound was predicted positive by the model it stood an 85% chance of causing BSEP inhibition with an IC$_{50}$ of $<300$ μM. The Kappa value for this SVM_AZdesc model was the highest (0.74), which provides an indication of the true accuracy of the model since it takes the probability of obtaining such agreement by chance into account. It is also interesting to note that the linear PLS model showed good negative precision and its sensitivity was almost as good as that of the SVM_AZdesc model.
Comparing the results from the two descriptor sets, it was clear that the non-linear models which combined fingerprint and a subset of the bulk property descriptors (AZFP_AZtop14) generally performed no better than the same methods using the AZdesc set. For this reason, these two models will not be discussed further. In comparison to the RP model, the SVM_AZdesc model has a better accuracy in terms of sensitivity and negative precision (0.90 vs. 0.84 in both cases). The common denominator in both these metrics, and hence the driving force behind this improvement, was the false negative count (compounds predicted negative but measured positive) which was reduced from fifteen to nine.

To check the robustness of these model generation strategies, a five-fold cross validation analysis was carried out on all 624 compounds for the non-linear and PLS modeling methods. The performance metrics for cross validation analysis is listed in the supplemental data (Table S2). Although SVM_AZdesc method still performs best in general, the difference between various non-linear modeling methods is actually very small and all non-linear methods perform better than the PLS_AZdesc method.

In situations such as this, where lipophilicity of compounds has such a strong bearing on their effect on BSEP activity, it is desirable to identify subtle structural changes which reduce activity without compromising other molecular properties. This is especially useful in cases where a chemical series is in late-stage optimization and working with limited opportunities to modify the physicochemical properties. WizePairZ was designed for precisely this purpose and its application to our BSEP data set identified a total of 103 unique molecular transformations when including four bonds of the local environment (counting each transformation and its reverse just once).

Of the four salicylic acid analogues depicted in Figure 6a, it was only Mesalazine (5-amino-2-hydroxybenzoic acid) that exhibited \textit{in vitro} BSEP inhibition. This compound was neither the most lipophilic in the group (ClogP = 1.06 vs. 2.44) nor that with the highest molecular weight (153 vs. 154), although WizePairZ was able to identify a structural motif which correlated with
reduced BSEP inhibition activity. It is the 5-amino group, which is absent from the remaining three structures. The presence of the amine marks this compound as the only one theoretically capable of forming zwitterions at physiological pH, which raises the intriguing possibility that this compound could be binding to BSEP in a higher-order state, analogous to that observed in its crystalline form (Banić-Tomišić Z, 1997). To explore this observation further it would be important to test 5-amino analogues of salicylic amide and thiosalicylic acid. Our analysis of BSEP inhibition activity across different ion classes revealed a low frequency of BSEP inhibitors amongst the zwitterionic compounds tested, which suggests that this may not be a common feature of compounds which inhibit the transporter.

We determined that the compound to the right of Figure 6b (Proxicromil) was a relatively potent BSEP inhibitor with an IC_{50} of 30 µM. Its matched molecular pair in the centre (from which only the hydroxyl group has been removed) is remarkable in that its BSEP inhibition activity was reduced by around 20-fold. This suggests that the hydroxyl group plays a role in the interaction of this compound with the transporter. Further support for this interpretation is provided by the BSEP inhibition potency of the structure on the left, in which three aliphatic carbon atoms had been removed and the hydroxyl group had been re-introduced, resulting in an overall modest decrease in ClogP when compared with the non-inhibitory compound in the centre. Previous attempts to model BSEP structure-activity relationship (SAR) identified hydroxyl counts as promoting inhibition (Saito et al., 2009). In their case the group was specifically bonded to aliphatic carbon atoms, whereas for our data the group was linked to an aromatic carbon atom.

Perhaps the striking SAR lies amongst the compounds paired with Mianserin, which are depicted in Figure 6c. Conversion of Mianserin to Mepyramine on the left reduces lipophilicity slightly, while Antazoline to the right features an increased ClogP although it is likely that this is offset by the increased basicity of the dihydroimidazole over the corresponding piperazine. The fairly subtle differences between the physicochemical properties of the three compounds
can be contrasted with the very large observed differences in BSEP inhibition activities and suggest that inhibition of BSEP activity by Mianserin is strongly influenced by the conformation of the molecule.

The final pair of structures provides what is potentially the most surprising result from the WizePairZ analysis (Figure 6d). The conversion of the 2-methylindole (Mepindolol) to its naphthyl equivalent (Propranolol) induced a near 30-fold drop in BSEP inhibition activity, despite an increase in ClogP of approximately 0.6. While this suggests that the indole in Mepindolol makes a significant contribution to BSEP inhibition, there were no further pairs of the active species in the dataset to corroborate or invalidate this finding.

It should be noted that the absence of multiple observations makes statistical analysis on the potential to generalise these transformations impossible. Thus the examples illustrated are provided on an anecdotal basis, intended to fuel hypothesis formulation.

**Discussion**

**Inhibition of BSEP is favored by high molecular weight hydrophobes**

How lipophilicity and molecular weight might relate to toxicity or adverse events has been reported earlier (Leeson and Springthorpe, 2007), although the precise property ranges over which this relationship takes hold are surprising. A recent study on 85 marketed drugs demonstrated the correlation between DILI in humans and *in vitro* BSEP inhibition suggesting 300 µM as a useful operational threshold (Dawson et al., 2012). Using a 300 µM cutoff, we see a very sharp onset of BSEP inhibition within a lipophilicity and molecular weight range that is typically considered desirable for many drug discovery projects (Lipinski et al., 1997; Wenlock et al., 2003). With over 90% chance of BSEP inhibition activity for compounds with ClogP >1.7 and MW >300, we would consider routine screening for BSEP inhibition of promising chemical series to be a prudent strategy. Thus, we would suggest a general guidance when working with compounds on the borderline of BSEP activity to reduce
molecular weight and/or lipophilicity. The contour plot (Figure 5b) indicates how a relatively modest reduction in either of these properties may be enough to tip the balance in the investigator's favor. Where tolerated by the primary pharmacological target, incorporation of increasingly basic functional groups is likely to be beneficial in reducing the level of BSEP inhibition, as is the systematic replacement of hydrogen bond acceptor features with donors. In particular circumstances, where compounds exhibit more significant inhibition and the proposed route of administration for the compound allows, introducing cationic groups such as quaternary amines appears to be an almost certain way of eliminating activity.

We have previously constructed a homology model of BSEP (unpublished) taking the X-ray structure of P-glycoprotein (Pgp) as a template (Aller et al., 2009) (Supplemental Figure S3). It was used to align the findings reported here with structural information available on this transporter. Aller et al. described two portals, allowing access to an inner cavity from either side of the transporter from within the inner leaflet. The strong dependence on ClogP observed for our compounds is fully consistent with this hypothesis, as the more lipophillic members of the dataset are more likely to become embedded within the vesicle, where they can then gain access to the BSEP cavity. Conversely, the more polar compounds in the set appear to be unable to access this site, regardless of their molecular weight. The correlations with calculated pKa and hydrogen bonding counts both point to the pore being an unfavourable environment for polar hydrogen atoms. This is likely due to the presence of the histidine and two arginine residues in the transmembrane domain (His$^{72}$, Arg$^{223}$ and Arg$^{1034}$), and the apparent absence of any anionic residues in this region (Supplemental Figure S3a), consistent with an active site environment that has evolved to facilitate the transportation of cholesterol derived acids from hepatocytes into bile.

**Integrating computational and experimental approaches**

Our proposal for the integration of computational and experimental approaches in BSEP screening is based on our experience with the QSAR model developed above
(SVM_AZdesc). If we were to prioritize only those compounds with high molecular weight and lipophilicity for experimental testing, there is a danger that active inhibitors will fail to be identified at an early stage (affecting 16% of active compounds). Our results show how the additional information encoded by this model facilitates the correct identification of positive compounds from the low molecular weight category.

Here we attempt to expose deeper insight into the origin of this improvement giving drug designers the confidence to use the model in prioritizing compounds for experimental testing. In Figure 7 we show ten structures intended to illustrate from where the crucial improvements originate. None of these compounds were involved in training the model. The first two compounds in this figure, Benzylpenicillin and Buphenine, contradict our hypothesis by exposing flaws in the model. Although they would both fall into the ‘high’ ClogP/molecular weight category, they are incorrectly classified as inactive by the model. However, these deficiencies are outweighed by the compounds in Figure 7b where exactly the reverse is true. All eight of these compounds would be incorrectly classified according to their physical properties alone, as according to the scheme no compound with a molecular weight below 296.2 can ever be classified as positive, regardless of its lipophilicity. Here we see how the additional information in the AZdesc set enables the model to correctly identify these compounds as active inhibitors of BSEP. Regrettably, the nature of some of the descriptors and the modeling method employed confine this computational approach to a black-box with limited interpretability. Future work should be focused on further developing transparent and predictive BSEP models which are beyond the lipophilicity and size factors.

**Lipophilicity dependence and inhibition of multiple transporters**

A preference for large lipophiles is an emerging theme from published analyses of cation transporter inhibition (Karlgren et al., 2012; Kido et al., 2011), with previous studies also demonstrating reasonable summaries of experimental datasets using only a handful of
simple descriptors (Ahlin et al., 2008). In contrast, it was reported that lipophilicity has very little effect on P-glycoprotein (P-gp) efflux (Hitchcock, 2012). In this study, only 17% of compounds remain misclassified following the simple partitioning scheme, yet they are the ones potentially holding the most information when it comes to understanding the specific recognition features that are important for BSEP. According to the published trends we would not expect small, polar compounds to inhibit other transporters, making the BSEP actives in this category ideal tool compounds for further study. Ultimately, the identification (and possibly even design) of compounds which are able to discriminate between proteins in this class will be important to fully understand the consequences of specific transporter inhibition in vivo.

**Limitations of the current analyses**

Currently, the relationship between in vitro potency of BSEP inhibition in membrane vesicle assays and compound concentrations within human hepatocytes in vivo which cause functional BSEP inhibition remains undefined. Our in vitro BSEP assay conditions were similar to those used in a study reported by Dawson et al. (2012), who evaluated BSEP inhibition by 85 drugs and found that a BSEP IC\textsubscript{50} threshold value of 300 \(\mu\)M provided useful discrimination between the majority of the tested drugs which caused cholestatic or mixed hepatocellular/cholestatic DILI and drugs which caused hepatocellular DILI, or did not cause DILI. An IC\textsubscript{50} cut-off value of 300 \(\mu\)M was therefore used in the current analysis. Notably, Morgan et al. (2011) found that numerous drugs associated with DILI in humans exhibit in vitro BSEP IC\textsubscript{50} values below 25 \(\mu\)M. Two of our potent BSEP inhibitors – Verapamil and Tamoxifen – have been reported by Morgan et al. not to exhibit significant BSEP inhibition. The differences in BSEP inhibition potency could be related to the different assay conditions (temperature, incubation time, substrate concentration) used in the two studies. Verapamil did not show significant inhibition of human BSEP mediated \[^{3}\text{H}]-taurocholate transport in \(S9\) insect vesicles up to 135 \(\mu\)M test concentration in the study by Morgan et al., whereas inhibition of \[^{3}\text{H}]-taurocholate uptake into canalicular membrane vesicles isolated from rat
liver has been demonstrated for Verapamil with an $K_i$ value of 92.5 $\mu$M (Horikawa et al., 2003), although a species difference cannot be excluded in this case. Similarly, Tamoxifen did not affect human BSEP mediated $[^3H]$-taurocholate transport in High Five insect cell derived membrane vesicles up to a concentration of 30 $\mu$M (Byrne et al., 2002), which is consistent with the findings by Morgan et al. However, in a study undertaken using SK-E2 cells expressing human BSEP, both Verapamil and Tamoxifen inhibited transporter activity which was determined using the fluorescent substrates Bodipy or dihydrofluorescein (Wang et al., 2003).

The $S_21$ insect cell expression system used in our BSEP inhibition studies has a low content of cholesterol (Paulusma et al., 2009). An increase in the cholesterol content of vesicles has been shown to increase taurocholate uptake activity, with moderate effects in insect cell derived membrane vesicles but stronger in vesicles derived from liver canalicular membranes. This typically results in an increase in the transport velocity $v_{max}$ whilst having no significant effect on the affinity of taurocholate for the BSEP transporter ($K_m$ value) (Paulusma et al., 2009; Kis et al., 2009). Importantly, for a limited number of drugs it has been demonstrated in the $S_21$ insect cell model that BSEP inhibition potencies are not affected by the membrane cholesterol content (Kis et al., 2009). We therefore consider that the BSEP inhibition potencies for the compounds evaluated in this study using the insect vesicles are likely to be indicative of values that would be observed in the presence of high concentrations of cholesterol.

The kinetics of BSEP inhibition may differ between drugs. Saito et al. (2009) have described a BSEP inhibition QSAR model which did not provide good prediction of inhibition by compounds which were non-competitive inhibitors of BSEP activity. Future studies would need to explore the effect of the mechanism of BSEP inhibition on the performance of QSAR models.
Conclusions

We have presented a thorough investigation of the physicochemical properties of compounds in relation to their inhibition of the BSEP transporter. We conclude that, for compounds where molecular weight is greater than 309, the most important parameter influencing BSEP inhibition potency is lipophilicity as calculated by ClogP. We also identified a statistically significant relationship with the acid dissociation constants of basic compounds, indicating that more basic compounds and those with more hydrogen bond donors tend to be less potent inhibitors. The two properties with the most marked effect on activity (molecular weight and ClogP) were used to construct a simple recursive partitioning scheme which allowed improved classification \((R^2 = 0.5\) in training, 0.36 in test) over any single property, and served as a benchmark against which we compared more elaborate modeling strategies.

Our evaluation of a collection of molecular descriptor sets and modeling algorithms yielded a further improvement in classification of a collection of 187 compounds to which the modeling algorithm had not previously been exposed. The source of this improvement appeared to originate from the ability of the QSAR model to identify low molecular weight actives where the partitioning scheme failed. These improved classifications appear to be the result of better molecular descriptions of the molecules as encoded by the AZdesc set, rather than the nature of the classification scheme used (i.e. linear vs. non-linear methods). We have provided the full dataset with this publication to allow others to emulate (or possibly surpass) this result.

Finally, we present a collection of interesting structural modifications giving rise to a reduction of BSEP activity. Although there is no statistical evidence to support the generalisability of these modifications, with further data they could serve as an interesting starting point for those wishing to further examine the SAR surrounding this transporter.
Acknowledgements

We are grateful to our AstraZeneca colleagues Sarah Dawson, John Cuff, Dearg Brown, Mike Rolf and the team from Reagents and Assay Development for excellent scientific and technical assistance. We also thank Prof. Dr. Bruno Stieger (University of Zürich, Switzerland) for the provision of the hBSEP baculovirus stocks. The data set used in the analysis was generated under contract by Ricerca Biosciences LLC (Bothell, WA, USA) and we thank Gonzalo Castillo for his excellent management of this activity.

Authorship Contributions


Conducted experiments: C. L. Walker

Contributed new reagents or analytic tools:

Performed data analysis: D. J. Warner, H. Chen

Wrote or contributed to the writing of the manuscript: D. J. Warner, H. Chen, L.-D. Cantin, J. G. Kenna, S. Stahl, C. L. Walker, T. Noeske
References


JMP, Version 8.0, SAS Institute Inc., Cary, NC.


Legends for Figures

Figure 1. Inhibition of BSEP mediated [3H]-taurocholate transport measured in inverted membrane vesicles from BSEP overexpressing Sf21 cells. Effects of selected pharmaceuticals on BSEP activity are shown: Bosentan (■), Bezafibrate (▲), Labetalol (◆), Acetaminophen (●).

Figure 2. Distribution of IC50 values for compound effects on BSEP activity. An IC50 value of 300 μM was used as the threshold for significant BSEP inhibition (Dawson et al, 2012). Data are presented as geometric means from at least three test occasions, with the compounds ordered by potency.

Figure 3. Logistic regression plots to demonstrate the influence of different physicochemical properties on the likelihood of BSEP inhibition for all 624 compounds. (a) ClogP, (b) pKa for 241 negatively ionized compounds, (c) number of hydrogen bond (HB) donors, (d) MW, (e) pKa for 338 positively ionized compounds, (f) number of hydrogen bond acceptors. Red points in the plots refer to BSEP positive compounds (POS class) and blue points refer to negatives (NEG class).

Figure 4. Pie charts depicting ion classes of 299 inactive and 325 active BSEP inhibitors (300μM cutoff).

Figure 5. (a) Recursive partitioning scheme for the training set (437 compound) based on molecular weight and ClogP (red bars correspond the ratio of BSEP actives and blue bars correspond to the ratio of BSEP inactives). (b) Probability contour plot of being in the BSEP POS class for the full dataset (based on the RP scheme).

Figure 6. Matched molecular pairs exhibiting unexpected activity profiles. Structures are displayed from left to right in order of increasing ClogP. The molecular weight and calculated
logP of each compound is expressed below the BSEP activities in the format MW / ClogP. The most potent BSEP inhibitor in each row is represented in red.

**Figure 7.** Improvement in false negative classification through use of the SVM_AZdesc model vs. simple property based recursive partitioning. (a) Two active compounds which are incorrectly flagged as negative by SVM_AZdesc but not by the RP method. (b) Eight active compounds which are incorrectly flagged as negative by the RP method but not by SVM_AZdesc. The molecular weight and calculated logP of each compound is expressed below the BSEP activities in the format molecular weight / ClogP.
Table 1. Performance metrics for all six BSEP classification models. Those indicating an improvement in performance of $\geq 0.05$ over the simple property based recursive partitioning scheme are highlighted in bold.

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<th>Model</th>
<th>Pos. prec.</th>
<th>Neg. prec.</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Fscore</th>
<th>Kappa</th>
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Figure 1

![Graph showing human BSEP activity (%)](image)

- **Bosentan**
- **Bezafibrate**
- **Labetalol**
- **Acetaminophen**

**Y-axis:** Human BSEP Activity (%)
**X-axis:** Concentration (µM)
Figure 4

Molecule ion Class
- Acid
- Base
- Cationic
- Neutral
- Zwitterion

BSEP Inactives
- 32.1%
- 34.8%
- 7.0%
- 22.7%
- 3.3%

BSEP Actives
- 37.8%
- 38.5%
- 3.1%
- 0.6%
- 20.0%
Figure 7

a) Benzylpenicillin
   IC$_{50}$ < 10µM
   334 / 1.7

   Buphenine
   IC$_{50}$ = 280µM
   299 / 3.0

b) Ramelteon
   IC$_{50}$ = 85µM
   259 / 2.5

   Metyrapone
   IC$_{50}$ = 229µM
   226 / 1.5

   Tropicamide
   IC$_{50}$ = 151µM
   284 / 1.2

   Ebselen
   IC$_{50}$ < 10µM
   274 / 3.7

   AZ45
   IC$_{50}$ = 98µM
   216 / 2.8

   Mianserin
   IC$_{50}$ = 47µM
   264 / 3.8

   Letrozole
   IC$_{50}$ = 225µM
   285 / 1.4

   Leflunomide
   IC$_{50}$ = 110µM
   270 / 2.3