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Towards Predicting Drug-Induced Liver Injury (DILI): Parallel Computational Approaches to Identify MRP4 and BSEP Inhibitors

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Running Title: Computational Modeling of MRP4 and BSEP to Predict DILI

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Abbreviations: BSEP, bile salt export pump; DILI, drug-induced liver injury; PFIC, progressive familial intrahepatic cholestasis; MRP, multidrug resistance protein

Abstract

Drug-induced liver injury (DILI) is an important cause of drug toxicity. Inhibition of MRP4, in addition to BSEP, might be a risk factor for the development of cholestatic DILI. Recently, we demonstrated that inhibition of MRP4, in addition to BSEP, may be a risk factor for the development of cholestatic DILI. Here, we aimed to develop computational models to delineate molecular features underlying MRP4 and BSEP inhibition. Models were developed using 257 BSEP and 86 MRP4 inhibitors and non-inhibitors in the training set. Models were externally validated and used to predict the affinity of compounds towards BSEP and MRP4 in the DrugBank database. Compounds with a score above the median fingerprint threshold were considered to have significant inhibitory effects on MRP4 and BSEP. Common feature pharmacophore models were developed for MRP4 and BSEP with LigandScout software using a training set of 9 well-characterized MRP4 inhibitors and 9 potent BSEP inhibitors. Bayesian models for BSEP and MRP4 inhibition/non-inhibition were developed with cross-validated Receiver Operator Curve (ROC) values greater than 0.8 for the test sets, indicating robust models with acceptable false positive and false negative prediction rates. Both MRP4 and BSEP inhibitor pharmacophore models were characterized by hydrophobic and hydrogen-bond acceptor features, albeit in distinct spatial arrangements; similar molecular features between MRP4 and BSEP inhibitors may partially explain why various drugs have affinity for both transporters. The Bayesian (BSEP, MRP4) and pharmacophore (MRP4, BSEP) models demonstrated significant classification accuracy and predictability.

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Introduction

Drug-induced liver injury (DILI) is an important cause of drug toxicity and a major reason for withdrawal of drugs from the market (Abboud and Kaplowitz, 2007) or attrition of drug candidates in late development stages, which can be extremely costly. Unfortunately, current *in vitro* screens or *in vivo* preclinical studies cannot accurately predict the potential of compounds to cause hepatotoxicity. DILI remains a major concern in drug discovery and clinical development. This obstacle has necessitated a search for alternative technologies, such as computational approaches to decrease the risk of DILI-associated late-stage failures.

Despite extensive research, the underlying mechanisms of DILI are not well understood. However, it is clear that compound-related properties as well as individual patient characteristics affect the occurrence of DILI. Formation of reactive metabolites, mitochondrial impairment, and inhibition of canalicular bile acid transport mediated by the bile salt export pump (BSEP) (e.g. troglitazone, bosentan, erythromycin) (Stieger et al., 2000; Fattinger et al., 2001; Kostrubsky et al., 2003) are known risk factors for the development of DILI in humans. This has been substantiated by large scale *in vitro* screening studies revealing that drugs that cause cholestatic DILI have higher potencies as well as frequencies of BSEP inhibition compared to drugs that are not liver toxic or that cause hepatocellular DILI. (Morgan et al., 2010; Dawson et al., 2012) BSEP is located at the canalicular membrane of the hepatocyte where it is involved in the excretion of bile acids into bile under physiological conditions. (Noe et al., 2002) The importance of this protein in bile acid homeostasis is emphasized by the observation that mutations in the BSEP gene *ABCB11* have been associated with progressive familial intrahepatic cholestasis type 2 (PFIC 2). Although BSEP inhibition may explain bile acid-mediated DILI liability for a large proportion of compounds, a subset of hepatotoxic drugs remains that cannot be explained by BSEP inhibition alone.

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In addition to canalicular BSEP, multidrug resistance protein 4 (MRP4) is a bile acid efflux protein localized at the basolateral membrane of hepatocytes. While hepatic expression is low under normal physiological conditions, MRP4 up-regulation has been demonstrated under cholestatic conditions. MRP4 is hypothesized to serve as a back-up system for bile acid efflux from hepatocytes into sinusoidal blood when the normal vectorial transport of bile acids from hepatocytes into bile is compromised. (Scheffer et al., 2002; Teng and Piquette-Miller, 2007; Gradhand et al., 2008; Chai et al., 2012) Recently, we screened 88 drugs (BSEP inhibitors and non-inhibitors) for inhibition of MRP4-mediated transport of the prototypical substrate [^{3}H]-dehydroepiandrosterone sulfate (DHEAS) and discovered potent MRP4 inhibition among cholestatic BSEP non-inhibitors. A statistically significant relationship was observed between the potency of MRP4 inhibition and the probability of cholestatic classification: for each 1% increase in MRP4 inhibition, the probability that a drug was cholestatic increased by 3.1%. Interestingly, many BSEP inhibitors also were MRP4 inhibitors. These data suggested that MRP4 inhibition may serve as a confounding factor in BSEP-mediated DILI, or in some cases lead to DILI in the absence of BSEP inhibition. Thus, MRP4 inhibition may be an additional risk factor for the development of cholestatic DILI.

The role of hepatic bile acid transport inhibition in the etiology of DILI emphasizes the urgent need to develop screening tools to accurately predict drug-bile acid transporter interactions. While *in vitro* membrane vesicle assays have been developed for BSEP and MRP4 screening, use of these assays early in drug development is time consuming, labor- and resource-intensive, and requires the physical availability of compounds (including metabolites) for testing. An alternative approach to *in vitro* testing is the use of computational models to predict drug-bile acid transporter interactions and aid in identifying transporter-associated DILI early in the drug

discovery process. For example, pharmacophore models have been used in ligand-based drug design to define the key structural characteristics that a molecule must possess in order to bind to the biological target (Ekins et al., 2012). Since models for BSEP have been reported recently (Pedersen et al., 2013; Ritschel et al., 2014), the aim of the current study was to develop a comprehensive model for MRP4 inhibition and evaluate its predictive ability. In addition, we developed Bayesian models to delineate molecular features underlying *both* MRP4 and BSEP inhibition. These *in silico* models were used to identify potential novel MRP4 inhibitors by virtual screening of an existing database, and to classify drugs as BSEP and MRP4 inhibitors in an effort to correlate these features with DILI incidence.

Materials and Methods

Dataset Composition. A dataset of 86 compounds derived from Köck and co-workers (Köck et al., 2014) was used for MRP4 inhibition modeling and a dataset of 257 compounds derived from Dawson et al. and Morgan et al. (Morgan et al., 2010; Dawson et al., 2012) was used for developing BSEP inhibition models. The compounds in these datasets were structurally diverse and from various therapeutic classes. They were classified as "cholestatic" or "non-cholestatic," according to DILI type reported in the literature. The compounds were further classified as "active" for the specified transporter if they had an $IC_{50} \leq 135$ μM for BSEP or a percent inhibition ≥ 21% compared to control at 100 μM for MRP4, otherwise they were classified as "inactive" against that transporter. The MRP4 classifications are based on findings by the Köck and co-workers that compounds that inhibit by at least 21% have a 50% chance of being cholestatic and the rationale for the BSEP classifications is to identify inhibitor compounds with both potent and moderate cholestatic risk, similar to Morgan et al. These classifications enable the identification of compounds that should be investigated further for their potential to cause cholestasis.

In addition to MRP4 and BSEP datasets, a database of 1,510 FDA-approved drugs was retrieved from DrugBank (http://www.drugbank.ca) (Law et al., 2014). The database was modified by removing ionic salts and large polymeric drugs and proteins, resulting in a catalogue of 1,488 drugs.

Training and Test Set Generation. The MRP4 and BSEP databases were separated into training and test sets by randomly dividing two-thirds of the compounds into the training set and the other third into the test set (Supplemental Table 1). Table 1 enumerates the number of

compounds in each set based on the respective transporter as well as the number of compounds that were classified as inhibitors and non-inhibitors.

The Bayesian modeling of MRP4 and BSEP used all the compounds in their respective training sets. In contrast, the pharmacophore models were developed using a subset of compounds from the training set. The MRP4 pharmacophore subset was based on clustering of the training set, which produced a subset of 9 compounds; analogously, the BSEP pharmacophore subset contained the strongest inhibitors, also producing a subset of 9 compounds. Details of subset generation and composition are explained further within the pharmacophore creation methods section.

The conformational models for pharmacophore creation were produced in LigandScout using the OMEGA conformer generator with the default best quality settings that produced a maximum of 500 conformations per molecule with an energy window of 10kcal/mol and RMS threshold difference of 0.4 to identify unique conformers. The common feature pharmacophore was generated using the default settings in LigandScout for ligand-based shared-feature pharmacophore creation with a feature tolerance scale of 1.0.

Principal Component Analysis (PCA) of Training, Test Set and DrugBank Molecules. The 3D molecular structures of 86 MRP4 inhibitors and non-inhibitors and the 257 BSEP inhibitors were obtained from PubChem (http://www.ncbi.nlm.nih.gov/pccompound). PCA plots of each transporters' training and test sets were produced in order to ensure that the two sets were representative of each other in terms of molecular descriptors. In addition, the training sets were compared to the modified DrugBank database (see above) to ensure that the training set was representative of currently approved drugs and had predictive power in that chemical space. The PCA plots were generated based on 8 molecular descriptors for each drug: ALogP, molecular

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weight, number of hydrogen bond donors, number of hydrogen bond acceptors, number of rotatable bonds, number of rings, number of aromatic rings, and molecular fractional polar surface area. The molecular descriptors and PCA plots were generated within Discovery Studio 4.0 (DS 4.0; Accelrys, Inc. San Diego, CA). The two-dimensional plots (Fig. 2A-D) represent only the first two principal components of each comparison for visual clarity.

Common Feature Pharmacophore Generation and Validation. Ligand-based pharmacophores and conformational models were generated using LigandScout (version 3.12 build 20130912, Inte:Ligand, Vienna, Austria) [1,2] with default settings. The pharmacophore models for MRP4 inhibition were generated from a subset of drugs produced by clustering the training sets based on similarity of the pharmacophore radial distribution function. Drugs with similar pharmacophore features were clustered together and the most potent inhibitors of each cluster were included in the subset to train the pharmacophore model. The rationale of clustering is to generate a pharmacophore from a smaller training set while still maintaining the structural diversity of the original training set. If the common pharmacophore creation failed or produced a pharmacophore with less than 3 features, the drug that failed to align was removed from the training set. Of the 10 pharmacophores generated per training set, the pharmacophore that aligned with the most compounds in the training and test set, and had the highest score, was selected for further testing.

The MRP4 common feature pharmacophore was validated within LigandScout through virtually screening the test set for its ability to distinguish actives (i.e. drugs with ≥21% MRP4 inhibitory activity) from inactives (i.e. drugs with <21% MRP4 inhibitory activity). The conformational models of the test set were generated in an identical manner as the training set.

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Drugs that aligned with all 3 pharmacophore features were predicted to be active MRP4 inhibitors.

The pharmacophore models for BSEP inhibition were generated from a subset of drugs from the BSEP training set that were the strongest inhibitors (<25 uM IC_{50}s) among the Morgan dataset (Morgan et al., 2010). This dataset was chosen because it contained the largest number of BSEP inhibitors. The clustering method utilized for the MRP4 inhibition pharmacophore was used initially; however, this resulted in a pharmacophore with poor predictive ability which is why the strongest inhibitors were used instead. The BSEP common feature pharmacophore was validated using the same methods as the MRP4 pharmacophore except that actives were drugs with an $IC_{50} \leq 135$ μM.

Building and Validation of Bayesian Models. Bayesian categorization involves simple and straightforward probabilistic classification by evaluating the frequency of structural features associated with a hypothesis of interest (Xia et al., 2004). The protocol "Create Bayesian Model" in DS4.0 was applied for model generation with the number of bins set to 10. In addition to 7 molecular descriptors, 'extended-connectivity fingerprints maximum diameter 6' (ECFP_6) and 'functional-class fingerprints maximum diameter 6' (FCFP_6) (Rogers et al., 2005) were calculated for all compounds. ECFP and FCFP differ such that, for example, a chlorine atom and a bromine atom, which are substituents in the same position on an aromatic ring, would be differentiated as different fingerprints with ECFP but not with FCFP. The models were built by using combinations of iterative sets of varying descriptors and cutoff values. Bayesian models were validated with 10-fold cross-validation-based 'receiver operator curve' area under the curve (XV ROC AUC) (Zweig and Campbell, 1993) associated with training set compounds. The predictive capacity of Bayesian models was validated with the same test set described for

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pharmacophore generation above. The activities of the test set compounds were predicted by the "Calculate Molecular Properties" protocol in DS4.0.

Evaluation of Model Performance. The Matthews correlation coefficient (MCC) was used to determine the relative predictive ability of the pharmacophore and Bayesian models. MCC ranges from –1 (no correlation) to +1 (full correlation) and is calculated as follows:

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}} \qquad \text{(Eq.1)}$$

where TP= number of true positives, FP=number of false positives, TN=number of true negatives, and FN=number of false negatives. Positive predictive value = TP/(TP+FP), sensitivity = TP/(TP+FN), and specificity = TN/(TN+FP).

The ROC curve is another method of evaluating models. It is a 2D plot that graphs the sensitivity of a model, its true positive rate, versus the reverse percentage of the specificity of the model, its false positive rate, by the ranked order of the pharmacophore-fit scores. One of the abilities of the ROC curve is the use of the area under the curve (AUC) when comparing the ability of different models to correctly classify true positives above false positives. Starting from the bottom left corner, the graph plots the percentage of the actives in the test set properly classified as active, which is defined as the sensitivity or true positive rate, versus the percentage of the inactives improperly classified as active, which is defined as the reverse specificity or false positive rate. In addition to the AUC, the ROC can be used to set a score cutoff which optimizes the tradeoff between sensitivity and specificity.

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RESULTS

Characteristics of the data set. The MRP4 inhibitor data were obtained from data previously generated in our laboratories (Köck et al., 2014) and the BSEP inhibitor data were compiled from two high-throughput screening studies (Morgan et al., 2010; Dawson et al., 2012). The BSEP studies were selected due to the large number of screened compounds from various therapeutic areas (Supplemental Table S1). Venn diagrams reveal the composition of the MRP4 inhibitor dataset contrasted with the BSEP data from Morgan and colleagues to illustrate compounds in the dataset that uniquely inhibit MRP4 or BSEP as well as compounds that inhibit both transporters simultaneously (Fig. 1). These diagrams demonstrate that most of the previously identified BSEP inhibitors tested by our laboratories were also MRP4 inhibitors (Fig. 1A). Among cholestatic compounds, most were dual BSEP and MRP4 inhibitors or MRP4-only inhibitors; only one BSEP-only inhibitor had been identified as cholestatic (Fig. 1B).

Structure Generation and Validation. The PCA plot is a useful tool to predict potential outliers by assessing similarity among training and test set compounds (Khandelwal et al., 2007). For the MRP4 dataset, PCA of 86 training and test set drugs with at least three principal components was performed based on 8 descriptors. There were 57 compounds from the training set and 29 from the test set. The first and second components accounted for 36.6% and 27.2% of the total variance. For the BSEP dataset, PCA of 257 compounds compared 171 and 86 compounds in the training and test sets, respectively. The first and second components accounted for 39.1% and 34.4% of the total variance, indicating that these components represented the majority of overall descriptor space occupied by the molecules. Figures 2A-B demonstrate that the test set drugs accommodate similar space compared with the training set compounds for their respective transporter. PCA plots of compounds in the training sets are overlaid on a PCA plot of DrugBank

drugs in Figures 2C-D, illustrating that training set compounds cover most of the descriptor space occupied by the compounds featured in the DrugBank database.

Building and Validation of Bayesian Models. Bayesian models for MRP4 inhibition were developed with a training set of 57 MRP4 inhibitors and non-inhibitors and the Bayesian models for BSEP inhibition were developed with a training set of 171 inhibitors and non-inhibitors. Eight structural descriptors as well as structural extended-connectivity or functional-class fingerprints (ECFP_6 or FCFP_6, see Methods) were incorporated for model development. Four Bayesian models were generated for MRP4 and BSEP inhibitors and non-inhibitors based on specified atom-type (ECFP) and functional class (FCFP) 2-dimensional substructure fingerprints.

The predictive performance of Bayesian models was evaluated by XV ROC AUC based on 10-fold cross-validation of training set compounds. XV ROC AUC reflects the relationship between sensitivity and specificity, ranging from 0 to 1, with a higher number indicating a better model (Zweig and Campbell, 1993; Obuchowski and Lieber, 1998). The Bayesian models also were validated with their respective test set, consisting of 29 drugs for the MRP4 model and 86 drugs for the BSEP model. Their predicted performance was established by sensitivity (SE), specificity (SP), overall prediction accuracy (Q) and Matthew's correlation coefficients (MCC values; a measure of the quality of binary classifications) calculated from the empirical true positive (TP), true negative (TN), false positive (FP), and false negative (FN) values (Ung et al., 2007; Khandelwal et al., 2008) (Table 2).

Table 2 shows the AUCs of Bayesian models based on the 10-fold cross-validation with training set compounds. AUC values range between 0 and 1, with 0.5 indicating 50% correct prediction and 1 indicating a perfect match between observed and predicted data (Fawcett, 2006). The AUC values associated with the four individual models indicated good internal

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consistency and prediction accuracy. While both the MRP4 inhibitor and the BSEP inhibitor Bayesian classification had similar ROC AUC scores for both the internal, leave-one-out cross-validation, and the external test set validation, the sensitivity and specificity varied more between the models. The MRP4 inhibitor models had significantly lower sensitivity, especially the MRP4 ECFP model, compared to the BSEP inhibitor models, but the trade-off was a higher specificity, minimizing false positives. Bayesian classification modeling of BSEP inhibitors resulted in more predictive models as demonstrated by their relatively higher Matthews' correlation coefficient compared to MRP4 inhibitor models, which could be due to the larger size of the training set (171 BSEP compounds vs. 59 MRP4 compounds). In addition to the external validation performed here, the BSEP Bayesian FCFP model was used to predict the classification of 5 strong inhibitors and 5 non-inhibitors from previous screen for BSEP inhibitors (Pedersen et al., 2013). The model was able to correctly classify nine of the ten compounds, only incorrectly classifying MK571 as a non-inhibitor.

Fingerprints can be defined as molecular fragments that characterize the structural features of drug molecules. Figure 3 and 4 displays the five most predictive structural fragments for both favorable and unfavorable inhibitory activity against MRP4 and BSEP using FCFP_6 fingerprints. Supplemental figure S2 contains an expanded figure of structural fragments favorable and unfavorable for inhibition of MRP4 and BSEP using both FCFP_6 and ECFP_6 fingerprints. Structural elements depicted in Figure 3 and 4 were identified in inhibitors and non-inhibitors amongst training set compounds, respectively. Oxygen atoms tended to be predictive of favorable inhibitory activity for both MRP4 and BSEP, however, negatively ionized oxygen atoms frequently occurred in the MRP4 model but not in the BSEP model even though both are considered anion transporters. This is in agreement with the study by Pedersen and

colleagues (Pedersen et al., 2013), who reported that BSEP substrates tend to be anionic but inhibitors were more likely to be neutral at physiological pH. Accordingly, positively charged secondary and tertiary amines frequently occur among the MRP4 fingerprints associated with non-inhibition. Thus, the identified fingerprints could be helpful in distinguishing inhibitors and non-inhibitors of MRP4 amongst novel compounds.

MRP4 Pharmacophore Development. The MRP4 training set of 57 drugs was imported into LigandScout 3.12 and clustered according to pharmacophore radial distribution function-code similarity with the maximum conformations set to 3 and the cluster distance set to 0.5. This algorithm clusters compounds that have similar individual 3D pharmacophore characteristics.

The following 9 drugs that represent the strongest inhibitors in their respective cluster were used to generate MRP4 inhibition pharmacophores: nitrenedipine, sulindac, sorafenib, clobetasol propionate, benzbromarone, glafenine, furosemide, finasteride, and simvastatin. The remaining 77 compounds not selected for the training set were moved to the test set for pharmacophore validation. The ligand-based common feature pharmacophore produced from the 9 compounds had 2 hydrophobic features and a hydrogen bond acceptor feature (Fig 5A). The two hydrophobic features were 5.01Å apart, while the hydrogen bond acceptor was 4.81Å from the neighboring hydrophobic feature, and 8.86Å from the distal hydrophobic group. All 9 drugs in the training set aligned with all 3 pharmacophore features. Two representative compounds were aligned to the pharmacophore to illustrate scale and similarity in how the molecules align with the respective molecular features comprising the pharmacophore (Fig 5B). These compounds were chosen because their steroid backbone renders them particularly rigid, increasing the likelihood that the representative conformer is close to its bioactive conformation; additionally, they contain few atoms that can engage in intermolecular interactions, which further confirms

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that these are the requisite features for MRP4 recognition. Exclusion volumes, i.e. spheres that cannot be occupied and represent steric hindrance, were generated initially using the non-inhibitors in the 57 compound training set; however, consideration of exclusion volumes rendered the models more likely to incorrectly classify MRP4 inhibitors as non-inhibitors during external validation and were subsequently omitted during database screening.

Quantitative validation of the MRP4 pharmacophore model. The MRP4 pharmacophore model was able to correctly classify 30 of the 42 actives in the test set and 22 of the 35 inactives, featuring model sensitivity of 71.4% and specificity of 62.8%. The area under the receiver operating characteristic curve was 0.70, which is considered a fair quality model (Fig 6). Based on the virtual screening results from the external test set validation, the model has its highest positive predictive value, the number of true positives over the sum of true and false positives, at a pharmacophore-fit score cutoff of 37.75. The positive predictive value of the model at this cutoff is 0.826, selecting 19 true positives, 45.2% of total actives in the set, but only 4 false positives, 11.4% of total inactives in the set. The pharmacophore-fit score cutoffs allow for selecting drugs with a higher likelihood of being classified correctly beyond those which align to the pharmacophore within the tolerance of the features.

The inactives that were incorrectly classified included dexamethasone, naloxone, clopamide, vinblastine, tolbutamide, probenecid, indinavir, flupirtine, chorpropamide, alprenolol, chlorpheniramine, fluorescein, and timolol. Interestingly, the false positive with the highest pharmacophore-fit score was dexamethasone, a glucocorticoid that had no significant inhibitory activity (5 ± 34%). This compound was aligned with clobetasol propionate, another glucocorticoid included in the 9 training set compounds, which was a strong inhibitor (101 ± 23%) (Fig 7).

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As can be seen in Fig. 7, clobetasol propionate (orange) and dexamethasone (gray) have a high degree of structural similarity. From this observation, molecular properties that could be mediating the significant difference in inhibitory activity were investigated. The molecular property that exhibited the most significant difference was calculated LogP which is 4.18 for clobetasol propionate and 1.68 for dexamethasone, rendering clobetasol propionate a more hydrophobic compound. The difference in calculated LogP values was evaluated for all 87 compounds tested by Köck and co-workers; actives trended towards higher LogP values than inactives. A Pearson correlation coefficient for the calculated LogP and a compound considered active (≥21% MRP4 inhibition) was 0.634 and the correlation coefficient for the percent MRP4 inhibition and calculated LogP was 0.508. Figure 8 represents a box plot of the calculated LogP values for the compounds classified as inactives and actives; the mean and median of the inactives' calculated LogP values were 0.38 and 0.69, respectively, and the actives' calculated LogP values were 3.64 and 3.84, respectively. It is interesting to note that numerous sulfonamides or sulfamides, such as clopamide, tolbutamide, probenecid, and chlorpropamide were classified as false positives. These molecules may either be a poor match to the models, or their features are incorrectly parameterized within the Bayesian and pharmacophore modeling algorithms.

The actives that were not properly classified as active by the model included 19-norethindrone, clozapine, desipramine, diphenhydramine, etoposide, maprotiline, nitrofurantoin, nortriptyline, oxybutynin, praziquantel, promethazine, and ticlodipine. Eight of these 12 drugs have similar structures containing an amine group, which is predicted to be positively ionized at physiological pH; in addition, six of these drugs contain two aromatic rings whose distance is comparable to the distance observed between the two hydrophobic features in the MRP4 model. Compounds

that fall into this category are clozapine, desipramine, diphenhydramine, maprotiline, nortriptyline, and promethazine. Oxybutynin and ticlodipine contain an amine predicted to be positively charged, but they have only one aromatic group. Nitrofurantoin is a compound that continually failed to match any structural similarity search to other known inhibitors; therefore, we speculate that it is binding in a different manner than any of the other inhibitors, perhaps at an allosteric site of the transporter.

Qualitative Validation of the MRP4 Inhibitor Model. In addition to the quantitative validation from virtually screening the test set, two compounds that qualitatively strengthen confidence in the model are DHEAS, the substrate used to generate the data, and felbinac, a potent MRP4 inhibitor from a separate screening of MRP4 inhibitors (Morgan et al., 2013). DHEAS was not included in either the training set or the test set, but the MRP4 pharmacophore model would be expected to align to the substrate that was used experimentally to generate the inhibition data. Figure 9A depicts how the two methyl groups on DHEAS align to the hydrophobic features in the pharmacophore and one of the oxygen atoms from the sulfate group aligns with the hydrogen bond acceptor feature. The alignment of the pharmacophore model to DHEAS is of particular interest because of its structural rigidity due to the steroid backbone structure. The only significant intramolecular motion that DHEAS can undergo is the rotation of the sulfate group. In addition to its rigidity, DHEAS contains few atoms that can participate in intermolecular interactions. From the DHEAS pharmacophore (Fig 9B), it appears that the two methyl groups can participate in hydrophobic interactions, while the ketone can be a hydrogen bond acceptor, and all the oxygen atoms in the sulfate group, which is negatively ionized at physiological pH, can act as hydrogen bond acceptors.

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Felbinac is of interest for the same reasons as DHEAS, namely its structural rigidity and minimal possible intermolecular interactions. In addition, felbinac is a potent inhibitor of MRP4-mediated transport of β-estradiol 17-(β-D-glucuronide) with an IC_{50} of 8.2 uM. (17) Felbinac aligns well with the MRP4 pharmacophore model and, as shown in Figure 9D, engages in only a limited number of intermolecular interactions; both hydrophobic and aromatic interactions with the phenyl groups in the biphenyl compound, and the oxygen atoms of the negatively ionized carboxylate group, are able to act as hydrogen bond acceptors. The two phenyl groups are locked rigidly on perpendicular planes and, therefore, only the carboxylate group is able to rotate.

LogP Filtering Improves Model Specificity. The MRP4 pharmacophore model's specificity can be significantly improved if the test set were to be filtered after screening with a calculated LogP cutoff of 2.92, which corresponds to the start of the lower quartile of the actives. The sensitivity would decrease to 52.38% (22 true actives of 42 total actives) but the specificity would increase to 91.43% (3 false actives of 35 total inactives). This marked improvement in specificity demonstrates the significant influence LogP plays in MRP4 inhibition.

BSEP Inhibitor Pharmacophore Development and Validation. The BSEP inhibitor pharmacophore was produced with a subset of drugs from the training set that represented the nine strongest BSEP inhibitors according to Morgan and co-workers that were also tested for MRP4 inhibition by our group previously. This subset included: nitrenedipine, fenofibrate, ritonavir, pioglitazone, rosiglitazone, valinomycin, simvastatin, benzbromarone, and lopinavir. The common feature pharmacophore produced with this set of nine compounds had three features: two hydrophobic features and one hydrogen bond acceptor (Supplemental Figure S1). This is similar to the MRP4 pharmacophore, which may explain the high degree of inhibition

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overlap, however, the distances between the features were different. The distance between the two hydrophobic features was 6.29 Å, the hydrogen bond acceptor and the proximal hydrophobic feature was 4.67 Å, and the hydrogen bond acceptor and the distal hydrophobic feature was 6.69 Å. All nine drugs that were used to train the pharmacophore hit all three features.

The BSEP inhibitor pharmacophore was validated by virtual screening of the test set and the model correctly classified 46 of the 56 inhibitors and incorrectly classified 120 of the 191 non-inhibitors. The model selectivity was 82.7% but the specificity was 37.2%. The poor model specificity is partly due to the higher proportion of non-inhibitors to inhibitors in the test set (191 vs. 56) but also indicative of the difficulty of modeling BSEP through a pharmacophore approach.

DISCUSSION

A ligand-based pharmacophore and Bayesian modeling approach is presented here, describing the molecular properties and chemical features necessary for human MRP4 and BSEP interaction. Since these transport proteins have been associated with DILI, these models may be useful in predicting DILI liability of novel compounds. The models were developed from our laboratories' previous work and data from other groups. An advantage of Bayesian classification modeling is the ability to easily interpret how the model weighs the various molecular properties, and which molecular properties are most predictive for classification. The Bayesian classification model was developed by creating up to 11 bins for each molecular property. For discrete properties such as the number of rings or the hydrogen-bond acceptor atoms, all the compounds with the same value were put into the same bin; for continuous properties such as molecular weight or ALogP, a bin was assigned a value range and all the compounds that fell within that range were put into that bin. The ranges for binning continuous values were created

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such that the number of compounds in each bin was evenly distributed. A normalized probability was then calculated for each bin according to the fraction of compounds in the bin that were active, i.e. if all the compounds in a bin were active, that bin was assigned a higher probability. The probability was normalized by adjusting for bins with few compounds. A Bayesian score was then calculated by summing the normalized probabilities of all the bins to which a compound was assigned.

By inspecting the normalized probabilities of individual bins, the molecular properties that contain bins with high probabilities indicate molecular properties that are well correlated with either inhibition or non-inhibition. For example, with the MRP4 model, a trend was observed with a higher ALogP correlating to MRP4 inhibition (14 of 15 drugs with an ALogP of 3.8 or higher were inhibitors while only 2 of 12 drugs with a ALogP of 0.94 or lower were inhibitors). A trend also was observed with large molecular weight drugs more likely to inhibit MRP4 compared to smaller drugs (23 of 25 drugs with molecular weight ≥356 Da). It should again be noted that the value range of a bin, for molecular properties that are continuous, is a result of evenly dividing the ordered drugs into 11 bins. In addition to those continuous properties, 23 of 25 drugs with 3 or 4 rings were inhibitors compared to 1 of 9 compounds with 0 or 1 ring.

Compared to the MRP4 Bayesian model, the BSEP Bayesian model was more predictive of negative properties, thus predicting more non-inhibitors than inhibitors. This is likely due to the fact that the training set contained more non-inhibitors relative to inhibitors compared to the MRP4 training set. For the BSEP model, only 7 of 90 drugs with a molecular weight less than 337 Da were inhibitors. Correspondingly, only 11 of 90 compounds with 4 or fewer rotatable bonds were inhibitors and, similar to the MRP4 model, only 3 of 72 drugs with an ALogP of 2.03 or lower were inhibitors.

Potential biological implications of the association between high calculated LogP values and MRP4 inhibition were considered. These data lead to speculation that molecules must partition first into the bilayer to be an inhibitor for MRP4, or that higher LogP values correspond with increased hydrophobic interactions within the protein environment, thus rendering these molecules stronger competitive inhibitors.

It is worth mentioning that these computational models are based on data collected from membrane vesicle assays and this *in vitro* system could have an influence on experimental transport inhibition results. The methods generally involve short incubation periods with the test compounds in which the degree of partitioning of the test compound into the membrane of the vesicles could be in flux. If the test compound exerts its inhibition while imbedded in the membrane, this could cause a skew in the data in which compounds with higher LogP values have a higher rate of partitioning into the membrane than those with lower LogP values.(Nagar and Korzekwa, 2012)

Comparison to Previous Models. Previous studies identified important molecular features for BSEP inhibition (Pedersen et al., 2013) and pharmacophore models have been proposed for BSEP (Ritschel et al., 2014) and MRP4 (Fukuda et al., 2013). While there was good corroboration between the important molecular features, there were several notable differences between the pharmacophores previously reported and those developed here, which can be ascribed primarily to differences in the training sets used to generate the pharmacophores.

Molecular properties that were reported previously to have a statistically significant difference between strong BSEP inhibition (>50% inhibition) and non-inhibitors were $LogD_{7.4}$, molecular weight, saturated nonpolar surface area, LogP, number of rotatable bonds, unsaturated nonpolar

surface area, number of hydrogen-bond acceptors, and net charge at pH 7.4. The molecular properties that remain statistically significant for weak inhibitors (27-50% inhibition) and non-inhibitors were only molecular weight and saturated nonpolar surface area. In comparison, our best Bayesian BSEP model (FCFP_6) ranked the top four feature bins based on their normalized probability in descending order: molecular weight, FCFP_6 fingerprint, number of rotatable bonds, and ALogP. However, since a compound can have only one value for a molecular property but multiple fingerprints, the fingerprints tend to be the predominate factor influencing the final Bayesian score for a compound. The pharmacophore model for BSEP is also in agreement with these previously reported molecular properties since they consisted of 2 hydrophobic features and a hydrogen-bond accepting feature. The hydrophobic features were associated with nonpolar surface area and high LogP while the hydrogen-bond accepting feature was associated with the number of hydrogen bond acceptors correlated with inhibition. The presence of only one hydrogen-bond accepting features in the pharmacophore model, however, suggests that a large number is not essential for inhibition, but could provide more opportunities for hydrogen bonding in the correct spatial arrangement.

Two common feature MRP4 pharmacophores were reported previously in the same paper; one, based on five protease inhibitors (PI) and, the other based on a more diverse set of ten drugs that were inhibitors based on literature reports. The pharmacophore based on five PIs resulted in a pharmacophore with four hydrogen bond acceptors (HBA), one hydrogen bond donor (HBD), and three hydrophobic features, and the pharmacophore based on ten drugs resulted in two HBAs and a hydrophobic feature. The PI-based pharmacophore featured a large number of features due to the small number of compounds in the training set and their high degree of structural similarity while the pharmacophore based on ten diverse compounds was based on the findings

from multiple labs. The MRP4 pharmacophore we developed is more appropriate for predicting potential MRP4 inhibitors in order to identify the cholestatic potential of compounds because it was based on data from a single laboratory utilizing one assay, was developed based on a diverse set of compounds, and contained a large number of inhibitors.

The other recently reported BSEP pharmacophore (Ritschel et al., 2014) was trained using five compounds of limited structural diversity, and resulted in a pharmacophore that had eight features; four hydrophobic features and two HBAs that had an associated vector feature. The authors found the pharmacophore too stringent so they modified it by making only four hydrophobic features in the core of the pharmacophore essential. The advantage of the BSEP pharmacophore presented in this paper is that it is derived from pharmaceuticals instead of a chemical library and developed with more diverse compounds, which results in a pharmacophore with fewer features, but one more equipped to deal with a larger chemical space. The BSEP pharmacophore reported in this paper, however, is able to align with the hydrophobic and hydrogen bond accepting features of the previously reported BSEP pharmacophore. This suggests that the pharmacophore reported in this paper may be convergent with previously reported pharmacophore, although less stringent.

In conclusion, ongoing studies will utilize these models in an ensemble fashion against drugs that are predicted to be MRP4 or BSEP inhibitors in order to further validate the models. These models, when used in combination, may aid in the *a priori* identification of potential cholestasis-inducing compounds during the early stages of drug development.

Authorship Contributions

Participated in research design: Brouwer, Köck, Swaan, Welch

Conducted experiments: Welch

Performed data analysis: Swaan, Welch

Wrote or contributed to the writing of the manuscript: Welch, Köck, Urban, Brouwer, Swaan

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Footnotes

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Figure Legends

Figure 1. A. Classification of the inhibitors used in development of the MRP4 models. Forty-five drugs were MRP4 inhibitors *only* and 31 drugs were BSEP inhibitors *only*, whereas 26 molecules inhibited both MRP4 *and* BSEP. **B.** Of the compounds in ***A***, 14 MRP4 inhibitors were cholestatic, whereas only one BSEP inhibitor was identified as a cholestatic drug. Of the 26 compounds classified as both MRP4 and BSEP inhibitors, 15 (58%) were clinically identified as cholestatic.

Figure 2. A. MRP4: Principal component analysis (PCA) of the training and test set compounds (257 total) were selected such that they occupy similar areas of the PCA plot. The PCA among training and test set compounds was generated with the following properties: ALogP, molecular weight, molecular fractional polar surface area, number of rings, aromatic rings, rotatable bonds, hydrogen bond acceptors and hydrogen bond donors. The first principal component explains 0.366 of total variance and the second principal component explains 0.272 of total variance; when combined these explain 0.638 of the total variance. The principal components are linear combinations of original descriptors. The dominate descriptors in the principal components are determined by the product of the descriptor coefficient while accounting for the magnitude of the descriptor. The first principal component is dominated by molecular weight, number of hydrogen bond acceptors, and number of rotatable bonds. The second principal component is dominated by molecular fractional polar surface area.

Component 1: = -3.8514 + 0.17609 * [ALogP] + 0.0029942 * [Molecular_Weight] + 0.19241 * [Num_H_Donors] + 0.12966 * [Num_H_Acceptors] + 0.11058 * [Num_RotatableBonds] + 0.21601 * [Num_Rings] + 0.26649 * [Num_AromaticRings] - 0.91018 * [Molecular_FractionalPolarSurfaceArea]
Component 2: = -0.91763 - 0.22028 * [ALogP] + 0.00060715 * [Molecular_Weight] + 0.30038 * [Num_H_Donors] + 0.1113 * [Num_H_Acceptors] + 0.0097512 * [Num_RotatableBonds] - 0.15452 * [Num_Rings] - 0.34347 * [Num_AromaticRings] + 4.3042 * [Molecular_FractionalPolarSurfaceArea]

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B. BSEP: PCA analysis of the training and test sets. The first and second principal components accounted for 0.391 and 0.344 of total variance, respectively. Together they explain 0.735 of the total variance. The first principal component (*x*-axis) is governed by the number of hydrogen bond donors/acceptors and number of rings, whereas the second principal component (*y*-axis) is governed by lipophilicity and the number of aromatic rings. Both principal components are strongly influenced by fractional polar surface area.

Component 1: = -4.0005 + 0.035399 * [ALogP] + 0.0031256 * [Molecular_Weight] + 0.16608 * [Num_H_Donors] + 0.14138 * [Num_H_Acceptors] + 0.11881 * [Num_RotatableBonds] + 0.23456 * [Num_Rings] + 0.23836 * [Num_AromaticRings] + 0.48987 * [Molecular_FractionalPolarSurfaceArea]
Component 2: = -0.12988 + 0.2367 * [ALogP] + 0.00047919 * [Molecular_Weight] - 0.20734 * [Num_H_Donors] - 7.2971e-002 * [Num_H_Acceptors] + 0.019248 * [Num_RotatableBonds] + 0.16242 * [Num_Rings] + 0.31811 * [Num_AromaticRings] - 3.6221 * [Molecular_FractionalPolarSurfaceArea]

PCA analysis comparing the training set of MRP4 (**C**) and BSEP (**D**) to the DrugBank database of FDA-approved drugs.

Figure 3. Favorable and unfavorable molecular features for interactions with MRP4. Each feature is a fragment-like fingerprint, up to 6 bond lengths in diameter, which occurs within the larger parent molecule. The squiggle and asterisks indicate that the bond extends further but does not specify the atom type. The favorable features or "good" features are labeled G1-G5 and the unfavorable features or "bad" features are labeled B1-B5. A feature is considered good if it frequently occurs within compounds that were classified as inhibitors and bad if it frequently occurs in compounds that are non-inhibitors. The large integer after the colon is the unique hash identifier for the shown fingerprint. The Bayesian score is the normalized probability assigned to that feature.

Figure 4. Favorable and unfavorable molecular features for interactions with BSEP.

Figure 5. Pharmacophore model of inhibitors of MRP4-mediated transport of DHEAS. **A.** The pharmacophore model with the measured distances between the 3 features. **B.** The pharmacophore model aligned with chemical groups of two drugs from the training set, clobetasol propionate (orange) and finasteride (lavender). Yellow spheres represent hydrophobic features and the red sphere represents a hydrogen bond acceptor. On the stick model, red represents oxygen atoms, blue represents nitrogen atoms, green represents halogen atoms, and the rest are carbons. Both hydrophobic features align with methyl groups and the hydrogen bond acceptor aligns with a ketone group. Hydrogen atoms are not displayed for clarity.

Figure 6. Receiver operating characteristic (ROC) curve of pharmacophore model of MRP4 inhibitors from virtually screening the test set (N = 77 compounds).

Figure 7. Structural alignment of glucocorticoids clobetasol propionate (orange) and dexamethasone (gray). Clobetasol propionate, a potent MRP4 inhibitor, inhibits MRP4-mediated transport of DHEAS by 101 ± 23%. In contrast, dexamethasone exhibits no significant inhibitory effect (5 ± 34% inhibition). The orange circles indicate identical chemical groups in proximity with each other. On the stick model, red represents oxygen atoms, green represents halogen atoms, and the rest represents carbon atoms.

Figure 8. MRP4: Comparison of calculated LogP of compounds classified as inactive (<21% MRP4 inhibitory activity; n=37) compared to those classified as active (≥21% MRP4 inhibitory activity; n=50). The mean and median LogP values of the inactives are 0.38 and 0.69, respectively, and 3.64 and 3.84, respectively, for the actives.

Figure 9. DHEAS, an MRP4 substrate, and felbinac, an MRP4 inhibitor, aligned with the MRP4 inhibitor pharmacophore. Both compounds also are depicted with their individual

pharmacophore, which shows all possible intermolecular interactions. **A**, DHEAS aligned to the MRP4 pharmacophore. **B**, DHEAS pharmacophore showing all possible intermolecular interactions. **C**, felbinac aligned to the MRP4 pharmacophore. **D**, felbinac pharmacophore showing all possible interactions. Yellow spheres represent hydrophobic features, red spheres represent hydrogen bond acceptor features, the red star represents a negatively ionizable feature, and the purple torus represents an aromatic ring feature. On the stick-models, red represents oxygen atoms, yellow represents phosphorus atoms, and the rest represents carbon atoms. Hydrogen atoms are not displayed for clarity.

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Table 1. Composition of Training and Test Set

Transport Model	MRP4	BSEP
Training Set Total (Inhibitors / Non-inhibitors)	57 (34 / 23)	171 (43 / 128)
Test Set Total (Inhibitors / Non-inhibitors)	29 (17 / 12)	86 (22 / 64)
Pharmacophore Training Subset[a]	9	9
Pharmacophore Test Set[b]	77	247

[a] Subset of drugs from the training set used to develop the pharmacophore
[b] Drugs not included in the pharmacophore training set were moved to the test set

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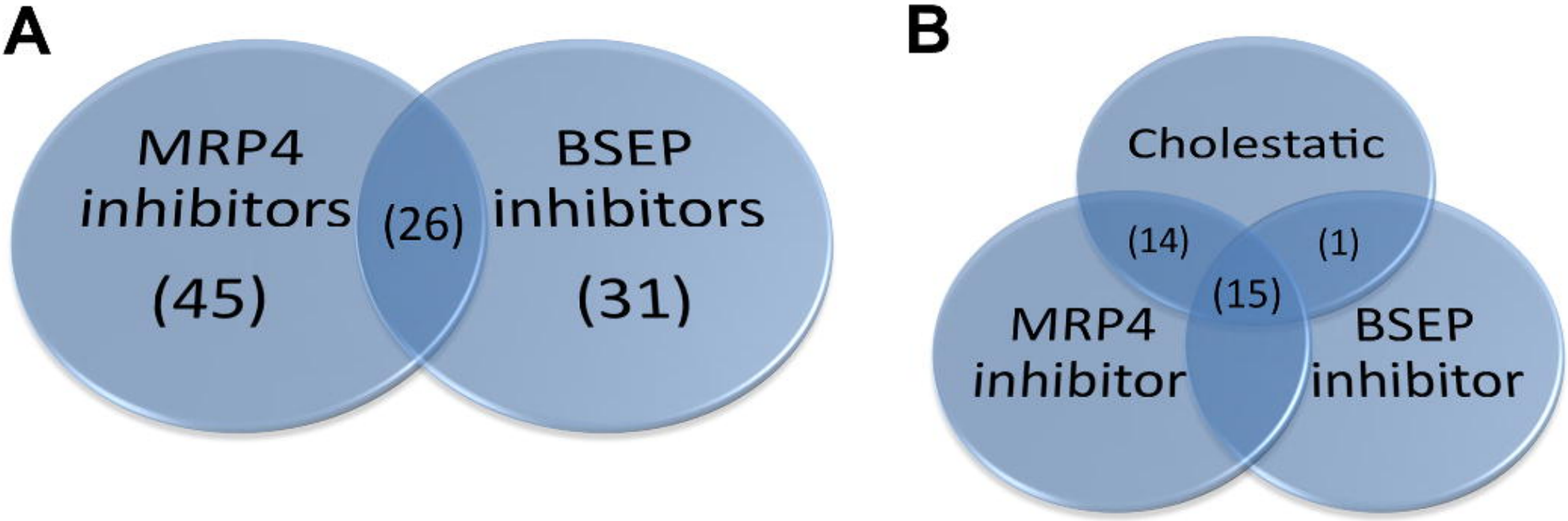
Table 2. Characteristics of Bayesian Models for MRP4 and BSEP Inhibition

Bayesian models	MRP4inhib-ECFP_6	MRP4inhib-FCFP_6	BSEPinhib-ECFP_6	BSEPinhib-FCFP_6
2D-fingerprints	ECFP_6	FCFP_6	ECFP_6	FCFP_6
10-Fold XV ROC AUC [a]	0.816	0.793	0.750	0.759
TP/FN/FP/TN [a]	33/1/1/22	33/1/1/22	43/0/3/125	43/0/5/123
External Validation [b]	0.819	0.838	0.845	0.871
TP/FN/FP/TN [b]	8/9/1/11	10/7/2/10	18/4/15/49	17/5/10/54
SE (%)[b]	47.1	58.8	81.8	77.3
SP (%)[b]	91.7	83.3	76.7	84.4
Q (%)[b]	65.5	69.0	77.9	82.6
MCC [b]	0.4123	0.4216	0.5238	0.5796

[a] Cross-validation-based 'receiver operator curve' area under the curve (XV ROC AUC) based on training set compounds (green shaded region).
[b] Predictive performance validation by test set compounds (blue shaded region). True positive (TP), true negative (TN), false positive (FP), false negative (FN), sensitivity (SE), specificity (SP), overall prediction accuracy (Q), and Matthew's correlation coefficient (MCC)(Ung et al., 2007; Khandelwal et al., 2008). SE =TP/(TP + FN), SP = TN/(TN + FP), Q = (TP + TN)/(TP + TN + FP + FN). MCC = $[(TP * TN) - (FN * FP)]/[(TP + FP)(TP + FN)(TN + FN)(TN + FP)]^{1/2}$

Figure 1


A
MRP4
inhibitors
(45)
(26)
BSEP
inhibitors
(31)
B
Cholestatic
(14)
(1)
(15)
MRP4
inhibitor
BSEP
inhibitor

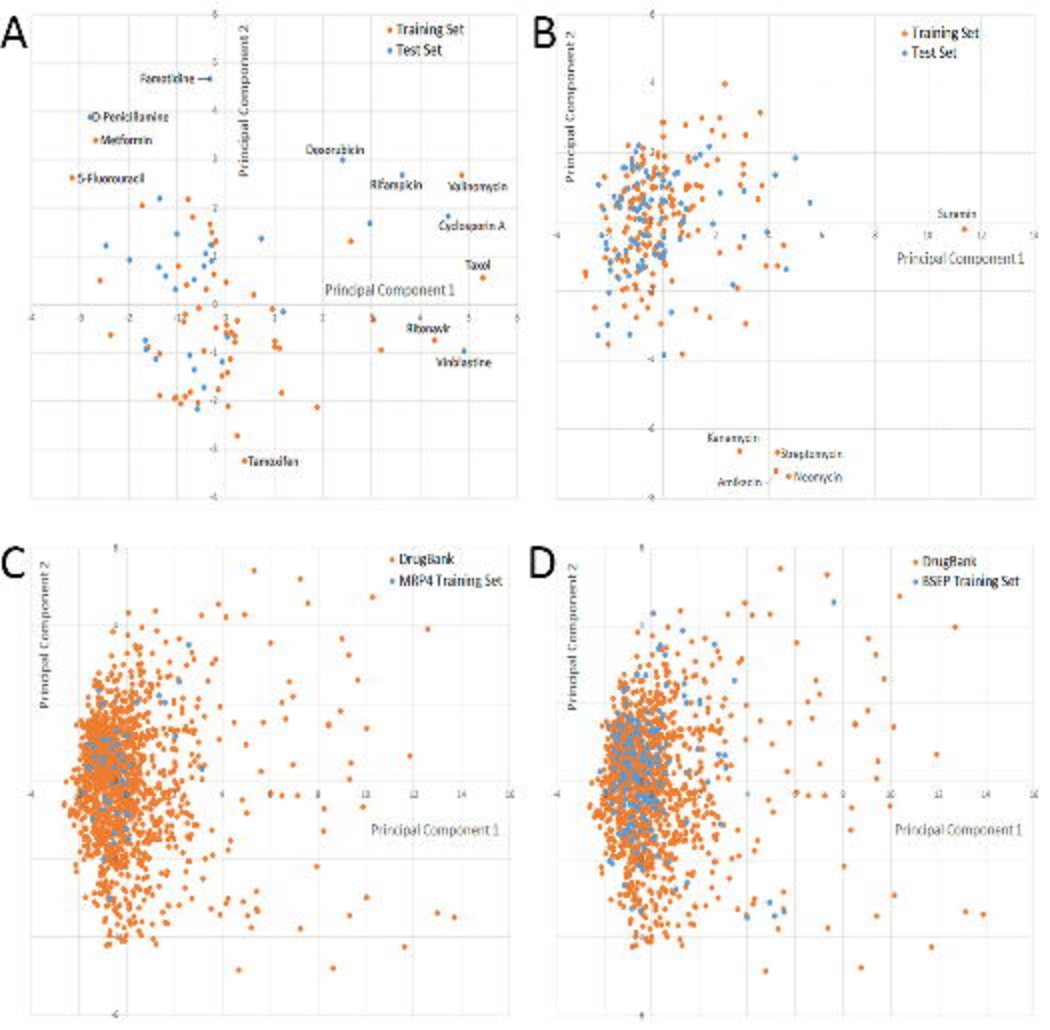
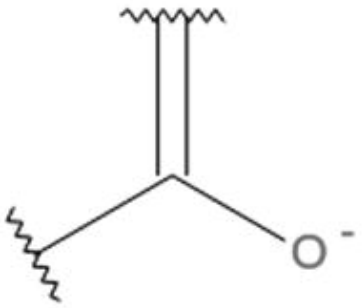
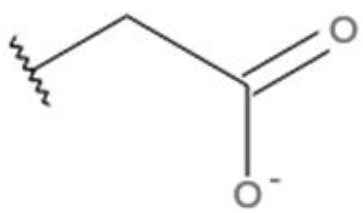

A
Training Set
Test Set
Principal Component 2
Principal Component 1
Famotidine
D-Penicillamine
Metformin
5-Fluorouracil
Doxorubicin
Rifampicin
Valinomycin
Cyclosporin A
Taxol
Rhonwle
Vinblastine
Tamoxifen
B
Training Set
Test Set
Principal Component 2
Principal Component 1
Suramin
Kanamycin
Streptomycin
Amikacin
Neomycin
C
DrugBank
MRP4 Training Set
Principal Component 2
Principal Component 1
D
DrugBank
BSEP Training Set
Principal Component 2
Principal Component 1

Figure 2

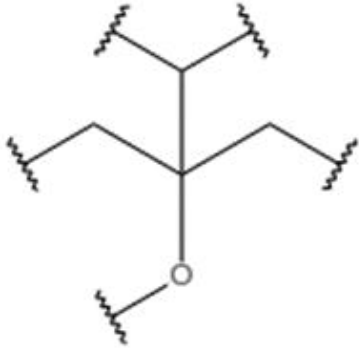
Category BayesianModel-MRP4_inhib_FCFP_6: good features from FCFP_6



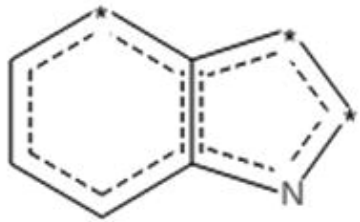
G1: 260714409
6 out of 6 good
Bayesian Score: 0.383



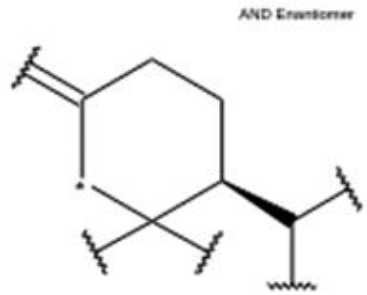
G2: -367494947
6 out of 6 good
Bayesian Score: 0.383



G3: -415216134
5 out of 5 good
Bayesian Score: 0.370

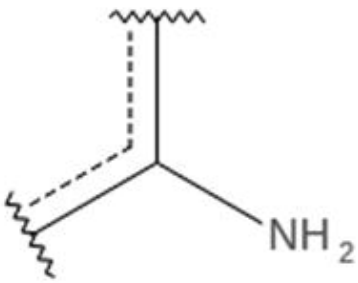


G4: 713358128
5 out of 5 good
Bayesian Score: 0.370

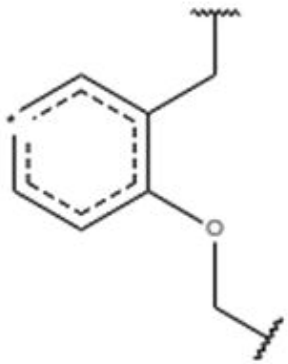


G5: -55265897
5 out of 5 good
Bayesian Score: 0.370

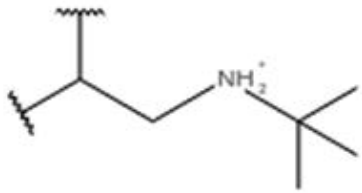
Category BayesianModel-MRP4_inhib_FCFP_6: bad features from FCFP_6



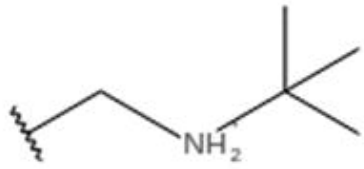
B1: 1069584379
0 out of 4 good
Bayesian Score: -1.257



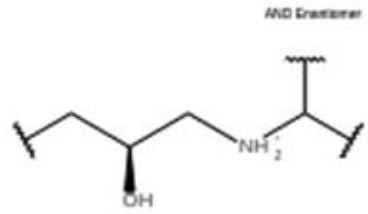
B2: -1099193755
0 out of 4 good
Bayesian Score: -1.257



B3: 692823813
0 out of 3 good
Bayesian Score: -1.060



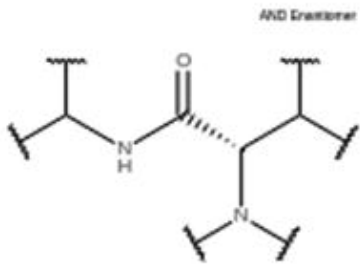
B4: 1995860413
0 out of 3 good
Bayesian Score: -1.060



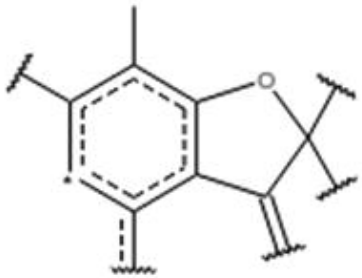
B5: 545355516
0 out of 3 good
Bayesian Score: -1.060

Figure 3

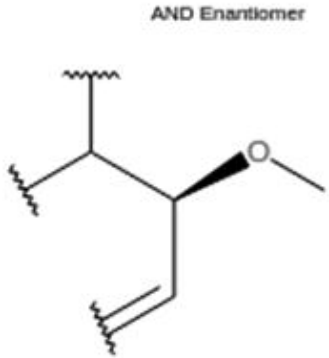
Category BayesianModel-BSEPinhib-FCFP_6: good features from FCFP_6



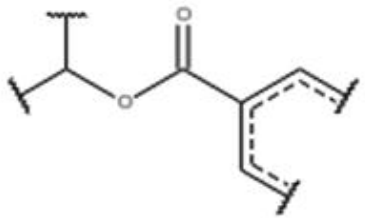
G1: 1862216547
5 out of 5 good
Bayesian Score: 0.816



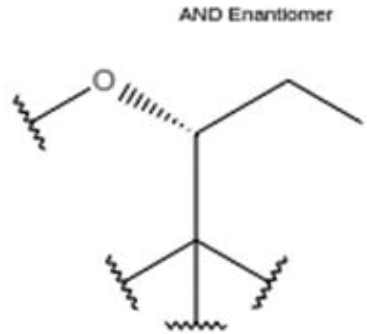
G2: -1096767684
4 out of 4 good
Bayesian Score: 0.767



G3: 346066116
4 out of 4 good
Bayesian Score: 0.767

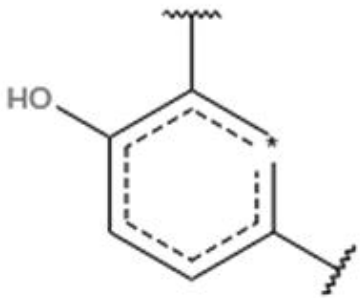


G4: 391786003
4 out of 4 good
Bayesian Score: 0.767

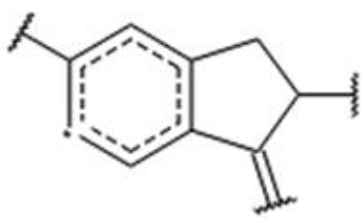


G5: -1576063084
3 out of 3 good
Bayesian Score: 0.697

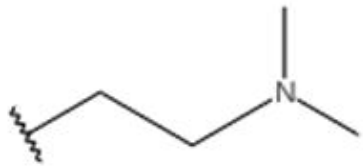
Category BayesianModel-BSEPinhib-FCFP_6: bad features from FCFP_6



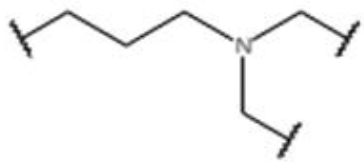
B1: 946589555
0 out of 12 good
Bayesian Score: -1.603



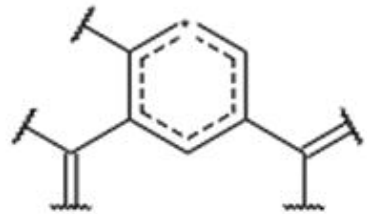
B2: -1943140669
0 out of 10 good
Bayesian Score: -1.460



B3: -14048077
0 out of 8 good
Bayesian Score: -1.293



B4: 309602933
2 out of 26 good
Bayesian Score: -1.163



B5: -451251206
0 out of 6 good
Bayesian Score: -1.093

Figure 4

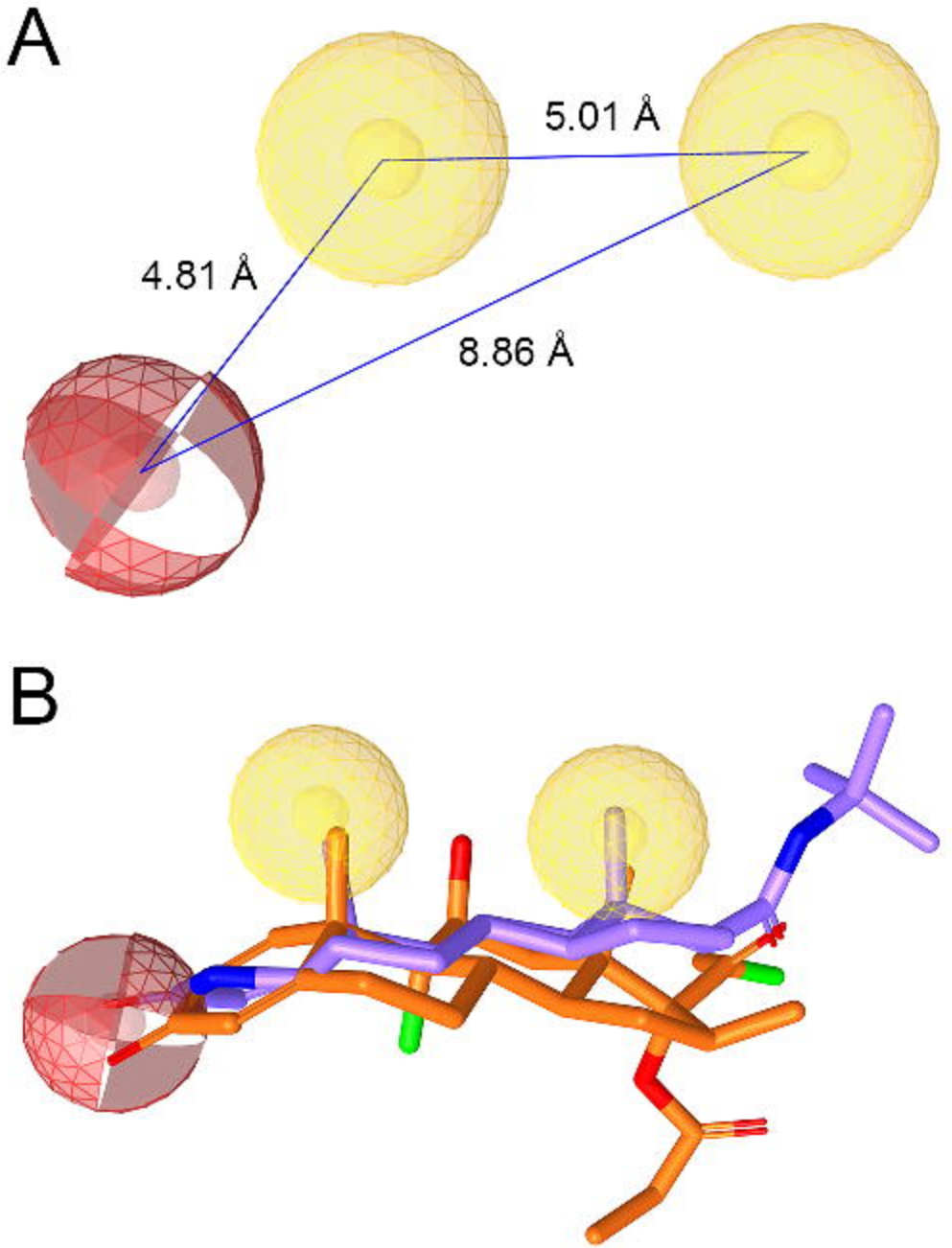


Figure 5

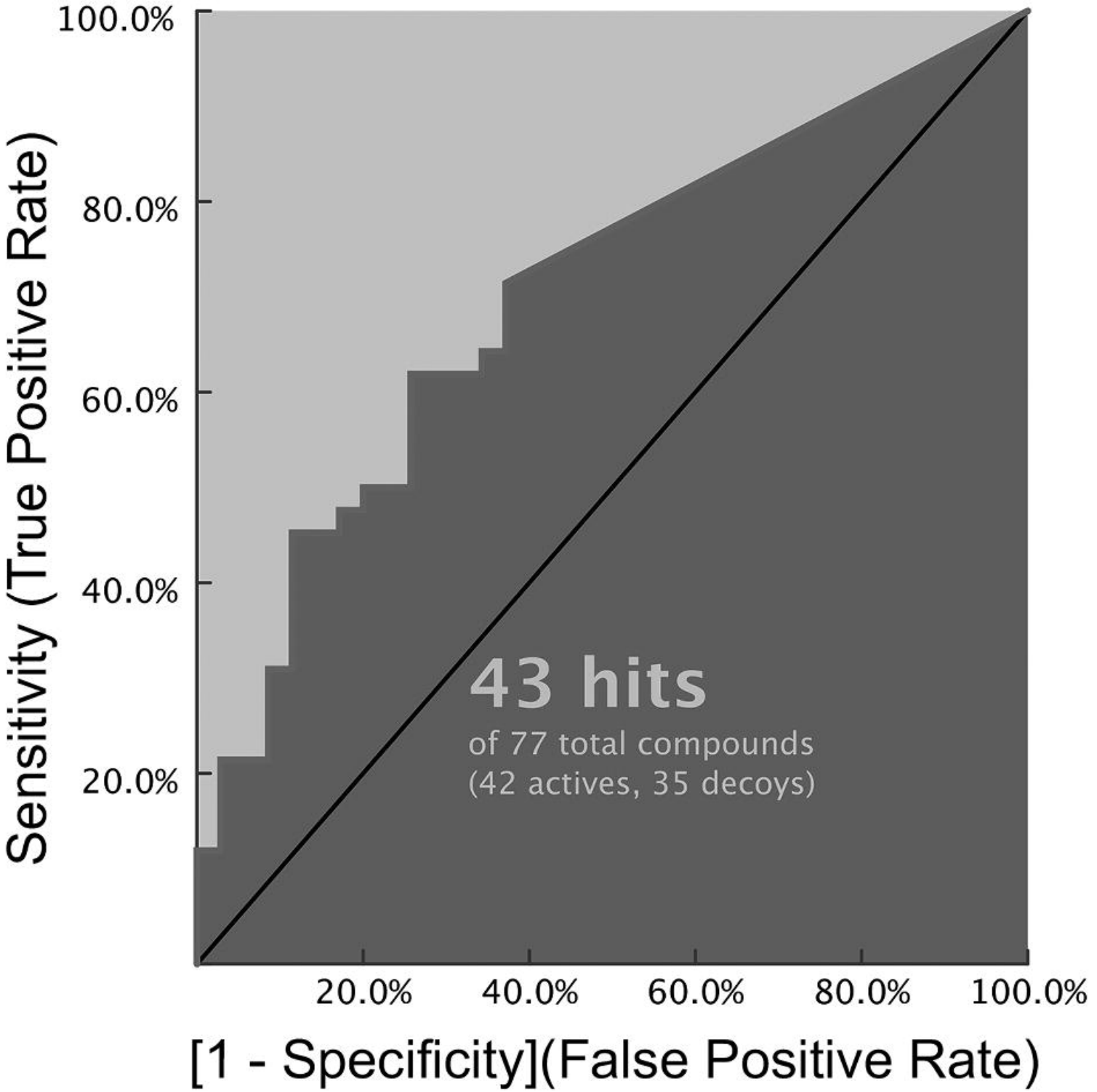

100.0%
80.0%
60.0%
40.0%
20.0%
Sensitivity (True Positive Rate)
43 hits
of 77 total compounds
(42 actives, 35 decoys)
20.0%
40.0%
60.0%
80.0%
100.0%
[1 - Specificity](False Positive Rate)

Figure 6

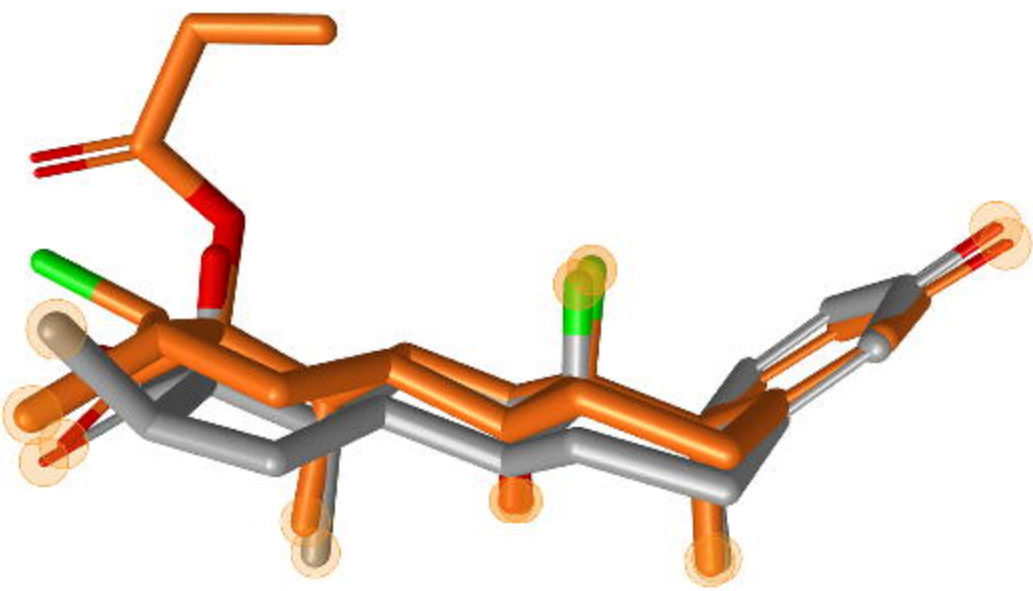


Figure 7

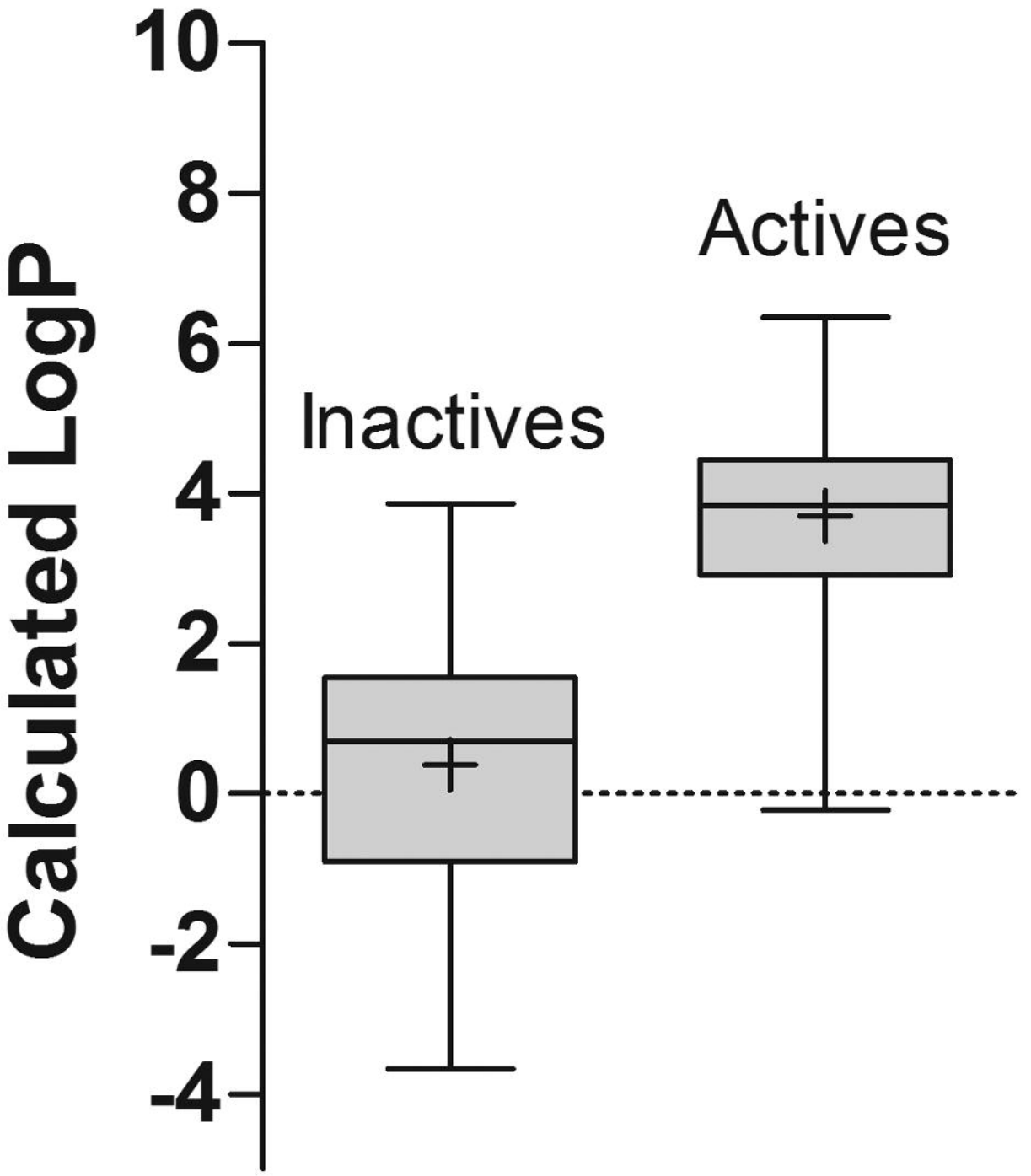


Figure 8

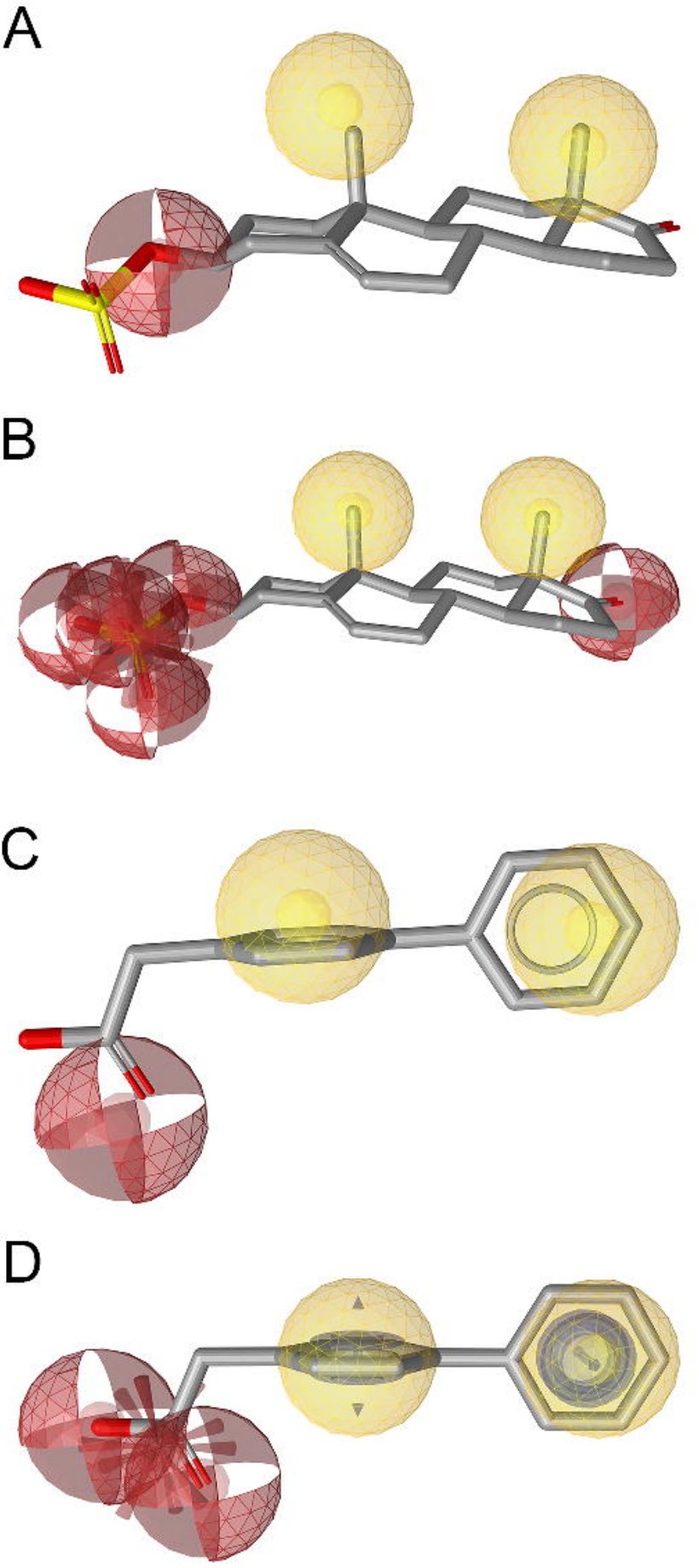


Figure 9