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Quantitative NTCP Pharmacophore and Lack of Association between DILI and NTCP Inhibition

Zhongqi Dong^a, Sean Ekins^b, and James E. Polli^{a,*}

^aDepartment of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Penn Street, Baltimore, Maryland 21201.

^bCollaborations in Chemistry, 5616 Hilltop Needmore Road, Fuquay Varina, NC 27526 USA.

Abstract

The human sodium taurocholate cotransporting polypeptide (NTCP) is a hepatic bile acid transporter. Inhibition of NTCP uptake may potentially also prevent hepatitis B virus (HBV) infection. The first objective was to develop a quantitative pharmacophore for NTCP inhibition. Recent studies showed that hepatotoxic drugs could inhibit bile acid uptake into hepatocytes, without inhibiting canalicular efflux, and cause bile acid elevation in plasma. Hence, a second objective was to examine whether NTCP inhibition is associated with drug induced liver injury (DILI). Twenty-seven drugs from our previous study were used as the training set to develop a quantitative pharmacophore. From secondary screening from a drug database, six retrieved drugs and three drugs not retrieved by the model were tested for NTCP inhibition. Tertiary screening involved drugs known to cause DILI and not cause DILI. Overall, ninety-four drugs were assessed for hepatotoxicity and were assessed relative to NTCP inhibition. The quantitative pharmacophore possessed one hydrogen bond acceptor, one hydrogen bond donor, a hydrophobic feature, and excluded volumes. From 94 drugs, NTCP inhibitors and non-inhibitors were approximately equally distributed across the drugs of most DILI concern, less DILI concern, and no DILI concern, indicating no relationship between NTCP inhibition and DILI risk. Hence, an approach to treat HBV via NTCP inhibition is not expected to be associated with DILI.

Keywords

Sodium Taurocholate Cotransporting Polypeptide (NTCP); quantitative pharmacophore; transporter; hepatotoxicity; drug induced liver injury (DILI)

1. Introduction

Human sodium taurocholate cotransporting polypeptide (NTCP) is a solute carrier protein (*SLC10A1*) that is primarily expressed on the basolateral membrane of hepatocytes. It is

*Corresponding author. Address: University of Maryland, School of Pharmacy, Department of Pharmaceutical Sciences, HSF2 room 623, Baltimore, MD 21201. Tel: 410-706-8292. Fax: 410-706-5017. jpolli@rx.umaryland.edu.

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responsible for transporting bile acids from portal blood into the liver in a sodium dependent manner, resulting in highly efficient reabsorption of bile acids during enterohepatic circulation. In addition to bile acids, NTCP also takes up estrone-3-sulfate and xenobiotics such as rosuvastatin and micafungin, which implies its potential role in drug disposition (Anwer and Stieger, 2013; Ho et al, 2006; Stieger, 2011; Yanni et al., 2010).

A recent study showed that human NTCP may also play an important role in hepatitis B (HBV) infection, which infects approximately 350 million people globally (Yan et al., 2012). Yan et al. found that hepatitis B infection in primary human hepatocytes was significantly reduced by knocking down NTCP. Additionally, exogenous expression of human NTCP in HepG2 cells, which are otherwise resistant to HBV infection, resulted in virus infection. These results indicate that NTCP may be one of the HBV receptors that contribute to human infection. Inhibition of NTCP uptake may potentially also prevent HBV infection (Seeger and Mason, 2013). However, no crystal structure of human NTCP has been elucidated, which hampers NTCP inhibitor identification and development.

Both Greupink et al. and our group have previously developed qualitative ‘common feature pharmacophore’ models. Greupink’s model was developed from four bile acids and estrone sulfate and possessed three hydrophobes and two hydrogen bond acceptors (Greupink et al., 2012), while our pharmacophore was derived from FDA approved drugs from a variety of therapeutic classes, which consisted of two hydrophobes and one hydrogen bond acceptor (Dong et al., 2013).

One objective of the current study was to extend the previous common feature pharmacophore work and develop a quantitative pharmacophore in order to predict inhibitor potency for human NTCP inhibition. This would also aid in elucidating potent NTCP inhibitors, which may be of value for preventing HBV infection. To our knowledge, the model presented here is the first quantitative pharmacophore for human NTCP inhibitors. The quantitative pharmacophore was found to possess one hydrogen bond acceptor, one hydrogen bond donor, a hydrophobic feature and four excluded volumes.

A second objective concerns drug-induced liver injury (DILI), which can terminate compounds in drug development (Ekins et al., 2010). DILI is the most common reason for drug withdrawal from the market (FDA, 2009; Schuster et al., 2005). DILI incorporates many mechanisms for hepatotoxicity. One mechanism for cholestasis and reduced bile acid excretion is inhibition of the Bile Salt Export Pump (BSEP) (Keppler, 2011). However, one study using the human sandwich-cultured hepatocyte model showed that several known (or suspected) hepatotoxic drugs inhibited taurocholate uptake, without inhibiting taurocholate efflux from hepatocytes (Wolf et al., 2010). Hence, hepatotoxicity of these drugs may be attributed to NTCP inhibition via a mechanism distinct from BSEP inhibition. Interestingly, hepatotoxicity that may be mediated via NTCP inhibition may be expected to show increased plasma bile acids, as NTCP takes up most taurocholate into the liver and inhibition of the NTCP may potentially lead to an increase of serum bile salt levels (Stieger, 2011). In fact, a recent study showed that a mutation of NTCP caused reduced taurocholic acid uptake and elevated plasma bile acid concentration (Vaz et al, 2014). Thus, given the observation from Wolf et al., the second objective of the current study was to examine if human NTCP

inhibition is associated with drug-induced hepatotoxicity. A relationship between human NTCP inhibition and DILI would have safety implications for drug candidates aimed at preventing HBV infection via NTCP inhibition.

2. Material and Methods

Figure 1 illustrates the approach to develop a quantitative pharmacophore for human NTCP and evaluate the relationship between drug inhibition of NTCP and drug induced hepatotoxicity. Briefly, 27 drugs with observed K_i values for NTCP inhibition were used to develop a quantitative pharmacophore (Dong et al., 2013). Using this quantitative pharmacophore, a database of 1316 approved drugs was computationally assessed, and *in vitro* secondary screening for NTCP inhibition was conducted for nine compounds (i.e. six compounds retrieved by the model and three compounds that were not retrieved). Tertiary screening involved an additional twenty five drugs that were subjected to the NTCP inhibition assay. A set of 94 drugs that are either orally or parenterally administered was assessed for their hepatotoxicity based on drug label information and were then further correlated with *in vitro* NTCP inhibition.

2.1 Materials

[^3H] Taurocholate (5.0 Ci/mmol) was purchased from PerkinElmer, Inc. (Waltham, MA). Taurocholate was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), penicillin-streptomycin, Geneticin, nonessential amino acid, trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, CA). WST-1 reagent was bought from Roche Applied Science (Indianapolis, IN). All drugs and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Enzo Life Sciences (Farmingdale, NY), AK Scientific

2.2 Inhibition study

Inhibition studies were conducted as previously described (Dong et al., 2013). Briefly human NTCP-HEK293 stable transfected cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum, 100 μM nonessential amino acid, 100 units/mL of penicillin, 100 $\mu\text{g/mL}$ of streptomycin and 1 mg/mL geneticin. Cells were grown at 37 $^{\circ}\text{C}$, 5% CO_2 atmosphere, with 90% relative humidity and fed every two days. After seeding in 24 well biocoated plates at the density of 300,000 cells/well for two days, cells were exposed to donor solution, which was composed of Hank's Balance Salts Solution (HBSS), cold taurocholate (10 μM), 0.5 $\mu\text{Ci/mL}$ [^3H]-taurocholate and test drug. Studies were conducted with and without sodium, as NTCP is a sodium-dependent transporter. After incubation for 5 min, buffer was removed, and cells were washed with ice cold sodium-free buffer where sodium chloride was replaced with tetraethylammonium chloride. Previous studies showed linear uptake of taurocholate into the cells between 0 and 20 min (data not shown). Cells were further lysed by acetonitrile. Lysate was dissolved in phosphate buffered saline (PBS). Radioactivity of each sample was subject to liquid scintillation counting. Taurocholate uptake was measured in parallel no-drug inhibitor studies using 200 μM taurocholate, in the presence and absence of sodium, and served as a positive control for NTCP-HEK293 cell functionality. Furthermore, the difference between

taurocholate flux in the presence and absence of sodium was taken to be the maximal flux of taurocholate without inhibitor, $J_{\max}$.

To determine the “observed K_i ” of NTCP inhibitors, seven drug concentrations were employed. The concentration range for the second series of studies (i.e. observed K_i study) was based on the first series of studies (i.e. estimated K_i value, per below).

Equation 1 was applied to calculate observed K_i

$$J = \frac{J_{\max} S}{K_t (1 + I/K_i) + S} + P_p S \quad \text{eqn 1}$$

where J is the taurocholate flux, $J_{\max}$ is maximal flux of taurocholate without inhibitor. $J_{\max}$ was estimated from taurocholate uptake studies at high taurocholate concentrations (200 μM) where transporter was saturated. On any one occasion, the typical value of $J_{\max}$ was 1.8 pmol/sec/cm^2 . I is the inhibitor concentration, S is the taurocholate concentration, P_p is the taurocholate passive permeability, K_i is the NTCP inhibition constant, and K_t is the NTCP taurocholate Michaelis-Menten constant which is $22.7(\pm 3.4)$ μM (Dong et al., 2013).

To determine the “estimated K_i ” of NTCP inhibitors, only one drug concentration was applied to inhibit taurocholate uptake, which was typically 100 μM . To determine the “estimated K_i ”, the ratio of active uptake with inhibitor versus active uptake without inhibitor was subjected to equation 2:

$$\frac{\text{active uptake with inhibitor}}{\text{active uptake without inhibitor}} = \frac{1 + \frac{[S]}{K_t}}{1 + \frac{[I]}{K_i} + \frac{[S]}{K_t}} \quad \text{eqn 2}$$

“Estimated K_i ” has been shown to be a resource-sparing approach to estimate “observed K_i ” (Dong et al., 2013; Gao et al., 2002; Zheng and Polli, 2010). It should be noted that eqn 1 and 2 were generally applied for the purpose of data reduction rather than mechanistic model identification (Wagner, 1993). Except for bendroflumethiazide, no effort was made to assess the various types of inhibition, which is otherwise difficult at best (Kolhatkar and Polli, 2010). For bendroflumethiazide, the mechanism of NTCP inhibition by the drug was examined by varying taurocholate concentration (5 μM , 10 μM and 20 μM) in the presence of varying bendroflumethiazide concentration (0 μM , 10 μM , 50 μM , and 200 μM).

2.3 Quantitative pharmacophore development and secondary screening

A quantitative pharmacophore was developed using Catalyst in Discovery Studio 3.5 (Accelrys; San Diego, CA). The conformation of the each molecule in the training set was generated using CAESAR (Conformer Algorithm based on Energy Screening and Recursive Buildup) algorithm, which yielded a maximum of 255 conformations per molecule. The calculated potential energy minimum was set to be 20 kcal/mol . Hydrophobic, hydrogen bond acceptor, hydrogen bond donor, and the positive and negative ionizable features were

selected to generate hypotheses. In addition, up to 10 excluded volumes were selected. All other parameters were set to default values. Ten hypotheses were generated and the one with best correlation and lowest RMS deviation was selected to screen a database of 1316 approved drugs (derived from the Approved Drugs mobile application <http://itunes.apple.com/us/app/approved-drugs/id534198253>; <http://chemoinfo.ipmc.cnrs.fr/MOLDB/index.html>) using the FAST algorithm, during which up to 100 conformations per molecule were generated. This yielded 514 drugs fitting to the pharmacophore. Six drugs were purchased and tested for inhibition while three additional drugs that were not retrieved by the model were also tested.

The pharmacophore was further challenged with a set of 42 molecules described in a recent paper from Greupink et al., of which 23 were either inhibitors or steroidal substrates of NTCP (Greupink et al., 2012). Briefly, the molecules were sketched with the Mobile Molecular Datasheet mobile app (Molecular Materials Informatics, Montreal, Canada <https://itunes.apple.com/us/app/mobile-molecular-datasheet/id383661863>). The sdf file was then converted to a 3D database in Discovery Studio using the FAST algorithm, during which up to 100 conformations per molecule were generated. The database was searched with our quantitative pharmacophore (reported here) and our previously reported common features pharmacophore (Dong et al., 2013).

2.4 Cytotoxicity studies

Cytotoxicity studies were conducted as previous described to assess whether drug was toxic to NTCP-HEK293 cells over the duration of an inhibition study, since cytotoxicity mistakenly can be interpreted as inhibition (Zheng et al., 2010). NTCP-HEK293 cells were seeded in 96 well biocoated plates at 50,000 cells/well for two days. After being exposed to drug solution for 10 min, donor solution was removed, and cells were then treated with 100 μ L HBSS buffer with 10 μ L of cell proliferation reagent WST-1. Cells were incubated at 37°C for 4 hr, followed by absorbance measurement at 440nm using a SpectraMax 384 plus plate reader (Molecular Devices; Sunnyvale, CA). MTT assay for 10 min was selected to cover the time course of the inhibition studies, as the goal was to assess if *in vitro* toxicity could be impacting *in vitro* results.

2.5 Assessing DILI potential

Drugs that were either orally or parenterally administered were assessed for their DILI potential, based on label information using method by Chen et al (Chen et al, 2011). Briefly, a set of keywords that are commonly associated with DILI (e.g. cholestasis, hepatotoxicity and jaundice) were searched for in the drug label. Drugs were then divided into four categories: BW (Box warning), WP (Warning and precaution), AR (Adverse reaction), and No mention, depending on the label section where these keywords were identified (or not present). Based on an 8-level system that was developed by Chen et al., DILI severity of each drug was assigned a value from 1 (denoting steatosis) to 8 (denoting fatal hepatotoxicity), or a value of -1 (denoting no DILI) (Chen et al, 2011). Finally, DILI potential was further evaluated based on both the label section that cites hepatotoxicity (e.g. box warning, warning and precaution) and severity of hepatotoxicity for each drug, resulting in assigning each drug as most DILI concern, less DILI concern and no DILI concern.

3. Results

3.1 Quantitative pharmacophore for NTCP inhibitors

Twenty seven drugs with previously reported observed K_i values were used as the training set to develop a quantitative pharmacophore (Dong et al., 2013). These drugs fell into a variety of therapeutic classes, such as antifungal, antihypertensive and anti-inflammatory. K_i values ranged from 7.6 to 599 μM (Table 1). The activity range of the data is considerably less than the 4 logs which is considered ideal for pharmacophore modeling and total cost subsequently was closer to the null cost than the fit cost (Table S1 in supplemental data). The correlation coefficient and normalized RMS deviation between the predicted K_i and observed K_i of the training set was 0.82 and 0.58, respectively (Table S1), which were optimal across all pharmacophores created. All predicted K_i values were within 4-fold of the observed K_i value. Overall, the quantitative pharmacophore was predictive for NTCP inhibitor potency. The quantitative pharmacophore is illustrated in Figure 2, which is composed of one hydrogen bond acceptor, one hydrogen bond donor, one hydrophobic feature, and four excluded volumes. As expected, the resulting model only required a sub-set of the considered factors (i.e. only four of the considered feature types).

One of the 27 drugs is bendroflumethiazide, which was subjected here to determine the mechanism of NTCP analysis. Figure 3 illustrates the Lineweaver-Burk plot for bendroflumethiazide inhibition of taurocholate uptake. Inhibition studies were conducted using various taurocholate concentrations (5 μM , and 10 μM , 20 μM) in the presence of various bendroflumethiazide concentrations (0 μM , 10 μM , 50 μM , and 200 μM). In Figure 3, all four lines intercept at the same y-axis point, suggesting that bendroflumethiazide is a competitive inhibitor for NTCP. In addition, fitting competitive, noncompetitive, and uncompetitive inhibition models indicated that competitive inhibition was the best fitting model (i.e. lowest AIC value). Hence, bendroflumethiazide was identified as a competitive inhibitor of NTCP.

3.2 Secondary screen

The quantitative pharmacophore was used to screen the database of over 1300 approved drugs and retrieved 514 molecules that mapped to the pharmacophore features (Fit values ranged from 0.22 to 4.63). Six retrieved drugs with Fit values ranging from 2.44 to 4.23 (i.e. pioglitazone, lapatinib, glyburide, bosentan, rifampicin, and sorafenib) were selected to test the quantitative pharmacophore, along with three drugs that were not retrieved by the model (i.e. metformin, telmisartan and flutamide; Table 2). Pioglitazone had the highest Fit value among the six drugs and was observed to be the most potent NTCP inhibitor with an observed K_i value of 4.0 μM (Figure 4). For each of the six drugs, the observed K_i was within 8-fold of the predicted K_i . For these six compounds, the correlation coefficient between predicted and observed K_i was 0.81. Among the three drugs that were predicted to not be inhibitors, flutamide and telmisartan inhibited NTCP, with an observed K_i of 113 μM and 60.0 μM , respectively. These results demonstrated the overall prospective predictability of the quantitative pharmacophore.

3.3 Pharmacophore challenge with previously identified NTCP inhibitors

Greupink et al. recently used a pharmacophore based on five steroidal compounds with hydrogen bond acceptors and three hydrophobic features to search databases of commercial molecules, tested them and refined their pharmacophore (Greupink et al., 2012). They identified 18 inhibitors of NTCP after testing 37 molecules (48.6% of compounds tested). All the molecules (N = 42) with discernible structures from the manuscript, of which 23 were inhibitors or substrates, were used as a test set for our quantitative pharmacophore here and our previously described common features pharmacophore (Dong et al., 2013). Our common features pharmacophore returned 37 molecules (20 actives, 54%; Table S2) while the quantitative pharmacophore retrieved 27 molecules (15 actives, 55%; Table S3). This suggests these pharmacophores have a similar overall selectivity as the Greupink pharmacophore, although developed from different training sets and may map different sites in NTCP. The anthraquinone derivative compound 2 from Greupink et al. also mapped to our quantitative pharmacophore (Fit value 3.55, Figure S1).

3.4 Tertiary Screening and Lack of association between NTCP inhibition and DILI

Previously, it was hypothesized that DILI may be associated with inhibition of bile acid uptake into the liver (Wolf et al., 2010). Hence, 25 drugs – a majority of which were known hepatotoxicants - were subjected to subsequent NTCP inhibition screening (i.e. tertiary screening). Inhibition results are listed in Table 3. Table 3 shows screening results of all 94 drugs from studies here and our previous study (Dong et al., 2013).

Additionally, Table 3 lists DILI information about all 94 drugs. DILI potential was determined from the package insert information for each drug. All drugs are orally or parenterally administered (e.g. skin-applied drugs were excluded). In Table 3, there are 20 drugs of most DILI concern, 62 drugs of less DILI concern, and 12 drugs of no DILI concern. These drugs cover 66 common therapeutic drug classes and include seven angiotensin II antagonists, five nucleoside and nucleotide reverse transcriptase inhibitors, four H₂-receptor antagonists, and four protein kinase inhibitors. Of the 94 drugs, 45 drugs were NTCP inhibitors (i.e. estimated K_i less than 300 μM), while 49 drugs were not NTCP inhibitors. Inhibitor and non-inhibitors were about equally distributed across the three levels of DILI concern (Pearson's chi-square p-value = 0.96), indicating no association between NTCP inhibition and DILI. Among most DILI concern drugs, 50% were inhibitors. Among less DILI concern drugs, 47% were inhibitors. Among no DILI concern drugs, 50% were inhibitors. Figure S2 illustrates this distribution. Overall the results indicate no general broad role of human NTCP in DILI based on an approach which combines label information and *in vitro* data.

3.5 Drug cytotoxicity study

In order to exclude the possibility that inhibition of taurocholate uptake by NTCP was caused by drug cytotoxicity, each test drug was subjected to cytotoxicity evaluation, using cell the proliferation reagent WST-1. Study conditions (e.g. drug concentration) simulated the inhibition study, but where cells in the cytotoxicity study were incubated with donor solution for 10 min, rather than 5 min. Results showed that no drug exerted appreciable toxicity to HEK293 cells (i.e. all compounds showed more than 80% cell viability; see Table

S4). On the other hand, HEK293 cells treated with 0.1% Triton X-100 as the positive control exhibited only 67% viability. Cytotoxicity findings support the observation that decreased taurocholate uptake by NTCP in the presence of drugs was due to drug inhibition on NTCP transport instead of drug cytotoxicity.

4. Discussion

4.1 Quantitative pharmacophore

A quantitative pharmacophore was developed from 27 FDA approved drugs using our previous data. In order to improve model prediction and reduce false positives, excluded volumes using NTCP non-inhibitors were incorporated into the quantitative model.

The model was able to predict K_i of NTCP inhibitors with predicted K_i values being within 8-fold of the observed K_i value. In addition, it ranked most of the test compounds (five out of six) correctly based on its Fit value, indicating that our quantitative model is reliable. While the statistics for the model were sub-optimal (Table S1), the best pharmacophore was further evaluated in this study.

We previously developed a qualitative common features pharmacophore (Dong et al., 2013). In the current study, a quantitative pharmacophore was developed. A comparison of these two pharmacophores shows that the hydrogen bond acceptor and one of the hydrophobic features were closely mapped across both pharmacophore approaches. However, the quantitative pharmacophore has an additional hydrophobe and one hydrogen bond acceptor (Figure 5). The slight difference stems from the different training set and algorithms used in model development. Of note, the two pharmacophores indicate that compound charge was not necessary for interaction with NTCP (Dong et al., 2013; Kolhatkar and Polli, 2012). Both these pharmacophores were able to map to compounds identified in a recent study by Greupink et al., even though that pharmacophore had three hydrophobic features and two hydrogen bond acceptors (Greupink et al., 2012). While these three pharmacophores are able to find NTCP inhibitors, they are not particularly selective pharmacophores, which suggests that multiple possibly overlapping pharmacophores can describe interactions with this protein, as has been observed with other transporters (Astorga et al., 2012). The structural diversity of NTCP inhibitors suggests that this transporter is relatively promiscuous and extends beyond bile acid substrates, FDA approved drugs and commercially available small molecules. This warrants further study to map all the likely possible pharmacophores for NTCP and to enable improved prediction accuracy.

4.2 Clinical implication of NTCP inhibitors

A recent study showed that human NTCP may be involved in HBV human infection (Yan et al., 2012). In addition, binding of the pre-S1 domain, the key determinant for entry of HBV, to human NTCP inhibited taurocholate uptake while bile acids inhibited HBV NTCP binding which suggested that the binding site of bile acids and HBV may overlap (Yan et al., 2014). Thus, inhibitors of bile acid transport by NTCP may potentially be drug candidates for preventing HBV infection via NTCP inhibition.

Collectively, from our previous study and the current work here, 45 drugs were found to be NTCP inhibitors including pioglitazone, cyclosporine A, irbesartan, indomethacin, L-carnitine, ezetimibe, and simvastatin. Interestingly, among the NTCP inhibitors identified, indomethacin was also previously shown to reduce hepatitis B e antigen (HBeAg) and HBV-DNA in sera of silent HBV carriers (Bahrami et al., 2005; Kapicio lu et al., 2000). Also, the known NTCP inhibitor L-carnitine showed higher ALT normalization rate in chronic HBV patients (Jun et al., 2013). In addition, a recent *in vitro* study showed that ezetimibe blocks hepatitis B virus infection by targeting a post-entry step(s) in the HBV life cycle, although minor effects on binding and uptake of viral particles were also observed (Lucifora et al., 2013). Moreover, cyclosporine A and simvastatin can inhibit HBV replication (Bader et al., 2010; Xie et al., 2007). These preliminary observations are consistent with the hypothesis that NTCP inhibitors may be potential drug candidates to prevent HBV infection.

4.3 NTCP and hepatotoxicity

In a study by Wolf et al. (2010), the human sandwich-cultured hepatocyte model showed that eight known (or suspected) hepatotoxic drugs inhibited taurocholate uptake, without inhibiting taurocholate efflux from hepatocytes. Hence, hepatotoxicity of these drugs may be attributed to NTCP inhibition via a mechanism distinct from BSEP inhibition. If that is the case, a therapeutic strategy to inhibit NTCP to prevent HBV infection would be suspect to potential hepatotoxicity risk. Thus, using a pharmacophore approach, drugs from a wide range of therapeutic class were screened for NTCP inhibition and subsequently evaluated for DILI potential. DILI potential assessment used the drug label information, which is both publically available and represents comprehensive liver toxicity information (Chen et al., 2011). Here, inhibitors and non-inhibitors were about equally distributed across the most DILI concern, less DILI concern and no DILI concern drugs, suggesting no statistically significant association between NTCP inhibition and DILI risk. Interestingly, if the K_i value to differentiate NTCP inhibitor and non-inhibitor was lowered to 100 μM , inhibitors and non-inhibitors exhibit different associations with DILI (Pearson's chi-square p-value = 0.036), where NTCP inhibitors were less likely to be no DILI concern drugs. However, this aspect needs further investigation since even most DILI concern drugs were still composed of mostly NTCP non-inhibitors (i.e. 55% NTCP non-inhibitors and 45% NTCP inhibitors). Of course, DILI is expected to be mediated by several mechanisms. C_{max}/K_i has been used to predict clinical relevance of P450 inhibition and OCTN2 (Bjornsson et al., 2003; Diao et al., 2009). Hence, the C_{max}/K_i ratio was assessed to predict drug hepatotoxicity (Table S5). The C_{max}/K_i values of the above 94 drugs were calculated, using literature or labeling C_{max} , as well as K_i reported here. Eleven of the 20 drugs with most DILI concern possessed C_{max}/K_i ratio larger than 0.01 (unitless). Twenty-seven of the 62 drugs with less DILI concern possessed C_{max}/K_i ratio larger than 0.01. Six of the 12 drugs with no DILI concern possessed C_{max}/K_i ratio larger than 0.01. Hence, as illustrated in Figure 6, C_{max}/K_i did not distinguish between most DILI concern versus less or no DILI concern (Pearson's chi-square p-value = 0.65) using a cut-off ratio of 0.01. C_{max}/K_i was also not significant using cut-off values of 0.001, 0.005, 0.02, 0.1, and 1.

In addition, the unbound C_{max}/K_i for 78 drugs with protein binding information available from labels were also calculated and evaluated using a cut-off value of 0.0002, 0.0003,

0.0004, 0.0006, 0.001, 0.01. Unbound $C_{\max}/K_i$ could not distinguish between most DILI concern versus less or no DILI concern either (Pearson's chi-square p-value >0.15). Figure S3 plots the distribution using a cut-off value of 0.0002. Overall, there was no general role of human NTCP in DILI. It is possible that DILI may involve other transporters such as OATPs, which can also take up bile acids but have different pharmacophore requirements compared to NTCP (Chang et al., 2005). Of note, there are few reports about diseases that are related to NTCP mutation. One recent clinical study indicated that a functional defect of NTCP caused conjugated hypercholanemia and resulted in high elevation of total bile salts in plasma (Vaz et al, 2014).

5. Conclusions

In summary, a quantitative pharmacophore possessing one hydrogen bond acceptor, one hydrogen bond donor, a hydrophobic feature and four excluded volumes was developed for NTCP inhibition. The pharmacophore showed good predictability and partially overlapped a hydrogen bond acceptor and one of the hydrophobic features of the previously described common features pharmacophore. Our analysis of 94 drugs suggested no relationship between NTCP inhibition and DILI risk, which would indicate that this may not be a concern if NTCP inhibitors were used as a potential treatment for HBV infection. A broader analysis of NTCP inhibitors against HBV infection is therefore warranted and the quantitative pharmacophore developed in this study provides a means to rapidly narrow down potential compounds to test from drug and vendor compound libraries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NTCP	sodium taurocholate cotransporting polypeptide
ASBT	apical sodium dependent bile acid transporter
HEK	human embryonic kidney
WST	water soluble tetrazolium
SCUT	Clinician's Pocket Drug Reference
DILI	drug induced liver injury

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Figure 1.

Flow diagram of approach to develop a quantitative pharmacophore for NTCP inhibition, as well as explore the possible relationship between human NTCP inhibition and drug induced liver injury (DILI). Initial and secondary inhibition studies were conducted yielding observed K_i values, which were then employed to develop and validate a quantitative pharmacophore. Using a larger drug pool with estimated K_i values, the relationship between NTCP and DILI was assessed.

Figure 2.

Quantitative pharmacophore of NTCP inhibitors. The pharmacophore employed 27 observed K_i values. Pharmacophore features one hydrogen bond acceptor (green), one hydrogen bond donor (pink), a hydrophobic feature (cyan), and four excluded volumes (grey). The structure of irbesartan is mapped to the pharmacophore.

Figure 3.

Lineweaver-Burk plot for bendroflumethiazide inhibition on taurocholate uptake. Three concentrations of taurocholate (5, 10 and 20 μM) and four concentrations of bendroflumethiazide (0, 10, 50 and 200 μM) were used. The four regressed lines intercept on the same y-axis point, indicating competitive inhibition.

Figure 4.

Inhibition of NTCP by pioglitazone. Taurocholate uptake into NTCP-HEK cells was reduced in the presence of pioglitazone in a concentration-dependent fashion. Inhibition studies were conducted using seven concentrations of pioglitazone (0–50 μM). Closed circles indicate observed data points, while the solid line indicates model fit. Observed K_i of pioglitazone was 4.04 ± 0.63 μM.

Figure 5.

Comparison of common feature and quantitative pharmacophore of NTCP inhibitors. RMSD was 2.11 Å. The hydrogen bond acceptor and one of the hydrophobic features were closely mapped to each other. The hydrogen bond acceptors were colored green, the hydrogen bond donor was colored pink, hydrophobic features were colored cyan, and excluded volumes were colored grey.

Figure 6. DLJDKDJk

Percent of compounds with a $C_{\max}/K_i$ ratio above 0.01 for each of the following groups: most DILI concern drugs, less DILI concern drugs, and no DILI concern drugs. Regardless of level of DILI concern, about half of drugs showed a ratio greater than 0.01, indicating no association between $C_{\max}/K_i$ for NTCP and DILI.

Table 1

The training set of 27 compounds for the development of the quantitative pharmacophore. Observed K_i values were measured from a range of inhibitor concentrations and found to vary from 7.6 to 599 μM . Predicted K_i value was estimated by the quantitative pharmacophore using CAESAR conformer method. Fit value reflects how well the compound mapped to the model, with high Fit value indicating better fit.

Compounds	Observed K_i (μM) ^a	Predicted K_i (μM) ^b	Pharmacophore Fit value
Bendroflumethiazide	53.0 $\pm$ 6.8	26	4.09
Budesonide	220 $\pm$ 45	200	3.20
Candesartan	233 $\pm$ 37	200	3.20
Cyclosporine A	7.6 $\pm$ 1.1	26	4.09
Diltiazem	599 $\pm$ 212	240	3.13
Doxazosin	35.3 $\pm$ 5.4	32	4.00
Ezetimibe	25.0 $\pm$ 3.2	22	4.16
Fenofibrate	129 $\pm$ 20	200	3.20
Indomethacin	173 $\pm$ 24	210	3.17
Irbesartan	11.9 $\pm$ 0.7	9.6	4.52
Ketoconazole	202 $\pm$ 48	210	3.19
Ketoprofen	321 $\pm$ 57	200	3.20
Losartan	72.1 $\pm$ 5.1	39	3.91
Methylprednisolone	238 $\pm$ 33	200	3.20
Nateglinide	200 $\pm$ 32	200	3.20
Nefazodone	126 $\pm$ 20	200	3.20
Nifedipine	62.6 $\pm$ 10	200	3.20
Nimodipine	190 $\pm$ 31	200	3.20
Nitrendipine	111 $\pm$ 10	200	3.20
Olmesartan	233 $\pm$ 43	120	3.43
Probenecid	544 $\pm$ 217	200	3.20
Prochlorperazine	209 $\pm$ 27	810	2.60
Raloxifene HCl	301 $\pm$ 131	130	3.40
Ritonavir	18.4 $\pm$ 1.6	29	4.04
Rosuvastatin	128 $\pm$ 13	200	3.20
Simvastatin	47.9 $\pm$ 3.7	23	4.15
Tioconazole	148 $\pm$ 34	200	3.20

^a Observed K_i was determined using seven concentrations in the range of 0 to 200 μM . Values denote mean ($\pm$ SEM).

^b Predicted K_i (μM) was predicted by the quantitative pharmacophore.

Table 2

Validation set of nine compounds for the quantitative pharmacophore. Nine observed K_i values were measured from a range of inhibitor concentrations. Predicted K_i value was estimated by the quantitative pharmacophore using FAST conformer method. Compounds are listed in order of Fit value.

Compounds	Observed K_i (μM) ^a	Predicted K_i (μM) ^b	Pharmacophore Fit value
Pioglitazone	4.04±0.63	18.6	4.23
Lapatinib	285±137	44.4	3.86
Glyburide	7.80±1.83	60.6	3.72
Bosentan	145±30	61.7	3.71
Rifampicin	416±98	901	2.55
Sorafenib	3000	1166	2.44
Metformin	3000	No fit	No fit
Telmisartan	60.0±14.6	No fit	No fit
Flutamide	113±14	No fit	No fit

^a Observed K_i was determined using seven concentrations in the range of 0 to 200 μM . Values denote mean ($\pm\text{SEM}$). A value of 3000 μM was assigned for non-inhibitors.

^b Predicted K_i (μM) was predicted by the quantitative pharmacophore using FAST conformer method. Drugs that were not fit by the quantitative pharmacophore were not predicted to inhibit (i.e. high K_i value or no K_i value).

Table 3

DILI characterization of 94 drugs. DILI was evaluated from the product label. Each drug was assigned a DILI severity based on an 8-level system developed by Chen et al. (2011). DILI potential was further assessed based on the label section that cites hepatotoxicity and severity of hepatotoxicity. Each of the 94 drugs are either orally or parenterally administration for human use. Compounds are listed alphabetically in the order of DILI potential (most concern, less concern and, finally, no concern). There are 20, 62, and 12 drugs that are of most DILI concern, less DILI concern, and no DILI concern, respectively.

Drug	Est K_i for NTCP (μM)	DILI-specific labeling ^a	DILI Severity ^b	DILI Potential ^c
Abacavir	1291 $\pm$ 4144	WP	8	Most DILI concern
Acarbose	3000	WP	5	Most DILI concern
Acyclovir	202 $\pm$ 52	AR	5	Less DILI concern
Ampicillin	182 $\pm$ 138	AR	3	Less DILI concern
Atenolol	167 $\pm$ 55	AR	4	Less DILI concern
Atracurium	176 $\pm$ 94	No mention	−1	No DILI concern
Aztreonam	3000	AR	5	Less DILI concern
Bendroflumethiazide	26.2 $\pm$ 1.0	AR	5	Less DILI concern
Bortezomib	3000	WP	7	Most DILI concern
Bosentan	92.9 $\pm$ 14.0	BW	7	Most DILI concern
Budesonide	264 $\pm$ 94	No mention	−1	No DILI concern
Candesartan	145 $\pm$ 44	AR	3	Less DILI concern
Captopril	3000	AR	6	Less DILI concern
Carbamazepine	914 $\pm$ 726	WP	7	Most DILI concern
Cefaclor	3000	AR	3	Less DILI concern
Ceftriaxone	1294 $\pm$ 4335	AR	3	Less DILI concern
Chloroquine	336 $\pm$ 101	AR	3	Less DILI concern
Chlorpromazine	767 $\pm$ 268	AR	2	Less DILI concern
Cimetidine	332 $\pm$ 73	AR	2	Less DILI concern
Ciprofloxacin	526 $\pm$ 399	WP	7	Most DILI concern
Cyclosporine A	10.3 $\pm$ 1.1	WP	2	Less DILI concern
Danazol	592 $\pm$ 187	BW	8	Most DILI concern
Daunorubicin	3000	No mention	−1	No DILI concern
Diltiazem	460 $\pm$ 232	WP	5	Most DILI concern
Doxazosin	41.2 $\pm$ 7.1	AR	2	Less DILI concern
Eletriptan	3000	AR	4	Less DILI concern
Enalapril	3000	AR	4	Less DILI concern
Eprosartan	3000	AR	3	Less DILI concern
Ethosuximide	513 $\pm$ 169	WP	3	Less DILI concern
Ezetimibe	53.8 $\pm$ 3.3	WP	3	Less DILI concern
Famotidine	387 $\pm$ 130	AR	3	Less DILI concern

Drug	Est K_i for NTCP (μ M)	DILI-specific labeling ^a	DILI Severity ^b	DILI Potential ^c
Fenofibrate	211 $\pm$ 60	WP	3	Less DILI concern
Flutamide	84.7 $\pm$ 2.2	BW	8	Most DILI concern
Furosemide	304 $\pm$ 132	AR	2	Less DILI concern
Gefitinib	250 $\pm$ 41	WP	3	Less DILI concern
Gemfibrozil	30.4 $\pm$ 2.9	WP	3	Less DILI concern
Glyburide	16.4 $\pm$ 1.1	AR	5	Less DILI concern
Imatinib	673 $\pm$ 130	WP	8	Most DILI concern
Indomethacin	141 $\pm$ 14	WP	3	Less DILI concern
Irbesartan	12.0 $\pm$ 1.6	AR	5	Less DILI concern
Isradipine	99.5 $\pm$ 14	AR	3	Less DILI concern
Itraconazole	77.1 $\pm$ 38	WP	8	Most DILI concern
Ketoconazole	98.8 $\pm$ 12	BW	8	Most DILI concern
Ketoprofen	361 $\pm$ 178	AR	5	Less DILI concern
ketorolac	254 $\pm$ 193	WP	3	Less DILI concern
Lapatinib	95.5 $\pm$ 38.9	BW	8	Most DILI concern
L-carnitine	181 $\pm$ 60	No mention	-1	No DILI concern
Losartan	105 $\pm$ 9	AR	4	Less DILI concern
Lovastatin	202 $\pm$ 32	WP	3	Less DILI concern
Mannitol	892 $\pm$ 501	No mention	-1	No DILI concern
Metformin	255 $\pm$ 118	No mention	-1	No DILI concern
Methylprednisolone	255 $\pm$ 53	AR	3	Less DILI concern
Metoprolol	340 $\pm$ 52	AR	5	Less DILI concern
Metronidazole	369 $\pm$ 83	No mention	-1	No DILI concern
Nafcillin	2006 $\pm$ 5836	No mention	-1	No DILI concern
Nalidixic acid	1008 $\pm$ 652	AR	2	Less DILI concern
Naproxen	217 $\pm$ 69	WP	3	Less DILI concern
Nateglinide	111 $\pm$ 7	AR	5	Less DILI concern
Nefazodone	100 $\pm$ 8	BW	8	Most DILI concern
Nicardipine	309 $\pm$ 144	AR	3	Less DILI concern
Nifedipine	144 $\pm$ 43	WP	3	Less DILI concern
Nimodipine	86.5 $\pm$ 5.4	AR	5	Less DILI concern
Nizatidine	929 $\pm$ 3318	AR	5	Less DILI concern
Olmesartan	422 $\pm$ 204	AR	4	Less DILI concern
Omeprazole	3000	AR	8	Less DILI concern
Oseltamivir	3000	AR	3	Less DILI concern
Pentamidine isethionate	615 $\pm$ 129	AR	3	Less DILI concern
Pioglitazone	8.30 $\pm$ 0.36	WP	3	Less DILI concern
Prednisolone	335 $\pm$ 177	AR	3	Less DILI concern
Probenecid	435 $\pm$ 114	AR	6	Less DILI concern

Drug	Est K_i for NTCP (μ M)	DILI-specific labeling ^a	DILI Severity ^b	DILI Potential ^c
Procainamide HCl	384 $\pm$ 185	AR	3	Less DILI concern
Prochlorperazine	280 $\pm$ 37	AR	2	Less DILI concern
Propafenone HCl	402 $\pm$ 116	AR	3	Less DILI concern
Quinine	1491 $\pm$ 581	AR	5	Less DILI concern
Raloxifene HCl	321 $\pm$ 139	No mention	-1	No DILI concern
Ranitidine	339 $\pm$ 433	AR	5	Less DILI concern
Reserpine	89.1 $\pm$ 65	No mention	-1	No DILI concern
Ribavirin	193 $\pm$ 223	AR	7	Less DILI concern
Rifampicin	103 $\pm$ 35	WP	8	Most DILI concern
Ritonavir	25.1 $\pm$ 1.6	WP	5	Most DILI concern
Ropinirole	3000	AR	4	Less DILI concern
Rosuvastatin	100 $\pm$ 12	WP	3	Less DILI concern
Simvastatin	57.3 $\pm$ 11	WP	3	Less DILI concern
Sorafenib	41.5 $\pm$ 8.1	WP	8	Most DILI concern
Tamoxifen	87.5 $\pm$ 53.8	WP	6	Most DILI concern
Telmisartan	65.0 $\pm$ 9.1	AR	3	Less DILI concern
Thiothixene	3000	AR	3	Less DILI concern
Triamterene	411 $\pm$ 139	AR	3	Less DILI concern
Valsartan	3000	AR	3	Less DILI concern
Vancomycin	488 $\pm$ 398	No mention	-1	No DILI concern
Vinblastine sulfate	159 $\pm$ 140	No mention	-1	No DILI concern
Warfarin	3000	AR	5	Less DILI concern
Zalcitabine	2035 $\pm$ 1506	BW	8	Most DILI concern
Zidovudine	351 $\pm$ 72	BW	8	Most DILI concern

^aDILI-specific labeling reflects where in the label, if at all, hepatotoxicity is cited. BW denotes box and warning. WP denotes warning and precaution section. AR denotes adverse reaction section.

^bEach of the 94 drugs was assigned a DILI severity from 1 (denoting steatosis) to 8 (denoting fatal hepatotoxicity), or a value of -1 (denoting no DILI).

^cDILI potential was categorized as most DILI concern, less DILI concern and no DILI concern based on 8-level system developed by Chen et al.