In Silico Identification of Potential Cholestasis-Inducing Agents via Modeling of Na+-Dependent Taurocholate Cotransporting Polypeptide Substrate Specificity

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Na+-dependent taurocholate cotransporting polypeptide (NTCP, SLC10A1) is the main transporter facilitating the hepatic uptake of bile acids from the circulation. Consequently, the interaction of xenobiotics, including therapeutic drugs, with the bile acid binding pocket of NTCP could lead to impairment of hepatic bile acid uptake. We pursued a 3D-pharmacophore approach to model the NTCP substrate and inhibitor specificity and investigated whether it is possible to identify compounds with intrinsic NTCP inhibitory properties. Based on known endogenous NTCP substrates, a 3D-pharmacophore model was built, which was subsequently used to screen two virtual libraries together containing the structures of 10 million compounds. Studies with Chinese hamster ovary cells overexpressing human NTCP, human hepatocytes, ex vivo perfused rat livers, and bile duct-cannulated rats were conducted to validate the activity of the virtual screening hits. Modeling yielded a 3D-pharmacophore, consisting of two hydrogen bond acceptors and three hydrophobic features. Six out of 10 structurally diverse compounds selected in the first virtual screening procedure significantly inhibited taurocholate uptake in the NTCP overexpressing cells. For the most potent inhibitor identified, an anthraquinone derivative, this finding was confirmed in human hepatocytes and perfused rat livers. Subsequent structure and activity relationship studies with analogs of this derivative indicated that an appropriate distance between hydrogen bond acceptor features and presence of one or two negative charges appear critical for a successful NTCP interaction. In conclusion, pharmacophore modeling was successfully used to identify compounds that inhibit NTCP. Our approach represents an important first step toward the in silico flagging of potential cholestasis-inducing molecules.

Key Words: NTCP; cholestasis; pharmacophore modeling; drug-induced toxicity.
well-known example being pruritus (Kuiper et al., 2010). Also, asymptomatic hypercholanemia during pregnancy, which is characterized by specifically elevated maternal serum levels of bile acids > 40µM, has been associated with a higher incidence of preterm labor, fetal asphyxia, and third trimester intruterine death (Abu-Hayyeh et al., 2010; Glantz et al., 2004; Williamson et al., 2004). Studies suggest bile acid-induced damage of cell membranes as a potential biochemical mechanism of the observed toxicity during cholestasis, whereas, additionally, bile acids have also been shown to cause mitochondrial dysfunction and modulate second messenger pathways involved in the regulation of cellular metabolism (Bouscarel et al., 1995; Schölmerich et al., 1984; Spivey et al., 1993).

Recently, some drugs and other xenobiotics have been identified as NTCP substrates, based on data obtained in isolated hepatocytes and NTCP recombinant overexpression systems. For example, various statins, the liver function diagnostics bromosulphophthalein and indocyanine green, as well as the antifungal drug micafungin are transported by NTCP (Greupink et al., 2011; Ho et al., 2006; Yanni et al., 2010). Hilgendorf et al. investigated the hepatic mRNA concentration of basolateral and canalicular transporters and found that NTCP expression is similar to that of the well-known and clinically relevant basolateral drug uptake transporter OATP1B1, which is among the most abundantly expressed transporters in human liver (Hilgendorf et al., 2007). Studies in human hepatocytes demonstrated that NTCP can indeed contribute substantially to the overall uptake of drugs in these cells (Ho et al., 2006; Yanni et al., 2010). This suggests that next to cholestasis, xenobiotic-induced inhibition of NTCP could also contribute to clinically relevant transporter-mediated drug-drug interactions and associated drug-induced toxicities.

In this article, we describe a pharmacophore modeling approach, which can assist in identifying chemotypes that may inhibit NTCP. During hit-to-lead and lead-optimization of new candidate drugs, the proposed in silico approach helps to flag unwanted chemotypes with intrinsic NTCP inhibitory activity during the early stages of drug discovery. To develop these models, we employed a computer-assisted ligand-based 3D-pharmacophore modeling approach to describe NTCP inhibitor specificity. The pharmacophore models were subsequently used to identify potential NTCP inhibitors from two virtual compound libraries, containing the chemical structures of close to 10,000,000 commercially available compounds. Subsequently, the candidate molecules that were identified in silico were evaluated experimentally for their activity against NTCP. To this end, we employed in vitro studies with Chinese hamster ovary cells expressing human NTCP (CHO-NTCP) and human hepatocytes, ex vivo rat liver perfusions, and in vivo experiments in rats.

MATERIALS AND METHODS

Chemicals. New chemical structures were identified from the CoCoCo database (maintained by the BioChemInformatics Laboratory, University of Bologna, Italy) and the CAP database (Chemicals Available for Purchase database, Accelrys, San Diego, CA) by screening the databases against the pharmacophore model. Once identified in silico, the selected chemicals were purchased from the registered chemical suppliers indicated in these databases. Both database resources contain only commercially available molecules and can be found through http://cococo.unimore.it and http://accelrys.com. Chemical structures of the compounds are provided in this article. In addition, full chemical names are listed in Supplementary table 1. Prior to conducting in vitro experiments, the purchased chemicals were dissolved in dimethylsulfoxide (DMSO), unless indicated otherwise. 1H-taurocholic acid (specific activity 4.6 Ci/mmol) was obtained from Perkin Elmer (Boston, MA). L-Glutamine and sodium pyruvate were obtained from Invitrogen, whereas DMSO and all other reagents were of the highest obtainable grade available and were purchased from local suppliers.

Experimental animals. Male Sprague Dawley rats (Harlan, Horst, The Netherlands) weighing 220–240 g (in vivo experiments) or 240–300 g (liver perfusions) were housed under a 12-h dark/light cycle at constant humidity and temperature. Animals had free access to tap water and standard lab chow. Prior to conducting experiments, animals were allowed to acclimatize for 1 week after arrival. All experiments were approved by the local committees for care and use of experimental animals and were performed according to strict governmental and international guidelines for the use of experimental animals.

Generation of a common feature pharmacophore and database screening. Molecular modeling studies were performed with the Discovery Studio Software package version 2.5.5 (Accelrys). A common feature, ligand-based pharmacophore model was built based on the molecular structure of five established NTCP substrates, namely the unconjugated bile acid cholic acid, the conjugated bile acids taurocholic acid, tauroursodeoxycholic acid and glycocholic acid, and the non–bile acid estrone-3-sulphate. The 3D chemical structures of the molecules were obtained from the PubChem online database (National Center for Biotechnology Information, Bethesda, MD). Due to the conformational freedom of the studied molecules, functional groups within molecules will not have fixed positions in 3D space. To account for the effect of rotational freedom, for each molecule up to 255 conformers were generated, probing low-energy 3D molecular conformations. To this end, the “best” conformer generation method of the Discovery Studio software package was used, allowing for a maximum energy difference of 20 kcal/mol between conformers. Ten pharmacophore hypotheses were generated, assigning hydrogen bond acceptors (HBAs), hydrogen bond donors, and hydrophobic features. The hypothesis that fitted the structures of the training set best was selected for subsequent database screening. A total of three in silico–screening and in vitro–testing cycles were performed. In the first screening round, the pharmacophore model was used to screen the Accelrys CAP database, containing the 3D structures of over 2.5 million commercially available compounds. The second and third screening rounds were performed in the context of model refinement and are described in a dedicated paragraph below.

Cell culturing of CHO-Parent and CHO-NTCP cells. CHO-NTCP cells stably transfected with the gene encoding human NTCP were obtained from Solvo Biotechnologies (Budapest, Hungary). CHO-parent cells, which were not transfected with the transporters of interest (CHO-P), were used as control cells to estimate non-NTCP cellular uptake. The cell lines were cultured in a humidified atmosphere at 37°C in the presence of 5% CO2. The culture medium consisted of Dulbecco’s modified Eagle’s medium (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum, 4mM l-glutamine, and 1mM sodium pyruvate without antibiotics.

Inhibition studies with CHO cells and human hepatocytes. As a first step to investigate whether molecules identified in silico could indeed interact with NTCP, it was studied whether the compounds could inhibit NTCP-mediated taurocholic acid transport. For inhibition studies, CHO-NTCP cells were incubated with 1µM 1H-taurocholic acid. Stock solutions of the various inhibitors were made in DMSO, and cells were coincubated with test compounds, the final DMSO concentration being 1% in all wells. The incubation period with 1H-taurocholic acid was 2 min because previous studies
have shown that uptake was linear over this time period (Greupink et al., 2011). Uptake of \(^{1}H\)-taurocholic acid in CHO-P cells served as a control to correct for uptake that was not related to NTCP-mediated transport. Data were expressed as the percentage uptake relative to CHO-NTCP cells incubated with vehicle and were plotted against the nominal inhibitor concentration. In the case of dose-response curves, a one-site binding model with variable slope was fitted to the data in order to estimate IC\(_{50}\) values. In case of uptake studies aiming to assess the apparent \(K_{m}\) and \(V_{max}\) of taurocholic acid uptake in the presence or absence of inhibitors, the data were analyzed using the standard Michaelis-Menten model, as described earlier (Greupink et al., 2011).

Studies in human hepatocytes were conducted with cryopreserved hepatocytes purchased from Life Technologies (Breda, The Netherlands). Hepatocytes were suspended in Krebs-Henseleit buffer to a final concentration of 1 million cells per ml. Cells were incubated at 37°C with 1µM of taurocholic acid, in the absence or presence of sodium for various time points, together with increasing concentration of the test compound. Incubations were stopped using the oil centrifugation method as described earlier (Yabe et al., 2011). Next to the incubations at 37°C, parallel incubations were performed at 4°C. The transporter-mediated uptake of radiolabeled taurocholic acid into hepatocytes was calculated by subtracting uptake at 4°C from the values observed at 37°C.

Reefinement of pharmacophore models and subsequent database screenings. The initial pharmacophore that was derived from known natural substrates/competitive inhibitors was refined using compounds 1–16. Inhibitory activities for compounds 1–16 were defined, classifying compounds 2, 15, and 16 as very active, compounds 1, 3, 5, 6, and 13 as moderately active, and compounds 4, 7–12, and 14 as poorly active/inactive. The original pharmacophore was refined by assigning excluded volumes to the model. The refined and the nonrefined pharmacophore were used to screen the CoCoCo database, containing close to 7 million commercially available compounds. For each pharmacophore, the 5000 best scoring compounds were retrieved from the screen and were subjected to a first filtering (selecting compounds with a MW < 700 and a cLogD < 3.5), using Pipeline Pilot Professional version 7.5 (Accelrys). Subsequently, the following three subsets were combined: the top 250 hits of the original pharmacophore, the top 250 hits of the refined pharmacophore, and all hits retrieved by both pharmacophores, regardless of their goodness of fit. All hits were mapped against the refined pharmacophore using flexible fitting. After Lipinski filtering, the top 150 compounds were selected for further analysis and testing. In addition to this first refinement, a second refinement of the initial pharmacophore using the exact same approach was conducted as described above but now using only the anionic compounds for model optimization. Database screening was also performed in a similar way as described for the first refined pharmacophore. Additionally, a criterion was added that retrieved hit compounds with a single or double negative charge at pH = 7.4.

Cholestatic effects of compound 2 in perfused rat liver. In brief, male Sprague Dawley rats were anesthetized with isoflurane and the portal vein, bile duct, and liver vein were cannulated. Liver perfusions were performed in a single-pass setting. The perfusate consisted of warm Krebs-Henseleit buffer, saturated with a 95%/5% mixture of O\(_2\)/CO\(_2\) and contained 10µM taurocholic acid, to maintain bile flow. In addition, \(^{1}H\)-taurocholic acid (1200 dpm/ml) was added to monitor the effect of the test compound on the clearance of taurocholate. Throughout the experiment, samples of the bile and venous outflow were collected in 5-min intervals. Livers were equilibrated for 20min, and then the test compound was added to the perfusate. Radioactivity in the perfusate, bile, and venous outflow were measured via scintillation counting. The amount of radioactivity in the bile and venous outflows was expressed as percentage of the amount entering the cannulated portal vein (perfusate). To assess the effect of protein binding, incubations were performed in the presence or absence of 1% bovine serum albumin (BSA) in the perfusate.

Cholestatic effects of compound 2 in rats in vivo. Compound 2 was also studied for its in vivo cholestatic potential in rats. Animals were anesthetized with O\(_2\)/N\(_2\)/O\(_2\)isoflurane, and the common bile duct was cannulated to allow monitoring of the excretion of compounds into the bile. The rats were kept under anesthesia for the duration of the experiment. A tracer dose of \(^{1}H\)-labeled taurocholic acid was administered via the penis vein (75 pCi/kg), with or without test compound (5 mg/kg). During the experiment, bile flow was spontaneous and the outflow of the cannula was collected in 5-min intervals. At the end of the experiment, animals were euthanized via cardiac puncture. During experiments, the body temperature of the animals was maintained at 37°C by placing the animals on thermostatic pads and covering them with isolating tissues. Bile samples were immediately stored at −20°C. For analysis, 10 µl of homogenized bile was mixed with 4 ml of scintillation fluid and analyzed for \(^{1}H\)-taurocholic acid content. Injection solutions were also analyzed, and the excretion of \(^{1}H\)-taurocholic acid was expressed as a percentage of the injected dose.

Statistical analysis. In all graphs, data are expressed as mean ± SEM. Subsequent statistical analysis and curve fitting were performed with the Graphpad Prism 5 software package (version 5.02, GraphPad Software Inc.). One-way ANOVA in conjunction with Dunnett’s post hoc test was performed to assess statistical significance of differences when multiple groups were compared. When two groups were compared, data were subjected to the two-sided Student’s t-test. Differences were considered statistically significant when \(p < 0.05\).

RESULTS

Pharmacophore Modeling

To identify the features that are relevant for a successful interaction with the bile acid binding site of NTCP, we built a ligand-based common feature pharmacophore model based on five known NTCP substrates (Fig. 1A). Note that these compounds are also inhibitors of each other’s NTCP-mediated uptake by virtue of binding to the same binding pocket. The resulting pharmacophore model (Fig. 1B) consisted of five features. Two of which are HBAs, the smaller sphere (green) indicating the point of origin of the H-bond and the larger sphere indicating the vector of the H-bond. The two HBA features are separated by three hydrophobic features (cyan spheres). In Figure 1C, the mapping of the prototypical NTCP substrate taurocholic acid to the pharmacophore can be seen. The sulfonic acid group that terminates the aliphatic side chain on the D ring and the hydroxyl group in the 7-position on the steroid skeleton map to the HBAs, whereas the carbon atoms of the steroid backbone correspond to the hydrophobic features.

Initial Database Screening

In the first virtual screening round, of the 2.5 million molecules present in the CAP database, approximately 10,000 molecules fitted the pharmacophore model well (mapping to all five features of the pharmacophore). This corresponds to around 0.4% of the total number of compounds present in the database. To assure that the molecules put forward for experimental validation would be soluble enough in physiological media, subsequent selection steps were performed to select only molecules with a cLogD < 3.5 and MW < 700Da. Next, the remaining molecules were clustered by chemical similarity into 70 clusters of 10 hits each. From these different clusters, an initial set of 10 chemically diverse molecules was manually selected to evaluate whether the molecules could interact with NTCP. The 2D chemical structures of the selected molecules...
are depicted in Figure 2A, full chemical names and goodness of fit to the pharmacophore model are listed in Supplementary table 1.

Inhibition Studies With CHO-P and CHO-NTCP Cells

As a first step to study whether the selected molecules could interact with NTCP, we investigated their inhibitory effect on NTCP-mediated transport of 3H-labeled taurocholic acid. In Figure 2B, results are displayed that were obtained in the CHO-NTCP cell model, and it can be seen that 6 out of 10 molecules significantly inhibited taurocholic acid uptake at a concentration of 100 µM. Compared with control cells, the three most potent inhibitors, 2-(4-((4-methyl-3-sulfamoylphenyl)phthalazinyl)amino)phenyl)acetamide (compound 1), 5-methyl-2-[[5-(4-methyl-2-sulfonatoanilino)-9,10-dioxoanthracen-1-yl]amino]benzenesulfonate (compound 2), and N,N′-dodeca-methylene-bis(hexahydrottaurocholic acid) (compound 3), reduced taurocholic acid uptake by 69 ± 3, 96.3 ± 0.6, and 54 ± 3%, respectively (p < 0.05). Interestingly, 2′,2″-(hexamethylenedioxy)-diazetanilide (compound 4) and 1-[1-(2,4-dimethoxybenzyl)-4-piperidiny]-4-(2-methoxyphenyl)piperazine (compound 9) stimulated taurocholate uptake to 144 ± 11 and 137 ± 19% of control cells, respectively.

For compounds 1–3, we investigated the mode of inhibition. To this end, the concentration dependency of 3H-taurocholic acid uptake by NTCP was studied in the absence or presence of compound 1, 2, or 3. Data revealed that in the presence of all three compounds the uptake curve of taurocholic acid shifted to the right (Figs. 3A–C). Coincubation with 100 µM of compound 1 increased the apparent $K_m$ for taurocholic acid from 13 ± 4 to 37 ± 7 µM (absence of test compound) to 37 ± 7 µM. Coincubation with 3 or 10 µM of compound 2 increased the apparent $K_m$ for the taurocholate-NTCP interaction from 14 ± 4 to 35 ± 2 and 88 ± 19.
Fig. 2. (A) Chemical structures of the 10 molecules selected for first round of *in vitro* testing. (B) Effect of test compounds 1–10 (100 µM) on NTCP-mediated 3H-taurocholic acid uptake (1 µM for 2 min) by CHO-NTCP cells. Open bars indicate absence of inhibition or stimulation of NTCP-mediated taurocholic acid transport; closed bars indicate a significant inhibition of taurocholic acid transport. Data are expressed as a percentage of control and represent the mean ± SEM of three separate experiments each performed in duplicate or triplicate. * indicates $p < 0.05$ compared with control. TC, taurocholic acid.
Finally, in the presence of 100 µM of compound 3, the apparent $K_m$ of NTCP for taurocholic acid increased to 42 ± 6 µM. In all cases, the observed maximum taurocholic acid transport rates were not decreased, which indicates that the molecules inhibit NTCP in a competitive manner.

**Structure Activity Relationship of Anthraquinone Derivatives**

To validate the pharmacophore, we next investigated whether analogues of compound 2 that failed to hit all features of the pharmacophore were also less-potent inhibitors of NTCP-mediated taurocholic acid uptake. Therefore, we studied whether successful mapping to both HBA features is required for molecules to be active. In Figure 4A, the chemical structures of the selected molecules are displayed. Figure 4B depicts a graphical representation of how the compounds map to the pharmacophore model.

For compound 2, the IC50 for inhibition of NTCP-mediated taurocholic acid uptake was 3.2 ± 1.0 µM (Fig. 5). The two analogues 5-methyl-2-[[4-(4-methyl-2-sulfoanilino)-9,10-dioxoanthracen-1-yl]amino]benzenesulfonic acid (compound 15) and 2,2'-[(9,10-dihydro-5,8-dihydroxy-9,10-dioxo-1,4-anthracenediyli)dimino]bis[5-methyl-]benzenesulfonic acid (compound 16) that had slightly different structures than compound 2 but did match with all features of the pharmacophore displayed a similar potency as compound 2. The IC50 values of compound 15 and compound 16 were determined to be 4.3 ± 1.7 µM and 5.5 ± 1.7 µM, respectively. In contrast, 1,4-bis(4-methylanilino)anthracene-9,10-dione (compound 11), which is very similar to compound 15, but is lacking the sulfonic acid substituents to cover the two HBA features of the pharmacophore model, was not active up to the highest concentration tested (100 µM). 9,10-dioxoanthracene-1-sulfonic acid (compound 12), containing only one sulfonic acid group conjugated directly to the anthraquinone skeleton, was inactive, and also 1,5-Anthraquinonedisulfonic acid (compound 14), which does possess two sulfonic acid groups but not at an appropriate distance to cover both HBA features, is inactive up to 100 µM. Also 5-methyl-2-[[4-(methylamino)-9,10-dioxoanthracen-1-yl]amino]benzenesulfonic acid (compound 13), which contains only one sulfonic acid group conjugated to a single benzyl substituent of the anthraquinone skeleton, was found to be less potent than the compounds that hit all features of the pharmacophore. The IC50 of compound 13 was 12.2 ± 1.4 µM, which is significantly higher ($p < 0.05$) than the IC50 values of compound 2, compound 15, and compound 16.

**Model Refinement**

Based on the new activity data obtained with compounds 1–16, the initial pharmacophore was refined by adding 22 excluded volumes (Fig. 6B). Both the old and the new pharmacophore were used to screen the CoCoCoCo database, and the top 5000 compounds for each pharmacophore were retrieved.
Additional filtering for compounds with a molecular weight < 700 g/mol and a cLogD < 3.5 reduced the number of hits by approximately 50%. Combination of the top 250 molecules obtained by screening with the original and the refined model, with the hits obtained with both pharmacophores, yielded 616 hits. Compounds that did not comply with Lipinski’s rule of five were filtered out from the top 150 compounds by manual selection. In this final step, compound selection was mainly driven by vendor availability (Fig. 6A). Surprisingly, only a very small number of compounds displayed inhibition of NTCP as tested using CHO-NTCP cells. Moreover, the compounds that were active also
only showed a limited efficacy at the tested concentration of 100 µM (Fig. 6C).

Upon analysis of the chemical structures obtained from the in silico screening round with this pharmacophore, we found uncharged molecules to be over-represented compared with negatively charged compounds. To explore whether the presence of 1 or 2 negative charges may result in a better interaction with NTCP, we conducted a second refinement of the original pharmacophore, now only taking into account the active molecules with a net negative charge at pH = 7.4. This yielded a third pharmacophore model, which is depicted in Figure 7B. The molecules obtained from the in silico screening round with this pharmacophore are depicted in Figure 7A. Compound 31 did not dissolve in the assay buffer and could not be tested. However, 4 out of the 7 remaining molecules exhibited significant inhibitory activity (p < 0.05, compared with control) against taurocholic acid uptake in CHO-NTCP cells, displaying more than 50% up to complete inhibition (Fig. 7C).

Effects of Compound 2 in Cryopreserved Human Hepatocytes

For the most potent NTCP inhibitor, compound 2, we investigated whether the observations made in the recombinant system expressing the transporter also translated to cryopreserved human hepatocytes. In line with the high NTCP affinity observed in the CHO recombinant expression model, we found that compound 2 also potently inhibited sodium-dependent taurocholic acid in human hepatocytes (Fig. 8). Incubation with 2 µM of compound 2 reduced taurocholic acid uptake from 77 ± 5 to 31 ± 5 pmol taurocholate per million hepatocytes (p < 0.05), whereas 20 and 200 µM of compound 2 reduced taurocholic acid uptake to 18.0 ± 0.4 and 13.0 ± 0.3 pmol taurocholate per million hepatocytes (p < 0.05), respectively. These are similar to the levels observed at 4°C or when incubated in the absence of sodium.

Cholestatic Effects of Compound 2 in Perfused Rat Liver and in Rats In Vivo

To further extrapolate the in vitro findings to a more physiological setting, we conducted an ex vivo rat liver perfusion study and an in vivo rat study to investigate whether compound 2 could reduce the biliary clearance of radiolabeled taurocholic acid. In Figure 9, the results of the liver perfusion experiments are shown. The effect of compound 2 on 3H-labeled taurocholic acid clearance was measured in a single-pass perfusion setting. After a stabilization period of 20 min, compound 2 was added to the perfusion medium at end concentrations of 10 and 100 µM. In Figure 9A, it can be seen that addition of compound 2 to the perfusion medium did not influence venous outflow, but that the amount of taurocholate in the venous outflow relative to inflow increased dramatically in the presence of 100 µM of compound 2 compared with control (Fig. 9B). Moreover, 100 µM led to cholestasis as reflected by a reduced bile flow (Fig. 9C) and a corresponding reduction in 3H-taurocholic acid excretion (Fig. 9D). In the liver perfusion experiments, we additionally found that addition of 1% BSA to the perfusion medium totally abolished the cholestatic effect of 100 µM of compound 2.

Intravenous administration of 5 mg/kg of compound 2 to bile duct–cannulated rats did not result in a statistically significant effect on bile flow (Fig. 10A) nor did it affect the biliary taurocholic acid excretion profile compared with control animals (Fig. 10B). Pilot studies showed that the plasma concentration of compound 2 approximated 100 µM (5 min after an iv bolus injection, we measured a total plasma concentration of 115 µM).
FIG. 6. (A) Compounds that were retrieved from the CoCoCo database using the refined pharmacophore and were tested for NTCP inhibitory activity in CHO-NTCP cells. (B) Graphical representation of the refined pharmacophore used to retrieve the molecules depicted in (A). In the refined model, 22 excluded volumes were added, based on experimental data obtained with molecules 1–16. (C) Effect of test compounds 17–29 (100 µM) on NTCP-mediated ³H-taurocholic acid uptake (1 µM for 2 min) by CHO-NTCP cells. Compound 27 did not dissolve in the uptake buffer and could not be tested. Open bars indicate an absence of inhibition or a stimulation of NTCP-mediated taurocholic acid transport; closed bars indicate a significant inhibition of taurocholic acid transport. Data are expressed as a percentage of control and represent the mean ± SEM of three separate experiments each performed in duplicate or triplicate. * indicates p < 0.05 compared with control. TC, taurocholic acid.
FIG. 7. (A) Chemical structures of the compounds that were retrieved from the CoCoCo database and were tested for NTCP inhibitory activity in CHO-NTCP cells. (B) Graphical representation of the third pharmacophore used to retrieve molecules depicted in (A). The model results from the second refinement step, using only activity data of the anionic compounds. (C) Effect of test compounds 30–37 (100 µM) on NTCP-mediated 3H-taurocholic acid uptake (1 µM for 2 min) by CHO-NTCP cells. Compound 31 did not dissolve in the uptake buffer and was not tested. Open bars indicate absence of inhibition or stimulation of NTCP-mediated taurocholic acid transport; closed bars indicate a significant inhibition of taurocholic acid transport. Data are expressed as percentage of control and represent the mean ± SEM of an experiment performed in triplicate. * indicates p < 0.05 compared with control. TC, taurocholic acid.
This should be a plasma concentration that results in Ntcp inhibition. Based on the observations from liver perfusion experiments, the lack of *in vivo* cholestatic activity is therefore likely to be the result of high protein binding, leading to unbound plasma concentrations of compound 2 that are substantially lower than the concentrations required for Ntcp inhibition.

**DISCUSSION AND CONCLUSIONS**

Here, we describe a pharmacophore model capable of identifying NTCP inhibitors from large virtual compound databases. Inhibition of NTCP function by xenobiotics will interfere with bile acid homeostasis and may lead to cholestasis. The flagging of chemical scaffolds with intrinsic NTCP inhibitory activity early during drug discovery is relevant because it can be used to identify chemotypes that may possess undesired off-target effects against this transporter. The substrate specificity of NTCP with regard to bile acid–like substrates has been studied, using a series of structurally similar bile acid analogs, but this did not culminate in the generation of a model that could identify non-bile acid–like structures (Kramer et al., 1999; Meier et al., 1997; Pauli-Magnus and Meier, 2006). To date, interaction studies with NTCP have therefore been mainly confined to *in vitro* screening of a large number of compounds (Kemp et al., 2005; Kim et al., 1999; Kouzuki et al., 2000). To our knowledge, our models are the first 3D ligand-based pharmacophore models to describe the substrate/inhibitor specificity of NTCP of human origin. Also, we are the first to apply and validate

**FIG. 8.** Effect of compound 2 on *H*-taurocholic acid uptake by cryopreserved human hepatocytes. Data represent the mean ± SEM of an experiment performed in fourfold. * indicates $p < 0.05$ compared with control.

**FIG. 9.** Results from *ex vivo* liver perfusion experiments. Effect of compound 2 on venous outflow (A) and on the amount of *H*-taurocholic acid measured in the venous outflow (B). The effect of compound 2 on bile flow (C) and on the amount of *H*-taurocholic acid excreted in bile (D). All data represent the mean of two liver preparations per condition ± the range of the observations, except the data obtained in the presence of 1% BSA, which is derived from one liver.
such models by screening large virtual compound databases to successfully identify currently unknown NTCP inhibitors that are not of bile acid origin.

Although the model only consists of two types of pharmacophoric features, namely three hydrophobic and two HBA features, we expected that in the first virtual screening quite a large number of compounds would fit the pharmacophore. To our surprise, only 0.4% of 2.5 million molecules screened fitted to the model. This low number is in line with only a handful of non–bile acid NTCP inhibitors currently known and suggests that the model was NTCP specific. Also, the three most potent compounds identified in the first screening round were found to be competitive inhibitors. This was in line with the nature of the pharmacophore model. The model was based on bile acids, which bind to the substrate binding pocket of NTCP and are also competitive inhibitors of the transporter (Meier et al., 1997). The identified inhibitors from the virtual screen were therefore expected also to bind to the same binding pocket and hence also be competitive inhibitors, which was indeed confirmed. Further confidence in the model was obtained from the finding that structural analogues of compound 2, which did not hit all of the pharmacophore features, were inactive or less potent than compounds that hit all features of the model. The analogues that did not map simultaneously to the two HBA features of the model were less active (compound 13) or inactive (compounds 11, 12, and 14). Moreover, compound 14 did possess two HBAs, but these were not of sufficient distance from each other in the molecule to map to the HBA features of the pharmacophore model. This suggests that an appropriate distance between HBAs in the molecule is important for a more successful interaction with the NTCP substrate binding pocket, and the presence of simply two HBAs is not sufficient for inhibitory activity. Also, from the model refinement studies, it appeared that negatively charged compounds were better inhibitors than uncharged compounds, which is in line with results obtained in earlier work (Kim et al., 1999; Kouzuki et al., 2000). Some of the compounds tested in the CHO-cell assay stimulated NTCP-mediated taurocholate transport. Stimulation has been observed for NTCP and other transporters before, but the underlying biochemical mechanism and clinical relevance of this phenomenon are currently unknown (Kim et al., 1999; Wittgen et al., 2011).

To strengthen the argument that NTCP inhibition may have clinically relevant implications, we analyzed how well the model is able to match known competitive inhibitory drugs or endogenous compounds. Unfortunately, for many compounds, the type of inhibition was not reported, which hampered this effort (Kim et al., 1999; Mita et al., 2006; Sandhu et al., 2005). Nevertheless, we found that the progesterone metabolites allopregnanolone sulfate and epiallopregnanolone sulfate are competitive NTCP inhibitors, with $K_i$ values of 8 and 6 $\mu$M, respectively (Abu-Hayyeh et al., 2010). Elevated plasma levels of these metabolites in pregnancy are potentially linked to clinical symptoms of cholestasis such as pruritus. Indeed, the structures of these compounds also mapped well to our models, covering both HBA features (see Supplementary figure 1).

Bosentan is a potent, noncompetitive inhibitor of rat NTCP ($IC_{50} = 0.7\mu$M) and a competitive but less potent inhibitor ($IC_{50} = 24\mu$M) of human NTCP (Leslie et al., 2007). The use of this endothelin antagonist has been associated with hepatotoxic effects. The structure of bosentan could be mapped to our models of human NTCP. Only one HBA feature was covered by the structure, which appears to be in line with its intermediate inhibitory potency against human NTCP (see Supplementary figure 1).

In the past, pharmacophore modeling studies have been performed for the rabbit and human apical sodium-dependent bile

**FIG. 10.** Effect of a 5 mg/kg iv bolus injection of compound 2 on bile flow (A) and biliary $^3$H-taurocholic acid excretion (B) in bile duct–cannulated Sprague Dawley rats. Bile flow was expressed as percentage of control, which was obtained during the stabilization period prior to injection of the test compound or vehicle. $^3$H-taurocholic acid excretion is expressed as percentage of the injected dose. All data represent the mean ± SEM of 3–5 rats per group.
acid transporter (ASBT, SCL10A2) (Baringhaus et al., 1999; Zheng et al., 2009). ASBT facilitates the reabsorption of bile acids from the gut lumen, displays 35% overlap in amino acids with NTCP, and possesses a similar membrane topology (Kosters and Karpen, 2008). ASBT pharmacophore modeling studies were born out of an interest as a potential target for cholesterol-lowering therapies. The pharmacophore model for human ASBT described by Zheng et al. has two HBA and a number of hydrophobic features. This is similar to our results and in line with the substrate overlap between the two transporters. However, the spatial orientation of the pharmacophoric features in the ASBT model is slightly different from the NTCP model, in which the two HBA features appear to be positioned closer together (Zheng et al., 2009). Instead of the two HBAs present in human NTCP (present data) and ASBT (Zheng et al., 2009), the rabbit ASBT model contains one acceptor and one donor feature, suggesting differences in substrate specificity between the rabbit and human proteins.

To study how the data obtained in silico and in the in vitro NTCP recombinant expression system translated to more physiologically relevant systems, we tested compound 2 in cryopreserved human hepatocytes and perfused rat livers and studied the effect on biliary taurocholic acid excretion in rats in vivo. Similar to the results obtained in CHO-NTCP cells, compound 2 exhibited a strong inhibition of taurocholic acid uptake in hepatocytes, with 2µM reducing taurocholate uptake by approximately 60%. This is similar to the inhibitory potency of cyclosporin A, which exhibits an IC₅₀ of 1µM and is one of the most potent NTCP inhibitors known to date (Kim et al., 1999). Also in rat liver perfusion experiments, compound 2 reduced ³H-taurocholic acid uptake from the perfusate and induced cholestasis, which is in line with NTCP inhibition; however, effects on other bile acid–transporting proteins cannot be excluded.

In perfused rat liver, the effect could be observed only at higher concentrations of compound 2, suggesting that the affinity for rat Ntcp may be lower than for the human protein. Such differences have been described before for the interaction between bile acids and NTCP/Ntcp and for non–bile acid compounds that interact with the transporter. For instance, the Kᵣ of taurocholic acid is higher for rat Ntcp than for the human ortholog (Meier et al., 1997). Another example is the more potent inhibition of rat Ntcp compared with human NTCP by the endothelin receptor antagonist bosantan (Leslie et al., 2007).

Interestingly, we found that compound 2 did not display any relevant effects on biliary taurocholic acid excretion in vivo, despite the fact that sufficiently high total plasma concentrations were obtained. This is likely due to a very high protein binding of this compound in vivo resulting in a low unbound plasma concentration, as our results obtained in rat liver perfusion experiments showed that addition of 1% BSA to the perfusate protected the liver from the cholestatic effects of compound 2. Unfortunately, iv injection of higher concentrations in order to test this hypothesis further was precluded due to solubility reasons. Nevertheless, these experiments demonstrated that although our model can successfully identify compounds with intrinsic NTCP inhibitory activity, it is helpful to combine our NTCP pharmacophore model with subsequent in silico selection steps that address in vivo protein binding.

In conclusion, we successfully applied pharmacophore modeling to identify new chemotypes with intrinsic NTCP inhibitory activity. Our approach represents a first step toward the in silico identification of potential cholestasis-inducing compounds during the early stages of drug discovery.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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