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Interaction of Digitalis-Like Compounds with Liver Uptake Transporters NTCP, OATP1B1, and OATP1B3

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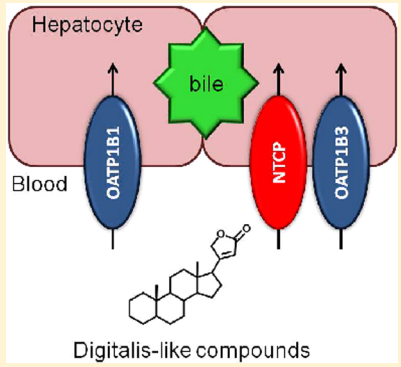
Supporting Information

ABSTRACT: Digitalis-like compounds (DLCs) such as digoxin, digitoxin, and ouabain, also known as cardiac glycosides, are among the oldest pharmacological treatments for heart failure. The compounds have a narrow therapeutic window, while at the same time, DLC pharmacokinetics is prone to drug−drug interactions at the transport level. Hepatic transporters organic anion transporting polypeptide (OATP) 1B1, OATP1B3, and Na^+-dependent taurocholate co-transporting polypeptide (NTCP) influence the disposition of a variety of drugs by mediating their uptake from blood into hepatocytes. The interaction of digoxin, digitoxin, and ouabain with hepatic uptake transporters has been studied before. However, here, we systematically investigated a much wider range of structurally related DLCs for their capability to inhibit or to be transported by these transporters in order to better understand the relation between the activity and chemical structure of this compound type. We studied the uptake and inhibitory potency of a series of 14 structurally related DLCs in Chinese hamster ovary cells expressing NTCP (CHO-NTCP) and human embryonic kidney cells expressing OATP1B1 and OATP1B3 (HEK-OATP1B1 and HEK-OATP1B3). The inhibitory effect of the DLCs was measured against taurocholic acid (TCA) uptake in CHO-NTCP cells and against uptake of β-estradiol 17-β-D-glucuronide ($E_2 17\beta G$) in HEK-OATP1B1 and HEK-OATP1B3 cells. Proscillaridin A was the most effective inhibitor of NTCP-mediated TCA transport (IC_{50} = 22 μM), whereas digitoxin and digitoxigenin were the most potent inhibitors of OATP1B1 and OAPTP1B3, with IC_{50} values of 14.2 and 36 μM, respectively. Additionally, we found that the sugar moiety and hydroxyl groups of the DLCs play different roles in their interaction with NTCP, OATP1B1, and OATP1B3. The sugar moiety decreases the inhibition of NTCP and OATP1B3 transport activity, whereas it enhances the inhibitory potency against OATP1B1. Moreover, the hydroxyl group at position 12 reinforces the inhibition of NTCP but decreases the inhibition of OATP1B1 and OATP1B3. To investigate whether DLCs can be translocated, we quantified their uptake in transporter-expressing cells by LC−MS. We demonstrated that convallatoxin, ouabain, dihydroouabain, and ouabagenin are substrates of OATP1B3. No transport was observed for the other compounds in any of the studied transporters. In summary, this work provides a step toward an improved understanding of the interaction of DLCs with three major hepatic uptake transporters. Ultimately, this can be of use in the development of DLCs that are less prone to transporter-mediated drug−drug interactions.



KEYWORDS: *bufadienolides, cardenolides, convallatoxin, digitalis-like compounds, dihydroouabain, ouabain, ouabagenin, NTCP, OATP1B1, OATP1B3, cell transport, drug-induced toxicity*

INTRODUCTION

Digoxin belongs to a large family of naturally derived compounds called digitalis-like compounds (DLCs). The DLCs are the oldest cardiac medications that have been prescribed because of their inotropic effect.[1] By inhibiting Na,K-ATPase, DLCs induce an increase in intracellular Na^+ concentration followed by an increased intracellular Ca^{2+} concentration in cardiomyocytes, leading to a stronger contraction of the heart muscle.[2,3] Currently, digoxin is applied for the treatment of heart failure and arrhythmia;[4,5] however, it has a narrow therapeutic window. Supra-therapeutic concentrations of digoxin in plasma rapidly lead to a range of adverse effects ranging from anorexia, fatigue, nausea, vomiting, and visual disturbances to ventricular fibrillation and ultimately death.[6]

Digoxin is partly metabolized in the liver, and digitoxin is mainly cleared via the hepatic route.[7−9] Drug−drug interactions (DDI) that result in lower clearance of digoxin from the body are among the most important factors causing toxicity. The

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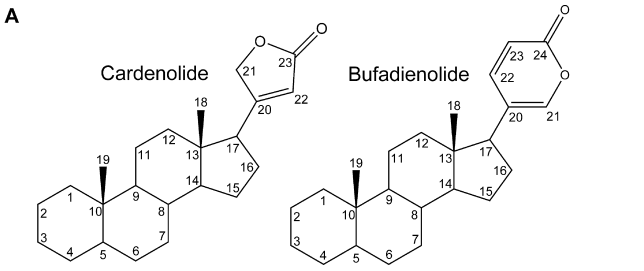
majority of bioavailable digoxin (60−80%) is excreted via the kidney as unchanged drug, whereas the rest is metabolized and excreted via the liver.[10] Inhibition of P-glycoprotein (P-gp), the efflux transporter of digoxin and digitoxin,[11,12] by coadministered medications such as quinidine, cyclosporine, and amiodarone leads to increased blood levels, causing toxicity.[6,10,11,13−16]

Next to the cellular efflux of DLCs mediated by P-gp, cellular influx through SLC transporters may play a role in the disposition of these compounds in the liver and kidney. Among the hepatic uptake transporters, two members of the organic anion transporting polypeptide (OATP) family, OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*), are predominantly expressed in human liver.[17−19] Moreover, Na^+-dependent taurocholate co-transporting polypeptide (*SLC10A1*, NTCP) is an uptake transporter of bile salts and a number of other substrates with a steroid-like structure.[20−22] Next to digoxin and digitoxin, many structurally related DLCs exist, yet the excretion route of these compounds is unknown. The majority of naturally occurring DLCs have not been investigated with regard to their interaction with hepatic uptake transporters. In fact, only the hepatic uptake of the cardiac glycosides digoxin and ouabain has been well-characterized in mouse, rat, and human. In rats, Oatp2 and Oatp4 were found to be hepatic uptake transporters.[23−28] In addition, murine and rat Oatp1 and Oatp2 were reported as ouabain uptake transporters.[22,29−31] In human hepatocytes, digoxin uptake was shown to be transporter-dependent;[32−34] moreover, digoxin and ouabain were reported as OATP1B3 substrates using *Xenopus laevis* oocytes,[35] and polymorphisms in OATP1B3 have been suggested to be associated with increased concentrations of digoxin in plasma.[36] However, the same result was not obtained by Taub et al. using OATP2-transfected cell lines.[37] Finally, it has been described that NTCP plays no role in ouabain transport, yet NTCP-mediated transport of Bamet could be inhibited by ouabain, indicating that the compound is capable of interacting with this transporter.[38,39]

Replacement of currently applied DLCs with alternative compounds that are less prone to drug−drug interactions occurring at the influx and efflux transporter level may be facilitated by charting the interactions of this compound class with various influx and efflux transporters.[40−42] In previous work, we addressed the interaction of a range of DLCs with P-gp. In the present study, we investigated the interaction of 14 DLCs (Figure 1) with human NTCP, OATP1B1, and OATP1B3 using cells overexpressing these transporters. First, we studied the inhibitory potency of DLCs on transporter activity and characterized structural features that are typical for inhibition of each transporter. We also determined if the DLCs were actually transported, and we identified convallatoxin, ouabain, dihydroouabain, and ouabagenin as new substrates for OATP1B3.

EXPERIMENTAL SECTION

Materials. High-glucose Dulbecco's modified Eagle's medium (DMEM) (NEAA, no glutamine), GlutaMAX high-glucose DMEM, Hanks balanced salt solution (HBSS), L-glutamine, and sodium pyruvate were purchased from Life Technologies (Breda, The Netherlands). [6,7-^{3}H(*N*)]Estradiol 17-*β*-D-glucuronide (34.3 Ci/mmol) and [^{3}H(G)]taurocholic acid (5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Groningen, The Netherlands). Fetal calf serum was purchased from Greiner (Alphen a/d Rijn, The



cardenolides	1	3	5	11	12	14	16	19	Other
convallatoxin		L-rhamnose	OH			OH		O	
cymarin		D-cymarose	OH			OH		O	
digitoxigenin		OH				OH			
digitoxin		tri-D-digitoxose				OH			
digoxigenin		OH			OH	OH			
digoxin		tri-D-digitoxose			OH	OH			
dihydroouabain	OH	L-rhamnose	OH	OH		OH		OH	20-22 saturated
gitoxigenin		OH				OH	OH		
ouabagenin	OH	OH	OH	OH		OH		OH	
ouabain	OH	L-rhamnose	OH	OH		OH		OH	
peruvoside		3-O-methyl glucose				OH		O	
strophanthidin		OH	OH			OH		O	
strophanthidol		OH	OH			OH		OH	
bufadienolides									
proscillaridin A		L-rhamnose	4-5 double bond			OH			

Figure 1. Structural features of DLCs. The cardenolides and bufadienolides contain *γ*-butyrolactone (*γ*-lactone) and *δ*-valerolactone (*δ*-lactone) at the 17 position, respectively (A). DLCs have a steroid ring as their core structure that contains different chemical substitutions (B).

Netherlands), and BD BioCoat poly-D-lysine 24-well plates were purchased from VWR (Leuven, Belgium). The protein concentrations were determined using a protein assay kit from Bio-Rad Laboratories (Veenendaal, The Netherlands). Convallatoxin (>70% purity), proscillaridin A (>80% purity), peruvoside and strophanthidin (>90% purity), cymarin, digitoxin, digoxin, dihydroouabin, gitoxigenin, oubagenin, ouabain and strophanthidol (>96% purity), digitoxigenin and digoxigenin (>98% purity), and sodium butyrate were purchased from Sigma (Zwijndrecht, The Netherlands). Ammonium formate and formic acid were purchased from Fluka (Steinheim, Germany). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany), and water was purified with a Millipore Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol was purchased from Lab Scan (Dublin, Ireland), and super gradient grade acetonitrile was obtained from VWR (Leuven, Belgium).

Cell Culture. Chinese hamster ovary cells stably expressing NTCP (CHO-NTCP) were obtained from Solvo Biotechnologies (Budapest, Hungary), and human embryonic kidney cells (HEK293) stably expressing OATP1B1 or OATP1B3 (HEK-OATP1B1 and HEK-OATP1B3) were kindly provided by TNO (Zeist, The Netherlands). For all experiments, CHO-parent (CHO-P) and HEK-parent (HEK-P) were used as the controls. CHO cells were cultured in high-glucose DMEM supplemented with 10% FCS, 4 mM L-glutamine, and 1 mM sodium pyruvate. HEK cells were cultured in GlutaMAX high-glucose DMEM supplemented with 10% FCS at 37 °C under 5% CO_2 humidified air.

Kinetic Characterization of NTCP, OATP1B1, and OATP1B3. CHO and HEK cells were seeded at a density of 600 000 cells per well (Greiner, Alphen a/d Rijn, The Netherlands) and 400 000 cells per poly-D-lysine-coated well (VWR, Leuven, Belgium) in 24-well plates, respectively. To

induce NTCP protein expression, 5 mM sodium butyrate was added to CHO cells. Twenty-four hours after CHO and 48 h after HEK cells were seeded, the medium was removed and the cells were washed twice with HBSS containing 10 mM HEPES (HBSS-HEPES), pH 7, at 37 °C. Subsequently, CHO and HEK cells were incubated with 150 μL of HBSS-HEPES containing [^{3}H]-labeled taurocholic acid or [^{3}H]estradiol 17-β-D-glucuronide, respectively, at 37 °C for the incubation times indicated in the figure legends. To stop the incubation, the cells were placed on ice and washed twice with ice-cold HBSS-HEPES containing 0.5% BSA followed by washing with ice-cold HBSS-HEPES. Next, the CHO and HEK cells were lysed using 1 M NaOH and 0.5% Triton X-100 for 1 h, respectively. After homogenizing, lysed cells were mixed with 4 mL of scintillation liquid, and cell-associated radioactivity was measured using a Packard Tri-Carb liquid scintillation counter (Ramsey, MN, USA). In all experiments, transporter-dependent uptake was calculated by subtracting the values measured using parent cells from the corresponding values measured in CHO and HEK cells expressing transporters. All experiments were performed in triplicate.

Inhibition Studies. To screen DLCs for inhibitory effects against NTCP, OATP1B1, and OATP1B3, the CHO and HEK cells were incubated with HBSS-HEPES containing trace amounts of [^{3}H]-labeled taurocholic acid (0.03 μM) and [^{3}H]estradiol 17-β-D-glucuronide (0.0043 μM), respectively. Experiments were performed at 37 °C in the presence of the various DLCs at 10 and 100 μM. Stock solutions of DLCs were made in DMSO, and the final concentration of DMSO was 1% in all incubates. The incubation solutions were supplemented with unlabeled estradiol 17-β-D-glucuronide or taurocholic acid to the concentrations indicated in the figure legends. In addition, 1% DMSO and 100 μM TCA (for NTCP) or 100 μM $E_2$17βG (for OATP1B1 and OATP1B3) were used as negative and positive controls, respectively. Furthermore, the IC_{50} of DLCs on NTCP-mediated TCA transport, OATP1B1-mediated $E_2$17βG transport, and OATP1B3-mediated $E_2$17βG transport was determined by coincubation with increasing concentrations of DLCs (Figure 4).

DLC Uptake Assays. To determine whether the DLCs are transported by NTCP, OATP1B1, and OATP1B3, the uptake of DLCs was determined in CHO-NTCP, HEK-OATP1B1, and HEK-OATP1B3 as well as in CHO-P and HEK-P as negative controls. The CHO and HEK cells were incubated with HBSS-HEPES containing 10 μM DLCs at 37 °C for 1 h at a 1% final concentration of DMSO in the absence and presence of the inhibitor rifampicin (100 μM). After the previously described washing steps with HBSS-HEPES (without and with 0.5% BSA), the cells were lysed using 0.5 mL of 99.9% acetonitrile and 0.1% formic acid for 30 min. After addition of the acetonitrile/formic acid mixture, proteins were allowed to precipitate for 30 min at −20 °C. After 5 min of centrifugation at 16 000*g*, the supernatants were transferred to new tubes and the acetonitrile/formic acid was evaporated under a gentle stream of N_2 gas at 37 °C. The pellets were dissolved in methanol containing 200 nM gitoxigenin as an internal standard to analyze the samples using LC−MS. Ouabain was used as an internal standard when gitoxigenin was the test compound.

Protein Concentration. In all inhibition and accumulation assays, the amount of cellular protein per well was determined in a parallel plate. Cells were lysed using distilled water and stored at −20 °C upon analysis. Protein concentrations were determined using the Bio-Rad protein assay kit. The uptake of substrates in CHO and HEK cells was expressed as picomoles per milligram of protein.

LC−MS Quantification of DLCs. The concentration of DLCs in the cell lysate samples was measured using an Accela UPLC (Thermo Scientific, San Jose, CA, USA) coupled to a TSQ Vantage (Thermo Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer. The compounds were separated on a HSS T3 analytical column (1.8 μm; 100 × 2.1 mm, Acquity UPLC, Waters, Ireland) coupled with a VanGuard HSS T3 precolumn (1.8 μm; 5 × 2.1 mm, Acquity UPLC, Waters, Ireland). The mobile phase consisted of solvent A (10 mM ammonium formate, pH 3.0) and solvent B (methanol). The elution gradient was 0 min, 90% A; 0.5 min, 90% A; 12.5 min, 15% A; 13.5 min, 90% A; and 20 min. 90% A for convallatoxin, cymarin, digitoxigenin, digoxigenin, dihydroouabain, gitoxigenin, ouabagenin, ouabain, peruvoside, strophanthidin, and strophanthidol and 0 min, 70% A; 0.5 min, 70% A;12.5 min, 15% A; 13.5 min, 70% A; and 20 min, 70% A for digitoxin, digoxin, gitoxigenin, and proscillaridin A. Gitoxigenin as the internal standard was measurable under both conditions. The column temperature was set at 60 °C, and the flow rate was 500 μL/min. In addition, the sample injection volume was 20 μL, the analysis run time was 20 min, and the samples were stored at a tray temperature of 8 °C. The effluent from the UPLC was passed directly into the heated electrospray ion source. The capillary temperature and the vaporizer temperature were set at 225 and 382 °C, respectively. With ionization voltage at +3.5 kV, heated electrospray ionization (HESI) was achieved using nitrogen gas as the sheath and auxiliary gases, with pressures of 30 and 35 AU (Arbitrary Units), respectively.

Positive ion mode was used with single ion monitoring (SIM) for the quantitative analysis of 14 different DLCs. The most abundant adducts (sodium $[M + Na]^+$ or potassium $[M + K]^+$) were used for quantification. Adducts with their corresponding mass/charge ratio (m/z) are shown in Table 1.

The LC−MS method was validated as follows. The linearity was evaluated by analyzing three batches of standard curves of all 14 DLCs over the concentration range of 1.0−400 nM. Good linearity was observed over the quantification range when a linear regression was used with least-squares regression ($1/\chi^2$). The correlation coefficients (r) were greater than 0.9900

Table 1. Mass Fragment of Most Abundant Adducts for the Detection of DLCs by LC−MS

compounds	$[M + Na]^+$ (m/z)	$[M + K]^+$ (m/z)
convallatoxin	573.3	
cymarin	571.3	
digitoxigenin	397.2	
digitoxin	787.5	
digoxigenin		492.2
digoxin	803.4	
dehydroouabain	609.3	
gitoxigenin		429.3
ouabain	461.2	
ouabagenin	607.3	
peruvoside	571.2	
strophanthidin	553.3	
strophanthidol		443.2
proscillaridin A	429.1	

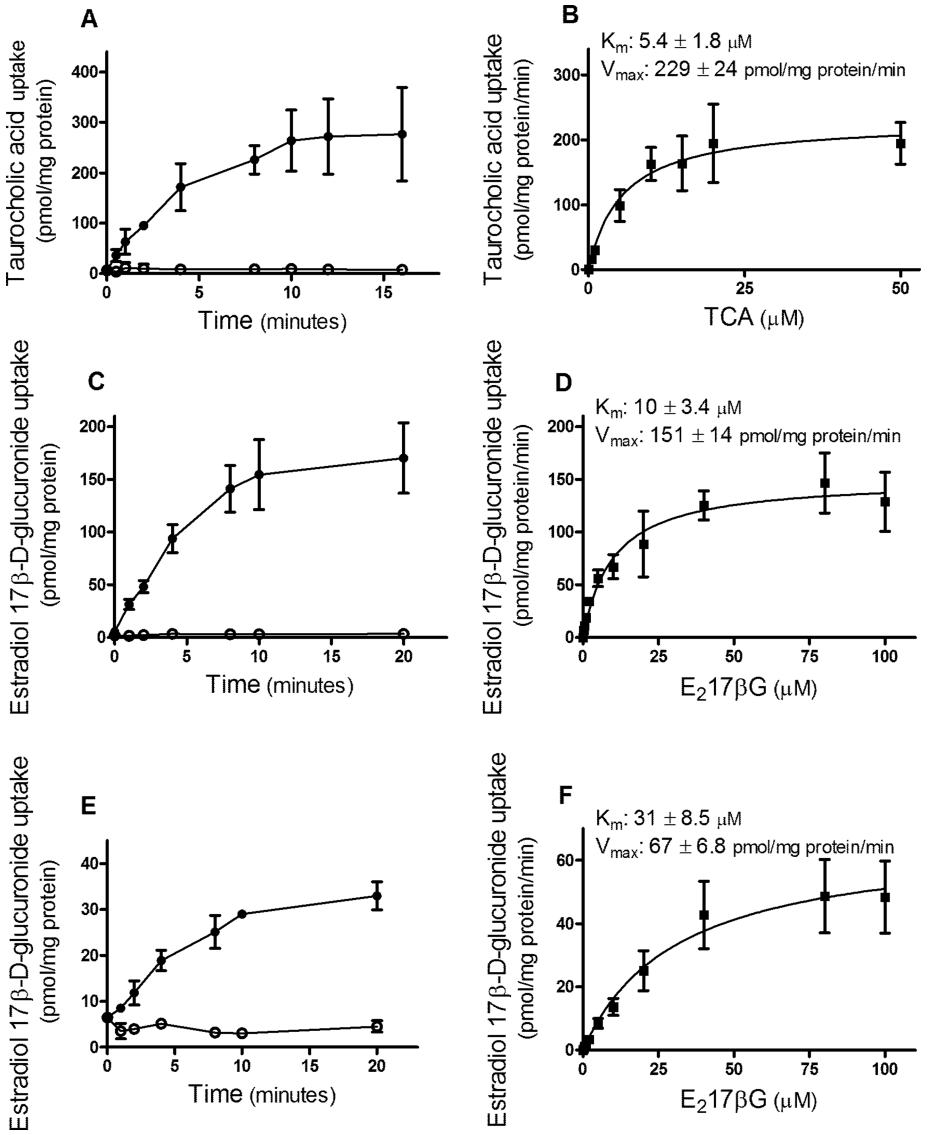


Figure 2. Time- and concentration-dependent transport of taurocholic acid and estradiol 17-β-D-glucuronide in CHO-NTCP (A, B), HEK-OATP1B1 (C, D), and HEK-OATP1B3 (E, F), respectively. The NTCP-mediated transport of taurocholic acid (TCA) in CHO-NTCP was measured in the presence of 1 μM TCA (A) and at increasing concentrations of TCA (B) during a 1 min incubation. Time course of the transport of 17-β-D-glucuronide ($E_2$17βG) by OAP1B1 (C) and OATP1B3 (E) in HEK cells was measured in the presence of 2 μM $E_2$17βG. The concentration-dependent transport of $E_2$17βG by OAP1B1 (D) and OATP1B3 (F) was measured during 2 and 3 min incubation times, respectively. CHO-P and HEK-P were used as controls (empty circles in A, C, and E), and their values were subtracted in panels B, D, and F. Data points represent the mean ± SD of experiments performed in triplicate.

for all analytical batches, with a bias within ±10%. The intra- and interassay accuracy and precision were determined at the LLOQ (1.0 nM), low (10 nM), medium (50 nM), and high (250 nM) QC levels by analyzing three separate analytical batches in five replicates for each concentration. The intra- and interassay precision was less than 10 and 11%, respectively, and the bias ranged from −4.8 to 9.0% and −9.2 to 11.1%, respectively. These results were within an acceptable range, and the analytical method proved to be reproducible in terms of intra- and interassay accuracy and precision.

Analysis. All of the data are expressed as the mean ± SD of three independent experiments after correction for the uptake in CHO-parent and HEK-parent cells. To determine K_m and V_{max} of the transporters, concentration-dependent uptake data were fitted to the Michaelis−Menten equation using Graphpad Prism software (version 5.02; GraphPad Software Inc., San Diego, CA). DLCs were screened for inhibitory properties using one-way ANOVA followed by Dunnett's post-hoc multiple comparisons. The unpaired Student's t test was used to compare groups in the DLC uptake assays.

Inhibition curves were analyzed with a one-site binding model fitted to the equation y = bottom + (top − bottom)/(1 + $10^{(\log IC_{50} - x) - \text{Hill slope}}$) using GraphPad Prism. In this equation, x and y indicate log values of inhibitor concentrations and uptake versus control, respectively.

Xcalibur software (Thermo Scientific, San Jose, CA, USA) was used to control the LC−MS system, and LCquan software (Thermo Scientific, San Jose, CA, USA) was used for sample data analysis.

The physicochemical properties of DLCs such as octanol/water partition coefficient (cLogP) and polar surface area (tPSA) were calculated using the ChemBioOffice software

package (CambridgeSoft, Cambridge, MA, USA), and H-bond donors and acceptors were obtained from the PubChem database (National Centre for Biotechnology Information, Bethesda, MD, USA).

RESULTS

Kinetic Characterization of NTCP, OATP1B1, and OATP1B3. Previously, CHO-NTCP, HEK-OATP1B1, and HEK-OATP1B3 cell lines were characterized at the functional and/or protein expression levels.[43−45] In the present study, to characterize the function of the transporters in the cell lines, the time- and concentration-dependent uptake of TCA by NTCP and $E_2$17βG by OATP1B1 and OATP1B3 was measured (Figure 2). Uptake of TCA by CHO-NTCP and CHO-P was measured in the presence of 1 μM TCA. NTCP-mediated uptake of TCA was linear over 4 min (Figure 2A). Concentration-dependent uptake of TCA by NTCP, measured after 1 min of incubation, was characterized by an apparent affinity (K_m) of 5.4 ± 1.8 μM and a maximum rate (V_{max}) of 229 ± 24 pmol/mg protein/min (Figure 2B), and this was comparable to the values previously shown by others.[20,22,43,46] Time-dependent uptake of 2 μM $E_2$17βG was measured in HEK-P and HEK-OATP1B1 cells (Figure 2C). Because the uptake activity of HEK-OATP1B1 was linear over a time period of 4 min, an incubation time of 2 min was selected to study the kinetic characteristics of $E_2$17βG uptake by OAPTP1B1. The K_m and V_{max} values of $E_2$17βG for OATP1B1 were 10 ± 3.4 μM and 151 ± 14 pmol/mg protein/min, respectively. The kinetics of $E_2$17βG uptake by OATP1B1 was in accordance with the literature.[19,47,48] Uptake of $E_2$17βG (2 μM) in HEK-P and HEK-OATP1B3 was also linear over the first 4 min. K_m and V_{max} of OATP1B3-mediated $E_2$17βG uptake measured at 3 min were 31 ± 8.5 μM and 67 ± 6.8 pmol/mg protein/min, respectively (Figures 2E,F), which is also in line with previously reported values.[47]

Effect of DLCs on NTCP-Mediated Uptake of TCA and OATP1B1- and OATP1B3-Mediated Uptake of $E_2$17βG. To investigate the influence of DLCs on transporter activity, uptake rates of TCA by NTCP and of $E_2$17βG by OATP1B1 and OATP1B3 were measured in the absence and presence of 14 DLCs. We can exclude a cell toxicity effect, as we did not observe a DLC inhibitory pattern that was similar in all three cell lines. Figure 3A represents the effect of 10 and 100 μM of the individual DLCs on NTCP-mediated TCA uptake. The uptake of TCA by NTCP in the absence of DLCs was set at 100% (Ctrl: 48 ± 7 pmol/mg protein/min). The most potent inhibitor, proscillaridin A, inhibited transport activity by 77% at 100 μM. Digoxin inhibited uptake by 25 and 46% at concentrations of 10 and 100 μM, respectively. Convallatoxin, digitoxin, and ouabain (100 μM) inhibited TCA uptake by approximately 20%. Digoxigenin and gitoxigenin stimulated NTCP-mediated TCA uptake at 10 μM by 32 and 43%, respectively, whereas they hardly affected transporter activity at 100 μM. TCA itself (100 μM unlabeled) inhibited NTCP by 90%.

The uptake activity of OATP1B1 in the absence of DLCs was set at 100% (Ctrl: 57.8 ± 20 pmol/mg protein/min) (Figure 3B). Digitoxin, the most potent inhibitor of OATP1B1, inhibited uptake of $E_2$17βG by 95% at 100 μM. In addition, 100 μM digitoxigenin, digoxin, and proscillaridin A inhibited OATP1B1 by 58 and 66%, respectively. The inhibitory effect of 100 μM convallatoxin, digoxigenin, gitoxigenin, peruvoside, strophanthidin, and strophanthidol was less than 50%. The inhibitory potency of DLCs on OATP1B1-mediated transport can be summarized as digitoxin > digoxin = proscillaridin A > digitoxigenin > peruvoside > gitoxigenin.

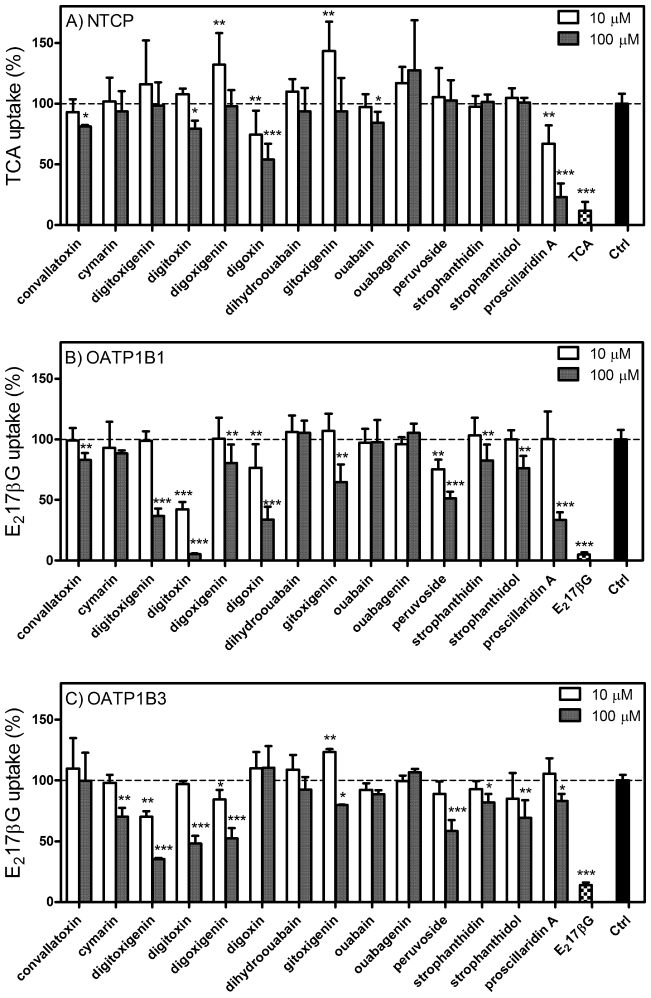


Figure 3. Effect of DLCs on TCA uptake by NTCP (A) and $E_2$17βG uptake by OATP1B1 (B) and OATP1B3 (C). Uptake was measured during 1 min at a concentration of 1 μM TCA for NTCP (A), during 2 min at a concentration of 2 μM $E_2$17βG for OATP1B1, and during 3 min at a concentration of 2 μM $E_2$17βG for OATP1B3. For all three transporters, uptake was measured in the absence (Ctrl) or presence of 14 DLCs at 10 and 100 μM. NTCP-, OATP1B1-, and OATP1B3-mediated uptake in the absence of DLCs (Ctrl) was set at 100% and was also measured in the presence of 100 μM of the corresponding unlabeled substrate. The mean ± SD values of three independent experiments is shown, and statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

OATP1B3-mediated $E_2$17βG uptake was 17.2 ± 4.5 pmol/mg protein/min (Ctrl: 100%) (Figure 3C). The most potent inhibitor of OATP1B3, digitoxigenin, inhibited uptake by 65% at 100 μM. Digitoxin and digoxigenin inhibited uptake up to 50% at 100 μM, but most of the tested DLCs, such as cymarin, gitoxigenin, strophanthidin, strophanthidol, and proscillaridin A, inhibited OATP1B3 by less than 30%. Gitoxigenin stimulated OATP1B3 activity by 23% at 10 μM and inhibited transport by 20% at 100 μM. At the tested concentrations, DLCs were less potent inhibitors of OATP1B3 than OATP1B1.

Concentration-Dependent Inhibition of NTCP, OATP1B1, and OATP1B3 by DLCs. To analyze the

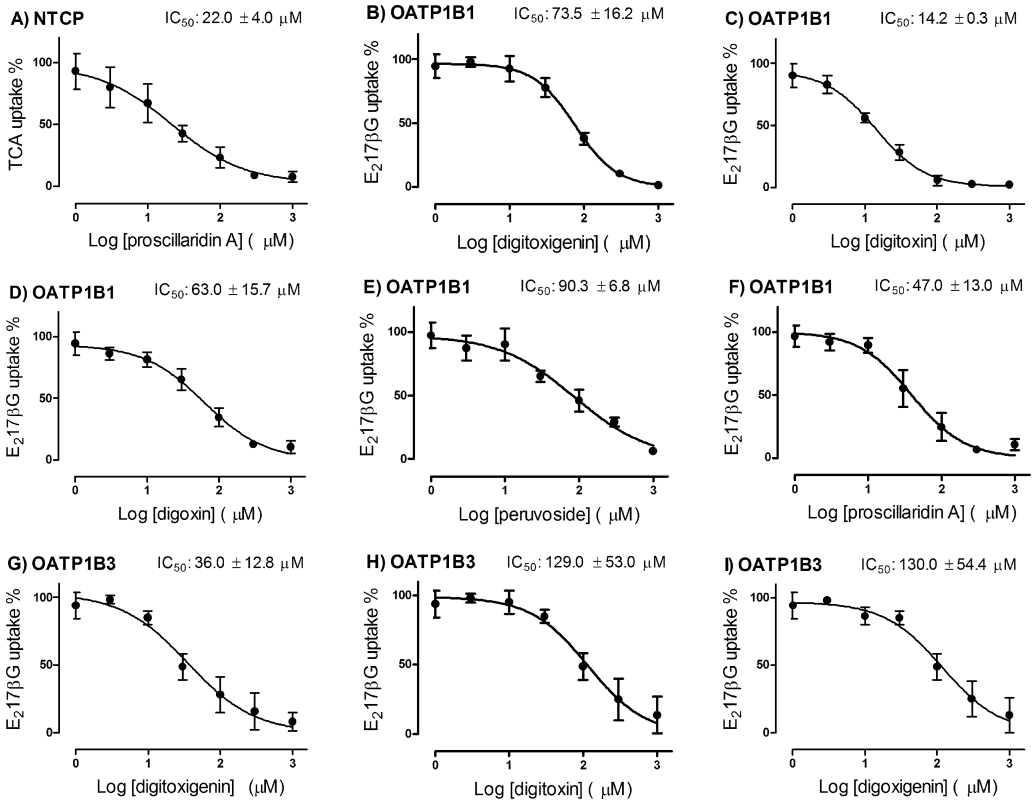


Figure 4. Concentration-dependent inhibition of NTCP(A), OATP1B1 (B–F), and OATP1B3 (G–I) by selected DLCs. CHO-P and CHO-NTCP were incubated with 1 μM TCA in the absence and presence of increasing concentrations of proscillaridin (A) for 1 min. $E_2$17βG (2 μM) was incubated with HEK-P and HEK-OATP1B1 in the presence of increasing concentrations (1–1000 μM) of digitoxigenin (B), digitoxin (C), digoxin (D), peruvoside (E), and proscillaridin A (F) for 2 min and with HEK-P and HEK-OATP1B3 in the presence of increasing concentrations of digitoxigenin (G), digitoxin (H), and digoxigenin (I) for 3 min. NTCP-mediated uptake of TCA and OATP1B1- and OATP1B3-mediated uptake of $E_2$17βG in the absence of DLCs was 48 ± 6, 50 ± 10.6, and 19 ± 8 pmol/mg protein/min, respectively, which were set at 100%. The mean ± SD of three independent experiments is shown.

inhibitory potency of DLCs against NTCP, OATP1B1, and OATP1B3 activity, DLCs with an inhibitory efficacy of 50% or more at 100 μM were selected for further study. The uptake rates of TCA by NTCP and $E_2$17βG by OATP1B1 and OATP1B3, expressed as the percentage of control, were plotted against the log value of increasing DLC concentrations (Figure 4). Proscillaridin A, the most potent NTCP inhibitor, exhibited an IC_{50} value of 22.0 ± 4.0 μM. Inhibitory potency against OATP1B1 could be ranked as follows: digitoxin (14.2 ± 0.3 μM) > proscillaridin A (47.0 ± 13.0 μM) > digoxin (63.0 ± 15.7 μM) > digitoxigenin (73.5 ± 16.2 μM) > peruvoside (90.3 ± 6.8 μM). Digitoxigenin was the most potent inhibitor of OATP1B3 (36.0 ± 12.8 μM), whereas the inhibitory potencies of digitoxin and digoxigenin were 3 times lower (129.0 ± 53.0 and 130.0 ± 54.4 μM, respectively).

Identification of NTCP, OATP1B1, and OATP1B3 Substrates. Absolute uptake of DLCs (10 μM) after 1 h in parent and transporter-expressing cell lines was measured using LC–MS. Significant differences in DLC uptake could not be detected when comparing CHO-NTCP and CHO-parent cell lines (Figure 5 and Table 2). Also, no significant differences in DLC uptake could be detected when comparing HEK-OATP1B1 and HEK-parent cell lines (Figure 6 and Table 2). OATP1B3 showed statistically significant uptake of convallatoxin (230 ± 40% compared to control cells, $p < 0.001$), dihydroouabain (381 ± 14% of control, $p < 0.001$), ouabain (1140 ± 210% of control, $p < 0.001$), and ouabgenin (212 ± 10% of control, $p < 0.001$) (Figure 7 and Table 2). The OATP1B3 inhibitor rifampicin[49] reduced the uptake of $E_2$17βG, convallatoxin, dihydroouabain, ouabain, and ouabagenin in HEK-OATP1B3 cells, whereas it had no effect on the uptake of these compounds in HEK-parent cells (Figure 8). Transport activity of OATP1B3 and its inhibition by rifampicin was confirmed by using $E_2$17βG as the probe substrate in our assay (Figure 8E).

■ DISCUSSION

To understand the variation in the pharmacokinetic profile of different DLCs, we studied the interaction of 14 DLCs with liver uptake transporters NTCP, OATP1B1, and OATP1B3. Whereas we found little interaction with NTCP, several DLCs inhibited OATP1B1 and OATP1B3 transport activity. Moreover, OATP1B3 transported four DLCs.

Looking more closely at the interactions of DLCs with NTCP, the presence of a sugar moiety appears to improve inhibition of NTCP activity (based on the comparison of sugar-conjugated DLCs digitoxin, digoxin, ouabain, and convallatoxin to that of unconjugated congeners digitoxigenin, digoxigenin, ouabagenin, and strophanthidin). This effect was most clearly observed for the digoxin/digoxigenin pair, whereas it was less prominent for other glycone/aglycone pairs. Furthermore, a hydroxyl group at position 12 (digoxin) increases the inhibitory efficacy compared to digitoxin (no hydroxyl group in the 12 position). Among the 14 tested DLCs, proscillaridin A was the

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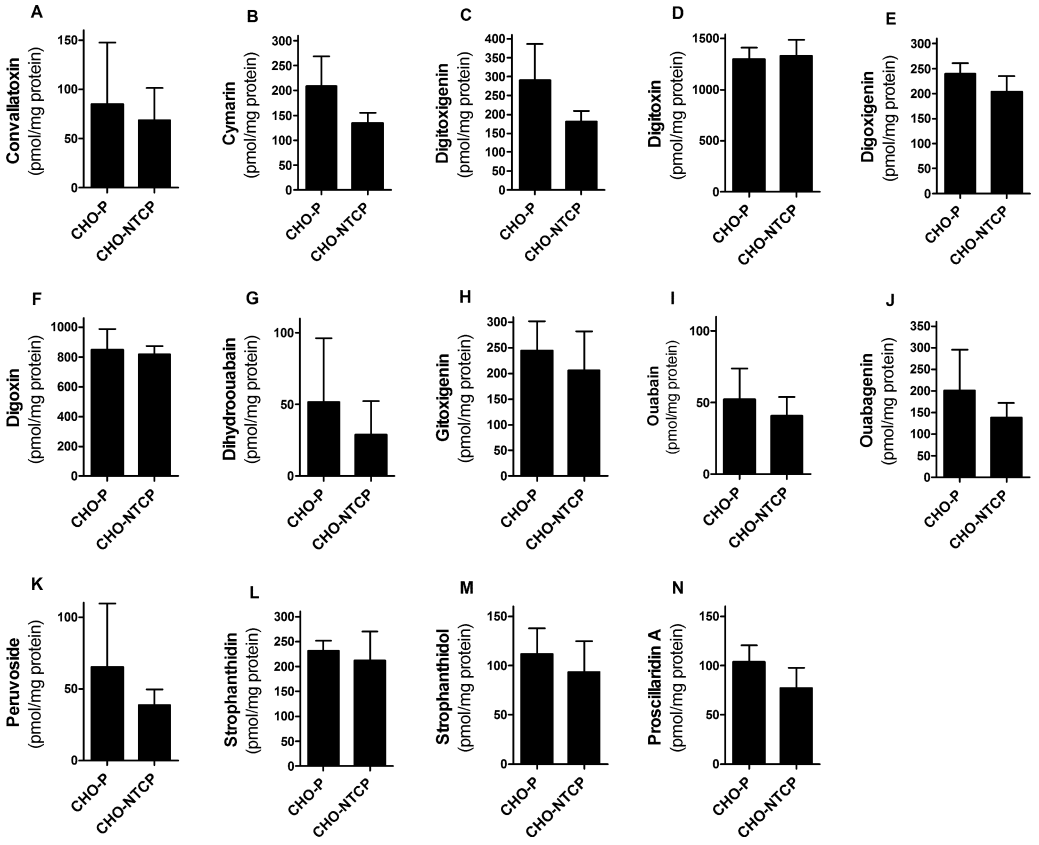

Figure 5. Uptake of DLCs in CHO-P and CHO-NTCP. Uptake of 14 DLCs was measured by LC–MS after a 1 h incubation of CHO-P and CHO-NTCP with convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N). Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown.

Table 2. Accumulation of DLCs in CHO-P, CHO-NTCP, HEK-P, and HEK-OATPs[a]

compounds	accumulation in NTCP vs control (%)	accumulation in OATP1B1 vs control (%)	accumulation in OATP1B3 vs control (%)
convallatoxin	75 ± 46 (0.75)	87 ± 16 (0.9)	231 ± 93 (2.3)[b]
cymarin	66 ± 17 (0.7)	123 ± 12 (1.2)	115 ± 13.5 (1.2)
digitoxigenin	66 ± 18 (0.7)	92 ± 20 (0.9)	107 ± 26 (1.1)
digitoxin	102 ± 12 (1.0)	56 ± 7.7 (0.6)	95 ± 13 (0.95)
digoxigenin	86 ± 18 (0.9)	104 ± 14 (1.0)	113 ± 9.7 (1.1)
digoxin	96 ± 6.6 (1.0)	129 ± 23 (1.3)	116 ± 14 (1.2)
dehydroouabain	62 ± 7.3 (0.6)	79 ± 18 (0.8)	381 ± 35 (3.8)[b]
gitoxigenin	82 ± 14.5 (0.8)	84 ± 5.3 (0.8)	102 ± 18 (1.0)
ouabain	83 ± 17 (0.8)	93 ± 38 (0.9)	1139 ± 503 (11.4)[b]
ouabagenin	76 ± 22 (0.8)	112 ± 20 (1.1)	212 ± 23 (2.1)[b]
peruvoside	75 ± 33 (0.75)	107 ± 14 (1.1)	157 ± 13 (1.6)
strophanthidin	91 ± 23 (0.9)	117 ± 6.2 (1.2)	104 ± 14 (1.0)
strophanthidol	82 ± 23 (0.8)	107 ± 6.7 (1.1)	113 ± 26 (1.1)
proscillaridin A	75 ± 20 (0.75)	100 ± 17 (1.0)	130 ± 11 (1.3)

[a]The accumulation of each DLC (10 μM) in CHO-P and HEK-P during a 1 h incubation was set at 100% (control), and the mean ± SD values of three independent experiments measuring the accumulation of DLCs in CHO-NTCP and HEK-OATPs were compared to that of the control using a one-way ANOVA test. [b]$p < 0.001$.

most potent inhibitor of NTCP. Proscillaridin A's IC_{50} (22 μM) exceeded plasma concentrations reported in the clinic (systemic concentration, 1 nM; portal vein concentration, 3.5 nM),[50] so no clinically relevant inhibitory effect on bile salt transport is expected for this compound. Although NTCP is known to be capable of transporting drug-like molecules, none of the tested DLCs was an NTPC substrate; hence, DDIs on the level of NTCP with DLCs as a victim drug are unlikely to occur.

OATP1B1-mediated uptake of $E_2$17βG was inhibited by digitoxin, digoxin, proscillaridin A, digitoxigenin, peruvoside, and gitoxigenin, whereas convallatoxin, cymarin, digoxigenin, strophanthidin, and strophanthidol hardly inhibited transporter activity. Inhibition of OATP1B1 by digoxin was also reported in previous studies.[51−53] Badolo et al. showed that 20 μM digoxin

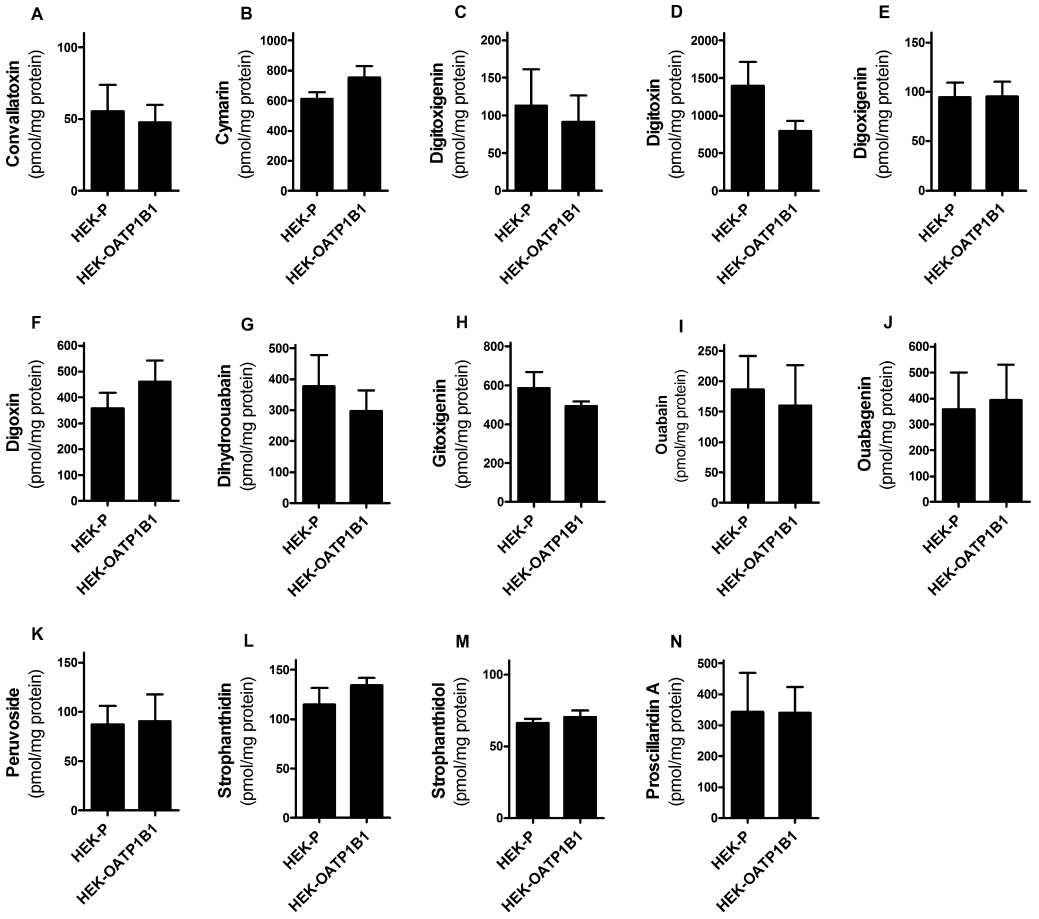

Figure 6. Uptake of DLCs in HEK-P and HEK-OATP1B1. The cell lines were incubated with convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N) for 1 h, and uptake was measured by LC–MS. Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown.

inhibited $E_2$17βG uptake in hepatocytes by 56% and in HEK-OATP1B1 cells by 36%,[51] which is in line with our study. Karlgren et al. reported a 50% reduction of OATP1B1 activity in HEK293 by 20 μM ouabain.[52] However, we did not observe any inhibition of OATP1B1 by ouabain (10 and 100 μM).

Reported plasma concentrations for digoxin and digitoxin are 3 and 20 nM, respectively (portal vein concentration of 1.5 nM for digoxin[54]), whereas we found their IC_{50} values to be ~1000-fold higher. The chance of these compounds causing a relevant inhibition of OATP1B1-mediated drug transport is therefore very small and is, in fact, also in line with the absence of clinical reports of such an event.[55,56] Whether the inhibitory potencies determined for the other DLCs against OATP1B1 are clinically relevant depends on the therapeutic plasma concentration windows for these compounds. To date, such clinical efficacy and safety data have not been reported to our knowledge.

In addition, the comparison of the inhibitory potencies against OATP1B1 by different DLCs also revealed structural features critical for interaction with the transporter. Digitoxin and digitoxigenin appeared to be more potent inhibitors than digoxin and digoxigenin, respectively, which demonstrated that the hydroxyl group at position 12 decreases inhibitory potency. Digoxin and digitoxin carry a sugar moiety at position 3, and they are better inhibitors of OATP1B1 than digoxigenin and digitoxigenin, which have a hydroxyl group at this position. This shows that the presence of a sugar moiety at position 3 could enhance the inhibitory effect of DLCs on OATP1B1 activity. This effect was not observed for the weak inhibitors, convallatoxin and strophanthidin. Moreover, convallatoxin and cymarin have identical steroid bodies and slightly different sugar moieties; still, they share a low inhibitory potency against OATP1B1. In addition, the presence of a hydroxyl group at position 16 seems to lower the inhibitory potency of DLCs (gitoxigenin vs digitoxigenin).

Among the tested DLCs, digitoxigenin, digitoxin, and digoxigenin were the most potent inhibitors of OATP1B3. We show that digoxin and ouabain hardly influence the transport activity of OATP1B3; however, Baldes et al. have shown that ouabain and digoxin inhibited OATP1B3-mediated Fluo-3 transport.[57] Because the inhibitory effect of inhibitors on a transporter could be substrate-dependent,[48,58,59] the different OATP1B3 substrates ($E_2$17βG vs Fluo-3) could provide an explanation for the differing results. Moreover, Karlgren et al. reported 20 μM ouabain to be an inhibitor of OATP1B3-mediated 1 μM $E_2$17βG uptake (with 20% inhibition), whereas 20 μM digoxin did not inhibit 2 μM $E_2$17βG uptake by OATP1B3 in the present study, which is possibly related to the different substrate concentration used.[53]

The absence of a sugar moiety at position 3 of DLCs appeared to improve binding to OATP1B3, because digitoxigenin, digoxigenin, and strophanthidin are better inhibitors than digitoxin, digoxin, and convallatoxin. Moreover, a

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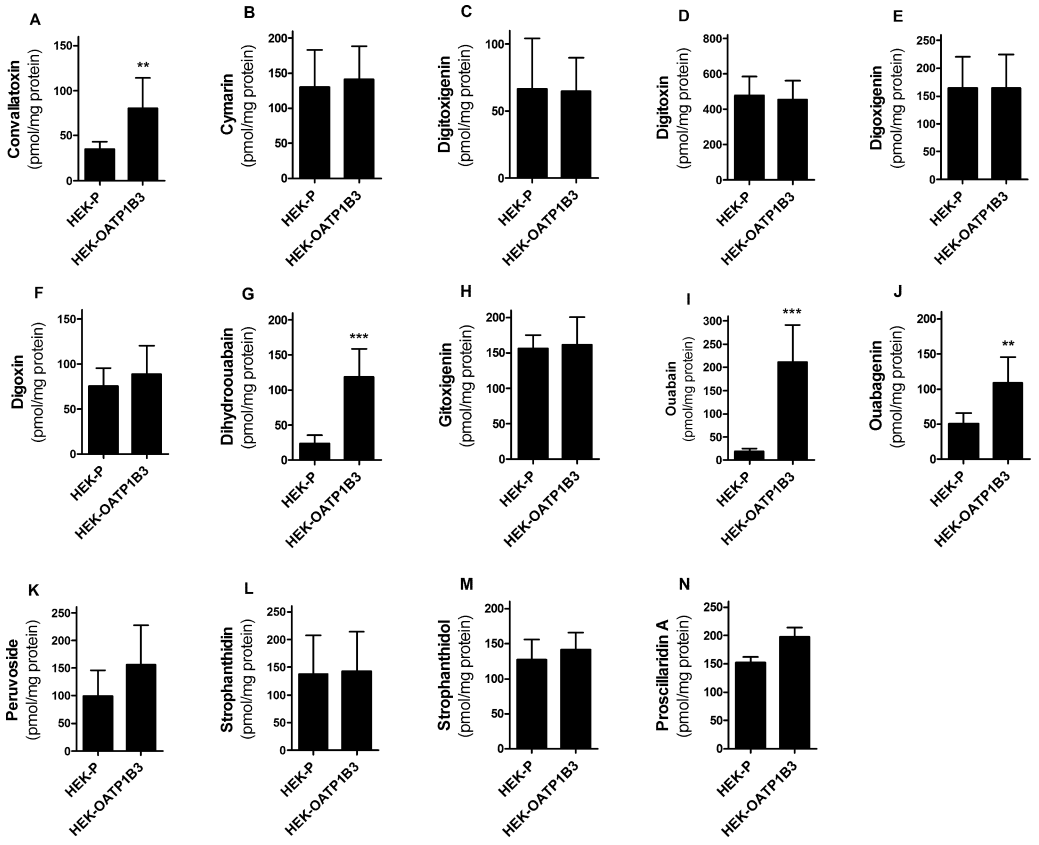

Figure 7. Uptake of DLCs in HEK-P and HEK-OATP1B3. Uptake of convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N) in HEK-P and HEK-OATP1B1 was measured after a 1 h incubation by LC−MS. Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown. An unpaired Student's *t* test was used to compare DLC uptake in HEK-OATP1B1 versus HEK-P (**, $p < 0.01$; ***, $p < 0.001$).

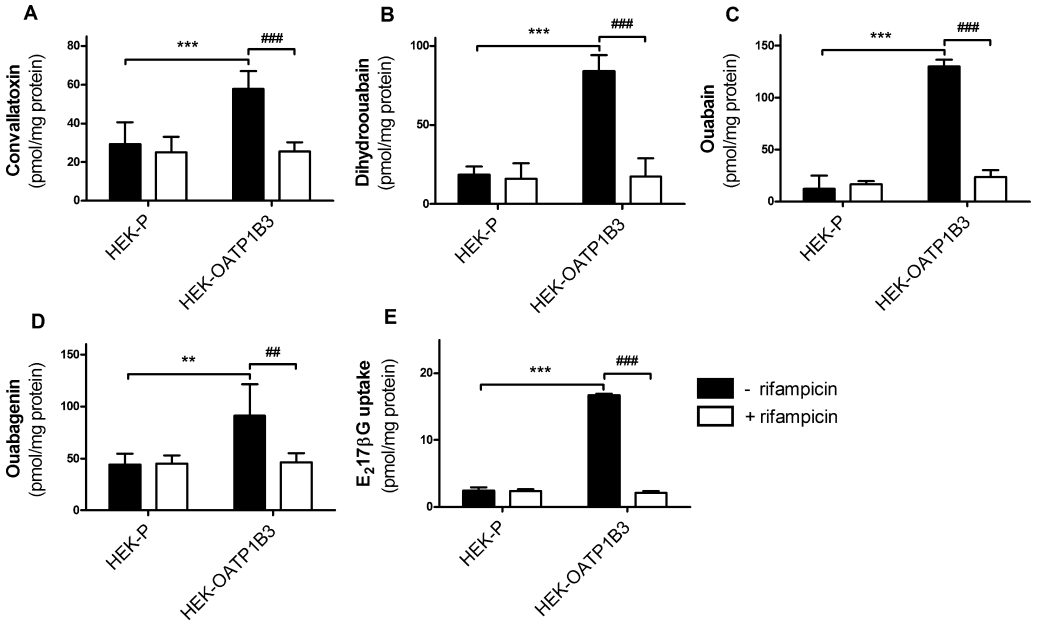

Figure 8. Inhibition of OATP1B3-mediated DLC transport. Uptake of DLCs was determined after incubation of 10 μM convallatoxin (A), dihydroouabain (B), ouabain (C), and ouabagenin (D) with HEK-P and HEK-OATP1B3 for 1 h in the absence (black bars) and presence (white bars) of rifampicin (100 μM). Uptake of $E_2 17\beta G$ (2 μM) in HEK-P and HEK-OATP1B3 was measured in the absence and presence of rifampicin (100 μM) after a 3 min incubation. The mean ± SD value of three independent experiments is shown. Uptake in HEK-OATP1B3 was compared to HEK-P (***, $p < 0.001$) and to the condition without rifampicin ($^{###}$, $p < 0.001$) using an unpaired Student's *t* test.

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cymarose sugar seems to be more favorable for binding than a rhamnose sugar (cymarin vs convallatoxin). In addition, the hydroxyl group at position 12 decreases the inhibitory potency (digoxin and digoxigenin vs digitoxin and digitoxigenin). Comparing gitoxigenin with digitoxigenin indicates that a hydroxyl group at position 16 reduces the inhibitory potency. Comparison of strophanthidin and strophanthidol indicates that a hydroxyl group at position 19 is better than a ketone for DLC binding.

It was shown previously that molecular weight, lipophilicity, polar surface, and presence of hydrogen-bond acceptors are the key factors for OATP1B1 and OATP1B3 inhibition.[34,51−53,60,61] In previous studies, we found a similar correlation between lipophilicity and inhibitory potency of various statins against NTCP.[43] Here, we did not observe an association between the inhibitory potency of DLCs against OATP1B1 and OATP1B3 and their physicochemical properties (molecular weight, octanol/water partition coefficient (cLogP), polar surface area (PSA), and number of H donors and acceptors). Although no statistically significant correlation was found, our findings did show a tendency of lipophilic DLCs to be better inhibitors of OATP1B1 and OATP1B3 (Supporting Information, Table 1).

Although none of the DLCs was a transporter substrate, we demonstrated that convallatoxin, dihydroouabain, ouabain, and ouabagenin are substrates of OATP1B3. It is the first time that convallatoxin, dihydroouabain, and ouabagenin have been reported as the substrates of OATP1B3. In a previous study by Kullak-Ublick et al., digoxin and ouabain uptake by OATP1B3 in *X. laevis* oocytes was shown,[35] but Kimoto et al. and Taub et al. did not observe any digoxin transport by OATP1B3.[32,37] Convallatoxin has been shown as an inhibitor of digoxin uptake in Caco-2 cells,[62] and its uptake by rat intestine seemed to be transporter-dependent.[63] The four DLCs that have been found as OATP1B3 substrates (convallatoxin, ouabain, dihydroouabain, and ouabagenin) do not inhibit the transporter under the conditions tested and are more hydrophilic than the other congeners (Table 2), which might be a characteristic of OATP1B3 substrates. Among newly found substrates of OATP1B3, ouabain (reported plasma concentration 0.7 nM) has been applied in therapy. Given the present data, ouabain pharmacokinetics may be affected by concomitant administration of potent OATP1B3 inhibitors.[64] Such DDI have, however, not been reported. We are not aware of clinical studies investigating the potential for DDI of convallatoxin, dihydroouabain, and ouabagenin either.

In summary, we identified new DLC inhibitors of the liver uptake transporters, NTCP, OATP1B1, and OATP1B3. We showed that structural features such as the sugar moiety and hydroxyl groups play different roles in the interaction with these transporters. The sugar moiety decreases the interaction with OATP1B3, whereas it enhances the interaction with NTCP and OATP1B1. The hydroxyl group at position 12 enhances the interaction with NTCP, but it decreases the interaction with OATP1B1 and OATP1B3. In addition, we found that convallatoxin, dihydroouabain, ouabain, and ouabagenin are novel substrates of OATP1B3.

■ ASSOCIATED CONTENT

Supporting Information

Calculated physicochemical properties of DLCs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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