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Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3

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Abstract

The pregnane X receptor is a ligand-activated transcription factor that is abundantly expressed in hepatocytes. Numerous drugs are pregnane X receptor ligands. To bind to their receptor they must cross the sinusoidal membrane. Organic anion transporting polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) are polyspecific transporters expressed at the sinusoidal membrane of human hepatocytes. They mediate transport of a variety of drugs including the pregnane X receptor ligands rifampicin and dexamethasone. To test whether additional pregnane X receptor ligands interact with OATP1B1- and 1B3-mediated transport, we developed Chinese Hamster Ovary (CHO) cell lines stably expressing OATP1B1 or 1B3 at high levels. OATP1B1- and 1B3-mediated estradiol-17β-glucuronide uptake was inhibited by several pregnane X receptor ligands in a concentration dependent way. IC_{50} values for rifampicin, paclitaxel, mifepristone, and troglitazone were within their respective pharmacological free plasma concentrations. Kinetic analysis revealed that clotrimazole inhibits OATP1B1-mediated estradiol-17β-glucuronide transport with a K_i of 7.7±0.3 μM in a competitive way. However, uptake of OATP1B3-mediated estradiol-17β-glucuronide was stimulated and this stimulation was due to an increased apparent affinity. Transport of estrone-3-sulfate was hardly affected while all other substrates tested were inhibited. Additional azoles like fluconazole, ketoconazole and miconazole did not stimulate OATP1B3-mediated estradiol-17β-glucuronide transport. In summary, these results demonstrate that pregnane X receptor ligands, by inhibiting or stimulating OATP-mediated uptake, can lead to drug–drug interactions at the transporter level.

Keywords: Organic anion transporting polypeptide; Hepatocyte; Drug–drug interaction; Nuclear receptor ligand

1. Introduction

Nuclear receptors are ligand-activated transcription factors that play an important role in xenobiotic disposition and human diseases like diabetes, obesity and cancer (Giguere, 1999; Wang and LeCluyse, 2003). Numerous clinically prescribed drugs including dexamethasone and rifampicin are ligands of the human pregnane X receptor, which is highly expressed in hepatocytes. By binding to their receptors, these drugs regulate the expression of many drug-metabolizing enzymes (Xie et al., 2004) and transporters (Guo et al., 2002). Therefore they can affect the metabolism of the majority of drugs. With the exception of rifampicin (Vavricka et al., 2002), paclitaxel (Smith et al., 2005) and lithocholate (Yamaguchi et al., 2006), it is largely unknown how pregnane X receptor ligands enter hepatocytes to bind to their receptor, but in general it is assumed to be by simple diffusion.

Organic anion transporting polypeptides (rodents: Oatps; human: OATPs) belong to a growing superfamily of transport proteins that mediate uptake of structurally diverse amphiphilic organic solutes (Hagenbuch and Meier, 2004). Among the 11 human OATPs, OATP1B1 and OATP1B3 are liver specific

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transporters that are multispecific and mediate the uptake of numerous drugs into hepatocytes (Hagenbuch and Meier, 2003). Previous work has shown that the glucocorticoid dexamethasone and the antibiotic rifampicin directly interact with OATPs. We demonstrated that dexamethasone inhibits OATP1A2-mediated dehydroepiandrosterone sulfate transport (Kullak-Ublick et al., 1998) in addition to being a substrate of rat Oatp1a1 (Bossuyt et al., 1996). Furthermore, we reported that rifampicin inhibits transport mediated by Oatp1a1 and Oatp1a4 (Fattinger et al., 2000). A more recent study showed that rifampicin not only inhibits human OATP1B1 and OATP1B3 but is directly transported by both OATPs (Vavricka et al., 2002).

Because drug–drug interactions can occur due to direct inhibition of uptake into liver in which OATP1B1 and OATP1B3 are expressed, we hypothesized that additional, readily available pregnane X receptor ligands including carbamazepine, clotrimazole, estradiol, lithocholate, metyrapone, mevinolin, mifepristone, paclitaxel, phenytoin, and troglitazone (Goodwin et al., 1999; Honkakoski et al., 2003) would interact with OATP1B1- and OATP1B3-mediated transport. To test this hypothesis, we developed Chinese Hamster Ovary (CHO) cells stably expressing either OATP1B1 or OATP1B3 and determined substrate uptake into these cells in the presence as well as absence of pregnane X receptor ligands.

2. Materials and methods

2.1. Materials

Radiolabeled [^{3}H]estradiol-17β-glucuronide (39.8 Ci/mmol) and [^{3}H]estrone-3-sulfate (57.3 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA), and [^{3}H]clotrimazole (7.5 Ci/mmol) from Moravek Biochemicals Inc. (Brea, CA). The cell-impermeant Fluo-3, pentapotassium salt was obtained from Invitrogen (Carlsbad, CA). Cell culture reagents were from Invitrogen (Carlsbad, CA) and fetal bovine serum from Hyclone (Logan, UT). All pregnane X receptor ligands were purchased from Sigma (St. Louis, MO) and stock solutions were prepared in ethanol or DMSO.

2.2. Cell culture, generation of stable cell lines and uptake experiments

Chinese Hamster Ovary (CHO) cells were grown at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium, containing 1 g/l D-glucose, 2 mM L-glutamine, 25 mM Hepes buffer and 110 mg/l sodium pyruvate, supplemented with 10% FBS (Hyclone, Logan, UT), 50 μg/ml L-proline, 100 U/ml penicillin and 100 μg/ml streptomycin. Open reading frames of OATP1B1*1b (Tirona et al., 2001) and OATP1B3 haplotype 1 (Smith et al., 2007) were PCR amplified using Pfu DNA Polymerase (Stratagene, La Jolla, CA) and cloned into the vector pIRESneo2 (Clontech, Mountain View, CA). After sequencing the plasmids, CHO cells were stably transfected by electroporation. Initially, the cell culture medium was supplemented with 1 mg/ml G-418. After single clones were isolated using cloning cylinders followed by limiting dilution, G-418 concentration was lowered to 500 μg/ml. Clones were characterized for transport and immunofluorescence and one clone for each OATP was used for further work.

For uptake experiments CHO-wild-type and OATP-expressing cells were plated at 40,000 cells per well on 24-well plates and 48-h later medium was replaced with medium containing 5 mM sodium-butyrate to induce nonspecific gene expression (Palermo et al., 1991). After another 24 h in culture, the cells were used for uptake experiments. Cells were washed three times with pre-warmed uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 20 mM Hepes, pH adjusted to 7.4 with Trizma base). Uptake was started by adding 200 μl of uptake buffer containing 0.3 to 0.6 μCi/ml of the radiolabeled substrate in the presence or absence of inhibitors. After 20 s, at previously determined initial linear rate conditions, uptake was stopped by removing the uptake solution and washing the cells four times with ice-cold uptake buffer. The cells were then solubilized with 500 μl of 1% Triton X-100. Three hundred microliters were used for liquid scintillation counting, and protein concentration was determined using the BCA assay with bovine serum albumin as a standard. Uptake of the anionic substrate Fluo-3 (Cui et al., 2001a) was determined essentially the same, except that the uptake was stopped after 10 min (the initial linear portion of uptake extended well over 10 min) and the cells were solubilized in the presence of 1 mM $CaCl_2$. Fluorescence was measured in a Bio-Tek Synergy HT microplate reader (Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Uptake rates were always calculated based on net uptake after subtracting the values obtained with wild-type CHO cells.

2.3. Immunofluorescence

For immunofluorescence, 30,000 cells were plated on culture slides (BD Biosciences, Bedford, MA) and induced with 5 mM sodium-butyrate 24 h before fixation. Cells were then fixed in 4% paraformaldehyde, 1% TX-100 in PBS for 1 h at room temperature. After washing, the cells were incubated with 5% BSA in PBS at room temperature for 1 h. After another wash with PBS, cells were incubated with anti-OATP1B1 (1:500) or anti-OATP1B3 (1:500) antibody in 1% BSA in PBS. Slides were then washed with PBS and incubated with Alexa Fluor 594 goat anti-rabbit IgG(H+L) (Invitrogen) for 1 h at room temperature. After a final wash with PBS, slides were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and used for confocal image analysis.

Fluorescent digital images were obtained using a Zeiss LSM510 confocal microscope equipped with a HeNe laser (1 mW) for the excitation (543 nm, 100% laser power) and detection (long pass 560 nm filter; LP560) of the Alexa Fluor 543/594. Images were acquired in Multitrack channel mode (sequential excitation/emission) with LSM510 (v 3.2) software and a Plan-Apochromat 63×/1.4 Oil DIC objective with a zoom factor of 1 (field size of 0.146 mm × 0.146 mm) and frame size of 1024 × 1024 pixels. Detector gain was set initially to cover

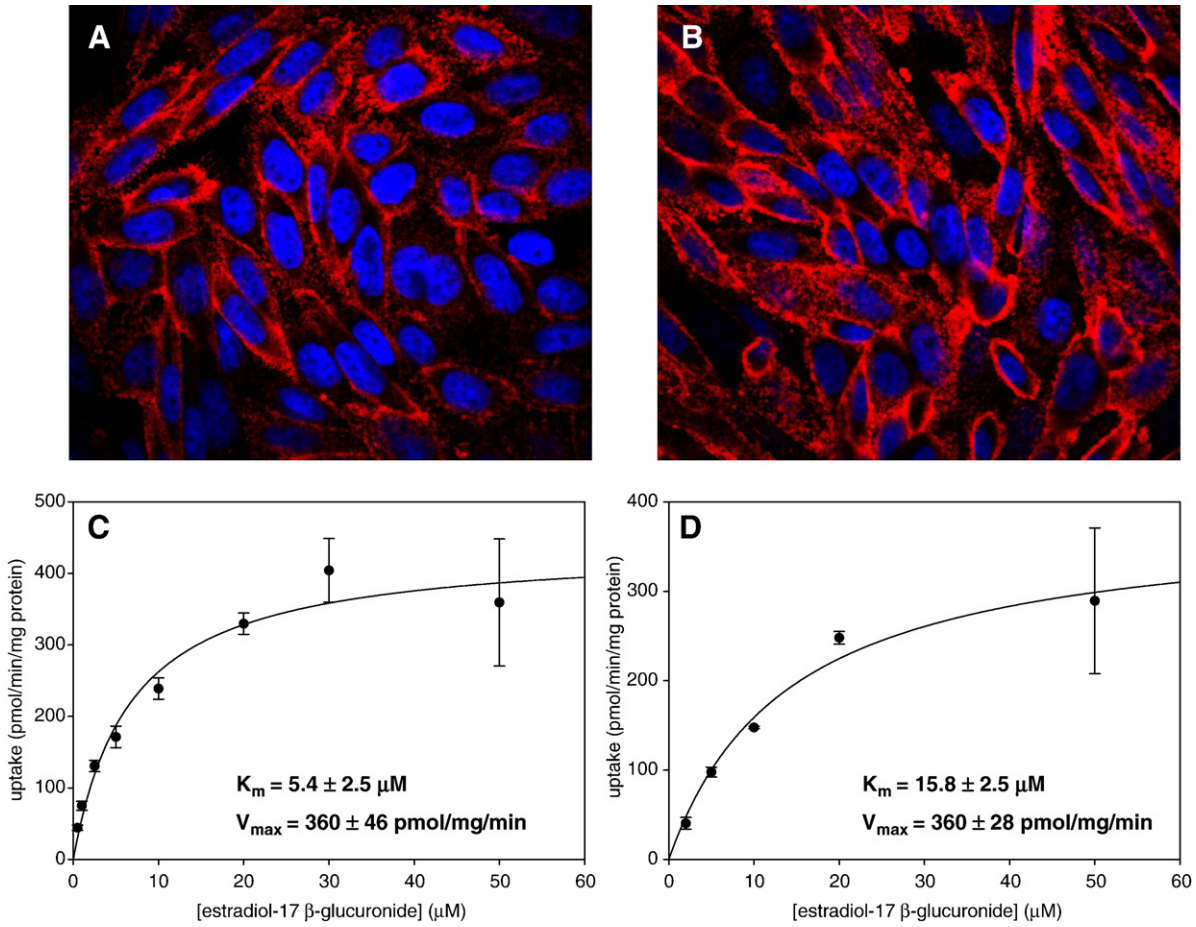

Fig. 1. Immunohistochemical (A, B) and functional (C, D) characterization of OATP1B1- and 1B3-expressing CHO cell lines. CHO cells expressing either OATP1B1 (A) or OATP1B3 (B) were grown on culture slides and stained with an anti-OATP1B1 or anti-OATP1B3 antibody (red color) while nulcei were visualized with DAPI (see Materials and methods). Uptake of increasing concentrations of [^{3}H]estradiol-17β-glucuronide was measured at 37 °C for 20 s with OATP-expressing and wild-type CHO cells. After subtracting the values obtained with wild-type cells, net OATP1B1 (C) and OATP1B3 (D) mediated uptake was fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Means±S.D. of triplicate determinations are given.

the full range of all the samples and background corrected by setting the amplifier gain, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. Control slides consisted of untransfected wild-type CHO cells.

2.4. Data analysis

IC_{50} and kinetic parameters were calculated using non-linear regression analysis and SigmaPlot (Version 9.01; Systat Software, Inc., Point Richmond, CA). To analyze whether the groups were different from the control, ANOVA was performed followed by the Bonferroni test (GraphPad InStat Version 3, GraphPad Software, Inc., San Diego, CA). The P value for statistical significance was set to <0.05.

3. Results

3.1. Characterization of OATP1B1 and OATP1B3 cell lines

We constructed CHO cell lines that stably express the most frequent variants of OATP1B1 (OATP1B1*1b or N130D) (Tirona et al., 2001) and OATP1B3 (haplotype 1 or S112A and M233I) (Smith et al., 2007). To demonstrate expression of OATP1B1 and 1B3 in the generated CHO cell lines, we used confocal microscopy. The results shown in Fig. 1(A and B)

Table 1
Kinetic parameters of OATP1B1- and OATP1B3-expressing CHO cells

Transporter	Substrate	K_m (μM)	V_{max} (pmol/mg/min)	V_{max}/K_m
OATP1B1	Estradiol-17β-glucuronide	5.4±2.5	360±46	66.7
	Estrone-3-sulfate	2.4±0.8	340±40	142
	Fluo-3	2.3±0.2	78±21 [a]	0.034
OATP1B3	Estradiol-17β-glucuronide	15.8±2.5	360±28	22.8
	Estrone-3-sulfate	58±20	1860±325	32
	Fluo-3	3.1±0.4	630±100 [a]	0.203

Initial transport rates (20 s for estradiol-17β-glucuronide and estrone-3-sulfate; 10 min for Fluo-3) at increasing concentrations were determined at 37 °C in OATP-expressing and wild-type CHO cells. Transport values were corrected with values obtained from wild-type cells and the resulting net carrier mediated uptake values were fitted by non-linear regression analysis to the Michaelis–Menten equation. Mean±S.E. are given for 2–7 experiments.

[a] fmol/mg/min.

demonstrate that the respective cell lines expressed OATP1B1 or 1B3 at the plasma membrane. To functionally characterize these OATP1B1- and 1B3-expressing CHO cells and measure some basic kinetic constants we first determined the initial linear portion of uptake. Uptakes of estradiol-17β-glucuronide and estrone-3-sulfate were linear for at least 20 s while uptake of Fluo-3 was linear over more than 10 min. Therefore, all experiments were performed at 20 s for estradiol-17β-glucuronide and

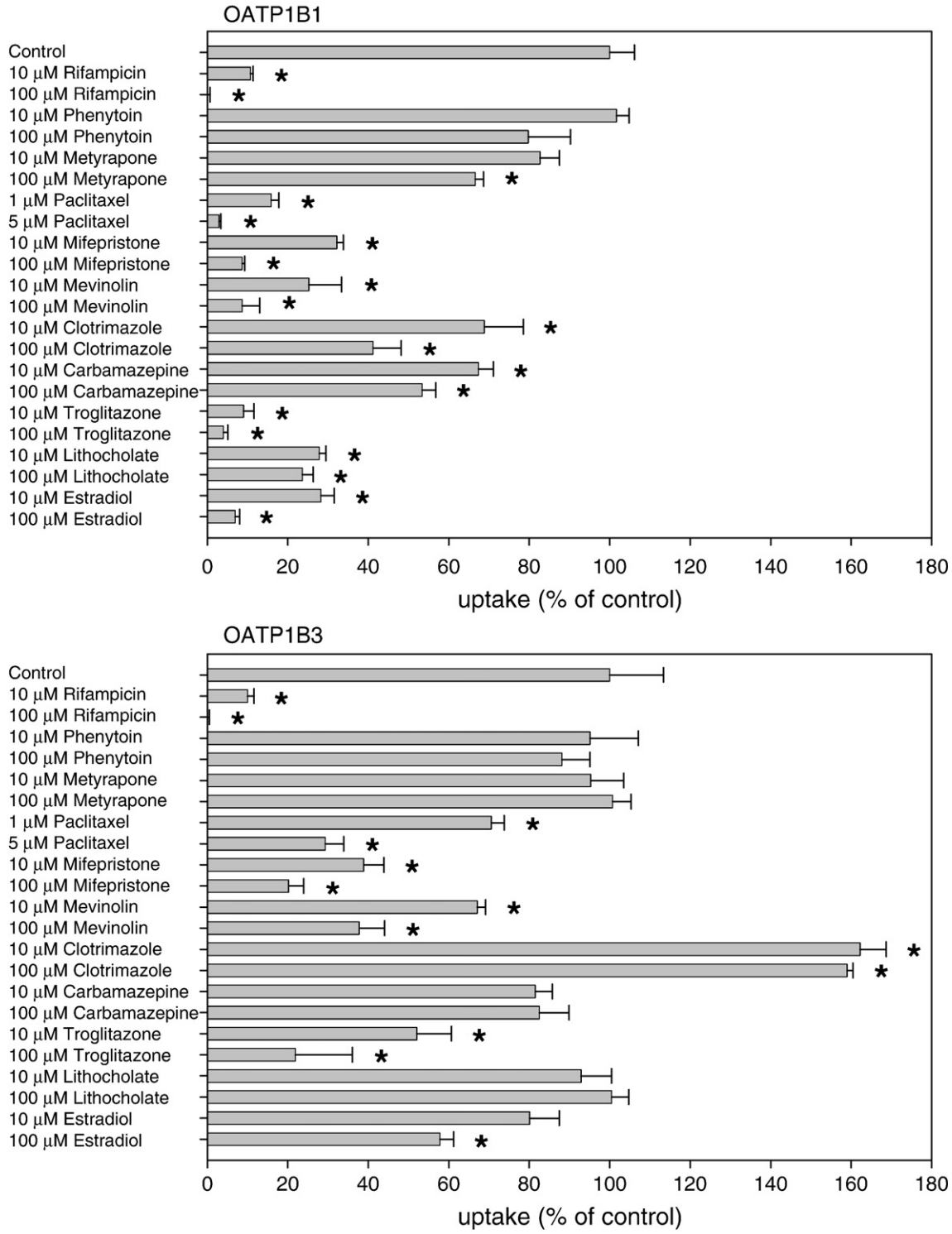

Fig. 2. Effect of pregnane X receptor ligands on OATP-mediated estradiol-17β-glucuronide uptake in stable transfected CHO cells. Uptake of 1 μM [^{3}H]estradiol-17β-glucuronide was measured at 37 °C for 20 s with OATP-expressing and wild-type CHO cells in the absence or presence of the indicated pregnane X receptor ligands. Values obtained with wild-type CHO cells were subtracted from values obtained with OATP-expressing CHO cells and are given as percent of the control. Means ± S.D. of triplicate determinations are given; *$P<0.05$.

estrone-3-sulfate and at 10 min for Fluo-3. Fig. 1(C and D) shows an example of saturation kinetics for estradiol-17β-glucuronide. Uptake was saturable with increasing concentrations of substrate for both OATP1B1- and 1B3-expressing cells. OATP1B1 exhibited an approximately 3-fold higher affinity than OATP1B3 with similar maximal transport rates (Fig. 1C and D; Table 1). Comparison of estrone-3-sulfate uptake mediated by both OATPs revealed that OATP1B1 represents a high-affinity low-capacity transporter, whereas OATP1B3 has low-affinity but high-capacity for estrone-3-sulfate uptake (Table 1). Fluo-3 was transported by both carriers with a similar affinity, but OATP1B3-mediated transport had an almost 10-fold higher capacity (Table 1).

3.2. Effect of pregnane X receptor ligands on estradiol-17β-glucuronide transport

To test whether pregnane X receptor ligands besides the known rifampicin interact with OATP1B1- and 1B3-mediated transport, we quantified uptake of 1 μM estradiol-17β-glucuronide in the absence and presence of 10 and 100 μM of selected commercially readily available pregnane X receptor ligands (Goodwin et al., 1999; Honkakoski et al., 2003) with the OATP1B1- and 1B3-expressing CHO cells. The results are summarized in Fig. 2. Rifampicin, a known substrate of OATP1B1 and of 1B3 (Vavricka et al., 2002) was used as a positive control and inhibited almost complete uptake mediated by both OATPs. Phenytoin had almost no effect, whereas all other pregnane X receptor ligands inhibited OATP1B1-mediated transport in a concentration dependent way. Paclitaxel has been shown to be a substrate for OATP1B3 but not for 1B1 (Smith et al., 2005). Nevertheless, it inhibited OATP1B1-mediated uptake stronger than uptake mediated by OATP1B3. In addition to paclitaxel, mifepristone, mevinolin, troglitazone, lithocholate and estradiol were strong inhibitors of OATP1B1-mediated estradiol-17β-glucuronide uptake (Fig. 2). Inhibition of OATP1B3-mediated estradiol-17β-glucuronide was less pronounced, but again paclitaxel, mifepristone, mevinolin and troglitazone were the strongest inhibitors. Interestingly, clotrimazole, which inhibited OATP1B1-mediated uptake, stimulated OATP1B3-mediated uptake at both concentrations (Fig. 2).

To estimate whether these pregnane X receptor ligands could inhibit uptake under normal pharmacological conditions, we determined the IC_{50} values for the compounds where free plasma concentrations are available. In Table 2 these results are summarized and compared to the respective free plasma concentrations. Based on the obtained IC_{50} values, rifampicin, paclitaxel, mifepristone and troglitazone are effective inhibitors of OATP1B1-mediated transport at concentrations that are reached in blood plasma. For OATP1B3 only rifampicin and mifepristone have IC_{50} values that would suggest potential interactions at the free plasma concentrations. We also examined the correlation between the IC_{50} values and the respective affinities to pregnane X receptor (Table 2) but we did not obtain any significant correlation, suggesting that the residues lining the transport/binding sites on OATP1B1 and 1B3 are quite different from those of the binding site(s) on pregnane X receptor.

3.3. Effect of clotrimazole and other azoles on substrate uptake

Because clotrimazole had such a different effect on OATP1B1- and 1B3-mediated transport of estradiol-17β-glucuronide, we further characterized this inhibition/stimulation and performed kinetic experiments. Fig. 3 shows that clotrimazole inhibited OATP1B1-mediated estradiol-17β-glucuronide uptake in a competitive way, affecting the apparent affinity but not the maximal transport rates. We calculated a K_i value of 7.7±0.3 μM from three independent experiments. Stimulation of OATP1B3-mediated estradiol-17β-glucuronide uptake by clotrimazole was due to increased apparent affinity. At 10 μM clotrimazole, the apparent K_m value decreased from 9.5 to 8.7 μM and at 100 μM clotrimazole to 4.7 μM without affecting the maximal transport rates. However, when we tested whether [^{3}H]clotrimazole is a transported substrate for OATP1B1 and/or 1B3, we did not find any uptake up to 10 μM clotrimazole, although the cells transported the positive control estradiol-17β-glucuronide.

We next compared OATP-mediated uptake of estradiol-17β-glucuronide, estrone-3-sulfate and Fluo-3 in the presence of increasing concentrations of clotrimazole. As shown in Fig. 4, clotrimazole inhibited OATP1B1-mediated uptake independent

Table 2
Comparison of IC_{50} values of pregnane X receptor ligands for inhibition of OATP1B1- and 1B3-mediated estradiol-17β-glucuronide uptake with unbound plasma concentrations and binding affinities to pregnane X receptor

Pregnane X receptor ligands	IC_{50} on OATP1B1 (μM)	IC_{50} on OATP1B3 (μM)	Unbound plasma concentration (μM)	Affinity for pregnane X receptor EC_{50} (μM)
Rifampicin	1.5	2.6	0.7–2.4 (Brunton et al., 2005)	0.71 (Moore et al., 2000)
Paclitaxel	0.03	0.5	0.085 (Brunton et al., 2005)	5 (Synold et al., 2001)
Mifepristone	3.3	5.3	1.9 (Sarkar, 2005)	5.5 (Moore et al., 2000)
Mevinolin	6.1	92.6	0.006 (Brunton et al., 2005)	1–5 (Lehmann et al., 1998)
Clotrimazole	9.0		2 (Tiffert et al., 2000)	1.6 (Moore et al., 2000)
Carbamazepine	188	510	9.7 (Brunton et al., 2005)	N.A.
Troglitazone	1.2	15.7	1–3 (Asano et al., 1999)	3 (Jones et al., 2000)
Lithocholate	0.7	6.8	0.05 (Castano et al., 2006)	9 (Staudinger et al., 2001)
Estradiol	3.9	12.9	0.001 (Persky et al., 1978)	N.A.

IC_{50} values were calculated using non-linear regression analysis and SigmaPlot; N.A.: not available.

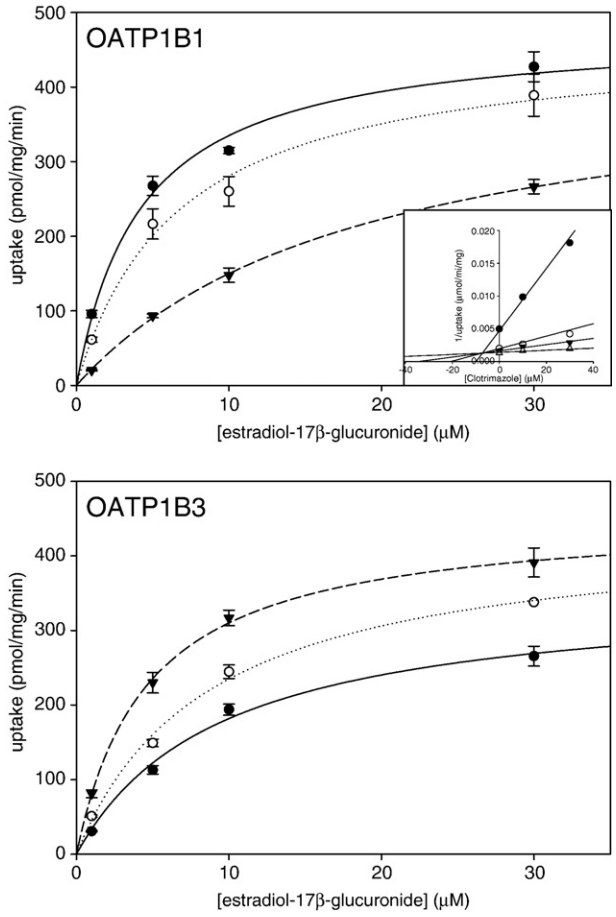

Fig. 3. Kinetic analysis of estradiol-17β-glucuronide transport by OATP1B1 and OATP1B3 in the absence and presence of clotrimazole. Uptake of increasing concentrations of [^{3}H]estradiol-17β-glucuronide was measured at 37 °C for 20 s with OATP-expressing and wild-type CHO cells in the absence or presence of clotrimazole. After subtracting the values obtained with wild-type cells, net OATP-mediated uptake was fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Means±S.D. of triplicate determinations are given. Clotrimazole concentrations in the case of OATP1B1 were 10 (open circles) and 30 μM (closed triangles) and in the case of OATP1B3 10 (open circles) and 100 μM (closed triangles). The inset shows a Dixon plot for the inhibition kinetics of OATP1B1-mediated transport.

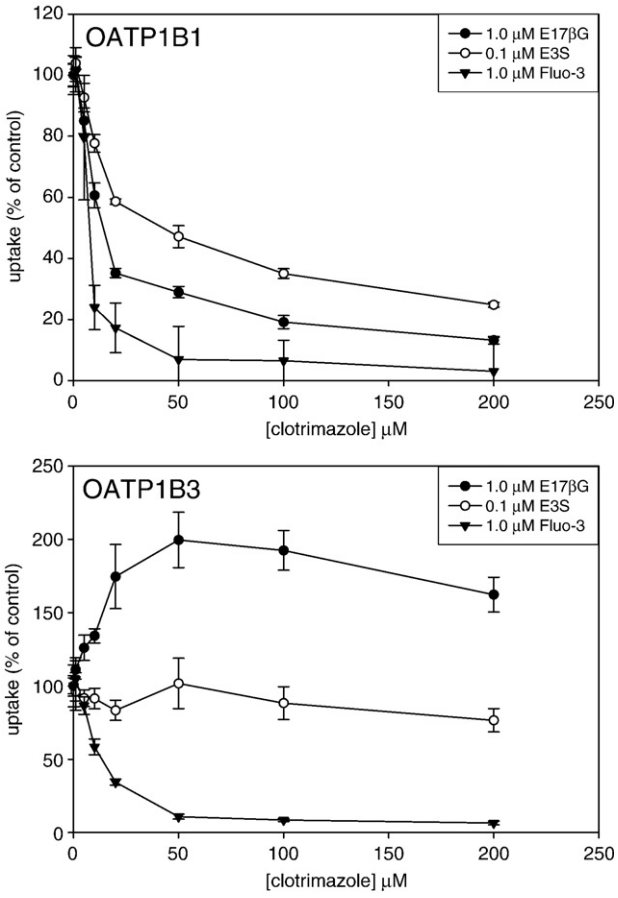

Fig. 4. Effect of clotrimazole on OATP-mediated substrate uptake. Uptake of the indicated substrates was measured at 37 °C with OATP-expressing and wild-type CHO cells in the absence and presence of increasing concentrations of clotrimazole. Values obtained with wild-type CHO cells were subtracted from values obtained with OATP-expressing CHO cells and are given as percent of the control. Means±S.D. of triplicate determinations are given.

of the transported substrate in a concentration dependent manner. However, the effect of clotrimazole on OATP1B3-mediated uptake was dependent on the transported substrate. Consistent with the results shown in Fig. 3, clotrimazole stimulated estradiol-17β-glucuronide uptake at all concentrations tested with a maximal effect at 50 μM. Estrone-3-sulfate uptake was minimally affected, whereas Fluo-3 transport was inhibited in a concentration dependent way. Six additional OATP substrates were tested in the absence and presence of 50 μM clotrimazole. As can be seen from the results summarized in Table 3, clotrimazole clearly stimulated OATP1B3-mediated estradiol-17β-glucuronide uptake. Uptake of estrone-3-sulfate was slightly higher in the presence of clotrimazole while transport of all other substrates was inhibited.

In order to examine whether additional azole derivatives would exert a similar effect on OATP-mediated estradiol-17β-glucuronide uptake, we measured transport in the presence of miconazole, ketoconazole and fluconazole at 10 and 100 μM. OATP1B1-mediated uptake was inhibited by all

Table 3
Effect of clotrimazole on OATP1B1- and OATP1B3-mediated substrate uptake

Substrate	OATP1B1 uptake (fmol/mg)		OATP1B3 uptake (fmol/mg)	
	Clotrimazole (50 μM)		Clotrimazole (50 μM)	
	−	+	−	+
Estradiol-17β-glucuronide	141.2±15.0	39.1±3.2	40.7±6.1	103.5±8.3
Estrone-3-sulfate	760.9±30.3	365.5±8.9	43.9±3.7	51.1±2.6
CCK-8	5.9±0.4	1.0±0.9	80.1±4.2	64.9±1.8
DPDPE	3.2±1.7	1.0±0.6	21.8±2.2	11.0±1.5
Etoposide	216.0±50.4	74.7±59.4	452.6±55.8	257.5±61.2
Paclitaxel	166.5±22.9	16.6±7.3	173.8±24.2	16.4±13.2
Taurocholate	5.2±1.5	1.4±1.5	30.1±4.1	16.5±1.8
Thyroxine	3.6±1.4	1.0±0.6	3.3±1.0	0.2±0.4

Uptake of tracer compounds was determined under initial linear rates (20 s) in the absence or presence (− or +) of clotrimazole. Mean±S.D. are given for one of two independent experiments.

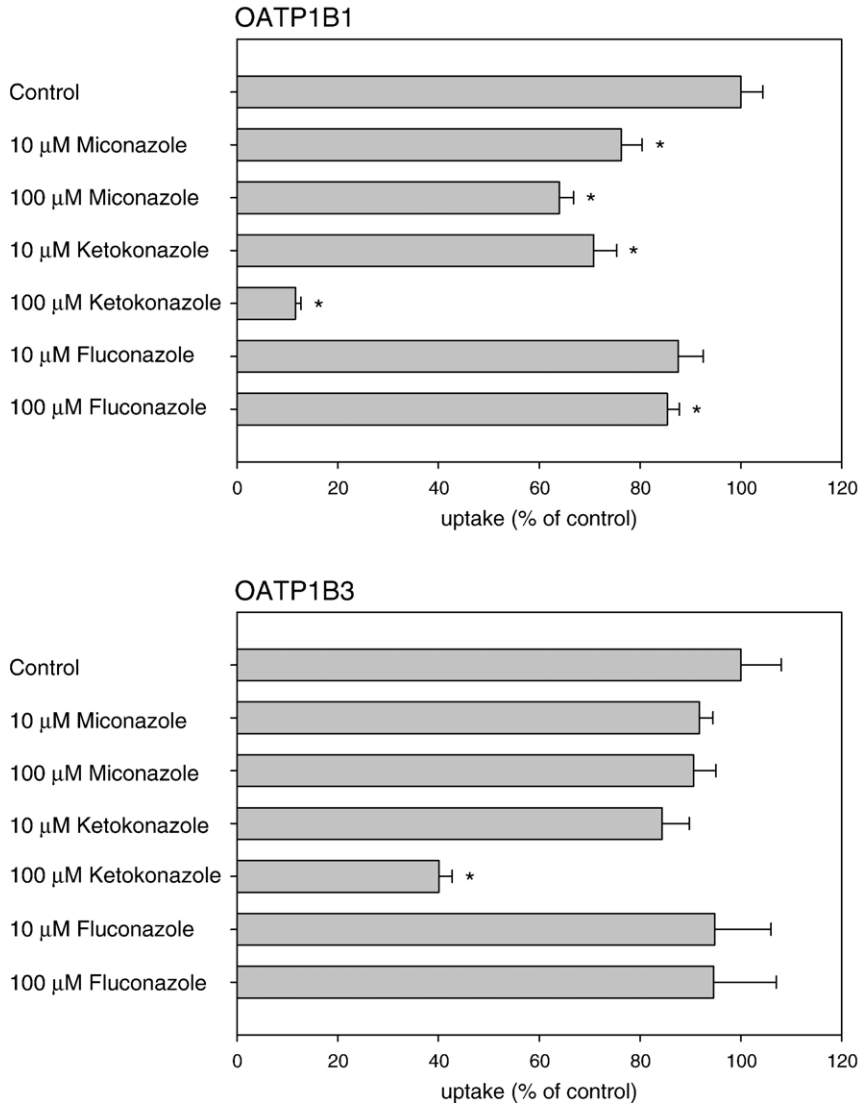

Fig. 5. Effect of azole derivatives on OATP-mediated estradiol-17β-glucuronide transport. Uptake of 1 μM [^{3}H]estradiol-17β-glucuronide was measured at 37 °C for 20 s with OATP-expressing and wild-type CHO cells in the absence or presence of the indicated azole derivatives. Values obtained with wild-type CHO cells were subtracted from values obtained with OATP-expressing CHO cells and are given as percent of the control. Means ± S.D. of triplicate determinations are given; *$P<0.05$.

three compounds, whereas OATP1B3-mediated uptake was only inhibited by ketoconazole (Fig. 5).

4. Discussion

The experiments presented here show that several pregnane X receptor ligands interact with uptake mediated by human OATP1B1 and 1B3 at pharmacological concentrations. Therefore, these pregnane X receptor ligands have the potential to cause adverse interactions with other drugs that are transported by the two liver specific OATPs. In agreement with previously reported studies for several drugs (Campbell et al., 2004; Hirano et al., 2006; Shitara et al., 2003; Vavricka et al., 2002) most tested pregnane X receptor ligands inhibit OATP1B1- and OATP1B3-mediated uptake. Clotrimazole is a common drug that is applied locally in the treatment of vaginitis and can reach estimated plasma concentrations similar to the concentrations used in our experiments (Ritter et al., 1982). At these concentrations clotrimazole inhibited OATP1B1 but stimulated OATP1B3, and thus can possibly alter the pharmacokinetic properties of drugs cleared by these two transporters in opposite ways. While inhibition of OATP1B1 could potentially lead to increased drug plasma levels, stimulation of OATP1B3 could lead to enhanced drug removal and thus to lower plasma concentrations.

Clotrimazole increased the affinity of OATP1B3 for estradiol-17β-glucuronide, probably through a conformational change of the protein, which does not seem to be allosteric. We are currently constructing chimeras and performing site-directed mutagenesis to further explain the exact molecular mechanism of this activation of substrate transport. Such stimulation could be a general mechanism to stimulate OATP-mediated uptake. This is supported by the stimulation of Oatp1a4-mediated taurocholate transport by estradiol-17β-glucuronide (Sugiyama et al., 2002), as well as the recent finding that progesterone can

stimulate OATP2B1-mediated transport (Grube et al., 2006). All these effects seem to be substrate dependent. While estradiol-17β-glucuronide uptake mediated by OATP1B3 was strongly stimulated by clotrimazole we could not reproduce the stimulation of taurocholate transport by estradiol-17β-glucuronide that was reported for rat Oatp1a4 (Sugiyama et al., 2002) with either OATP1B1 or 1B3 (data not shown). Nevertheless, this finding supports the hypothesis that substrates, as in the case of OCT1 (Gorboulev et al., 2005), interact with OATP1B3 at different binding sites. These comparable findings for several OATPs (OATP1B3, this study; Oatp1a4 (Sugiyama et al., 2002); OATP2B1 (Grube et al., 2006)) suggest that this could be a common phenomenon among all multispecific OATPs, explaining at least in part the molecular basis for the multispecificity. Additional evidence for multiple binding sites comes from the inhibition experiments shown in Fig. 2. OATP1B1-mediated estradiol-17β-glucuronide uptake can be inhibited completely with 100 μM rifampicin. Nevertheless, both, 10 and 100 μM lithocholate inhibited this uptake only by about 70%, suggesting different estradiol-17β-glucuronide transport/binding sites that are not completely inhibited by lithocholate.

Besides clotrimazole, we also tested the effect of additional azole derivatives (miconazole, ketoconazole and fluconazole) on OATP1B1- and OATP1B3-mediated uptake. None of these compounds stimulated OATP1B3-mediated uptake, suggesting that it is not the azole structure that is responsible for the stimulation effect of clotrimazole. However, additional experiments are needed to identify the molecular components of these azole derivatives that are responsible for inhibition or stimulation by interacting with OATPs.

Our results further demonstrate that the OATP1B1- and OATP1B3-expressing CHO cells described in this study are useful tools to screen for interactions between the expressed OATPs and pregnane X receptor ligands or other potential interacting compounds. These cell lines exhibit the highest V_{max} values published so far. The K_m values for estradiol-17β-glucuronide uptake are comparable to previously published data for both, OATP1B1- and OATP1B3-mediated transport (Hirano et al., 2004; König et al., 2006; Nakai et al., 2001). Similarly, the values for OATP1B1-mediated estrone-3-sulfate transport agree well with previously published values (Hirano et al., 2004; König et al., 2006; Nozawa et al., 2002). However, so far no K_m values have been published for estrone-3-sulfate transport mediated by OATP1B3. Two groups reported that there was no detectable estrone-3-sulfate transport in their HEK293 cells expressing OATP1B3 (Cui et al., 2001b; Hirano et al., 2004), whereas two other reports documented estrone-3-sulfate transport in OATP1B3 expressing MDCKII and HEK293 cells (Letschert et al., 2004) and in *Xenopus laevis* oocytes (Kullak-Ublick et al., 2001). This discrepancy can be explained by lower expression levels (several fold lower V_{max} values) obtained in HEK293 cells compared to the expression levels in the CHO cells used in our studies. With respect to Fluo-3, two previous reports showed that this calcium indicator is a substrate of OATP1B3 (Baldes et al., 2006; Cui et al., 2001a). Our studies extend those findings and show that Fluo-3 is also a substrate of OATP1B1, only with a much lower transport capacity.

Among the tested pregnane X receptor ligands, paclitaxel exhibited the strongest inhibition for both, OATP1B1- and OATP1B3-mediated uptake. A recent study demonstrated that paclitaxel is transported by OATP1B3-expressing *X. laevis* oocytes, but not by OATP1B1-expressing oocytes (Smith et al., 2005). Preliminary results obtained with our cell lines indicate that paclitaxel is indeed transported by both OATP1B1 and 1B3, although with different affinities (data not shown). Further experiments are required to elaborate these results and determine the affinity constants.

In summary, we have demonstrated that many pregnane X receptor ligands interact with OATP1B1- and 1B3-mediated substrate uptake. Clotrimazole which is not a transported substrate is able to stimulate OATP1B3-mediated uptake of certain compounds. Therefore, co-administration of drugs with compounds that interact with OATP-mediated transport has the potential to increase or decrease the bioavailability of these drugs and result in adverse drug–drug interactions. In addition, our OATP1B1- and 1B3-expressing CHO cells are excellent tools for the investigation of the multispecificity of these two important transporters. Furthermore, the presented results suggest that a detailed understanding of the molecular mechanisms of OATP-mediated transport will help to avoid adverse drug–drug interactions at the transporter level in the process of drug development.

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