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Research paper

Development of a fluorescence-based assay for screening of modulators of human Organic Anion Transporter 1B3 (OATP1B3)

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Abstract

The organic anion transporting protein 1B3 (OATP1B3), formerly termed OATP8, is responsible for uptake and subsequent elimination of multiple amphipathic drugs by the liver. *In silico* methods for the prediction of transport rates for drugs and drug-like molecules might provide an important tool in drug development. Most prediction methods however require a large training set of *in vitro* experimental data in order to yield reliable results. To obtain these data, we have developed a fluorescence-based assay that allows screening a relatively high number of substances for their transporter affinity.

HEK293 cells overexpressing OATP1B3 (HEK-OATP8) [Y. Cui, J. Konig, D. Keppler, Vectorial transport by double-transfected cells expressing the human uptake transporter SLC21A8 and the apical export pump ABCC2, Mol. Pharmacol. 60 (2001) 934–943.] were tested for transport of Fluo-3. Fluo-3 uptake could be seen in a concentration-dependent manner. Uptake can be inhibited completely by the addition of the known OATP1B3-inhibitor rifampicin proving that Fluo-3 is transported by OATP1B3. To verify the suitability of the system to identify modulators of OATP1B3, we tested known substrates for competitively inhibiting the Fluo-3 transport by giving them simultaneously with a 2 μM Fluo-3-solution to the cells. The transport of Fluo-3 was decreased by all test substrates in a concentration dependent manner.

Keywords: active transport; bioavailability; hepatic uptake; OATP; human; substrate specifity

1. Introduction

Besides their permeability through the intestinal epithelium, the bioavailability of orally administered drugs depends on their clearance by the cells of the intestine and the liver (first-pass effect). One of the major functions of the liver is the removal of a variety of endogenous and xenobiotic compounds from the blood circulation and their secretion into the bile. Uptake transporters expressed on the basolateral (sinusoidal) membrane of hepatocytes facilitate the cellular accumulation of drug substrates, prior to efflux transporter-mediated excretion through the apical (canalicular) membrane into the bile.

In human hepatocytes, the sodium-independent uptake of amphiphilic organic anions is mediated mainly by the human organic anion transporters OATP1B1 (SLC21A6, also known as OATP-C or OATP2) [1–4], OATP1B3 (SLC21A8 or OATP8) [1,5] and OATP2B1 (SLC21A9 or OATP-B) [6]. All three transport proteins belong to the subgroup 21 of the solute carrier (SLC) superfamily. OATP1B1 and OATP1B3 are expressed exclusively in human hepatocytes, while OATP2B2 is also expressed in other tissues, including placenta, intestine, kidney and lung [6].

The substrate-specifity of OATP1B3 is partly overlapping with other OATPs and includes bile salts like taurocholate and glycocholate, bromosulfophtalein (BSP), steroid hormone conjugates, thyroid hormones, the cardiac glycosides digoxin and ouabain and anionic peptides, like BQ-123 or [D-penicillamin2,5] enkephalin [5–8].

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The fluorescent Ca^{2+} − indicator Fluo-3 was described by Cui *et al.* [1] to be a substrate for OATP1B3 in MDCKII-cells double-transfected with OATP1B3 and MRP2. Most of the transport experiments conducted so far for OATPs used radioactively labeled substrates. In this study, we examined the uptake of the fluorescent substrate Fluo-3 by OATP1B3 and developed a fluorescence-based assay to screen a large number of substances for potential substrates of OATP1B3.

2. Materials and methods

2.1. Materials

1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′N′-tetraacetic acid tetra ammonium salt (Fluo-3) was obtained from Calbiochem (Bad Soden, Germany). G418 (Geneticin) sulfate was purchased from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma (Deisenhofen, Germany). Poly-D-lysine coated 24-well-plates were from greiner bio-one (Frickenhausen, Germany) .

2.2. Cell culture

Human embryonic kidney cells overexpressing human recombinant OATP1B3 (HEK-OATP8) were kindly provided by Prof. Dr. Dietrich Keppler (Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Heidelberg, Germany). Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and 650 μM G418 sulfate at 37 °C, 85% humidity and 5% CO_2.

2.3. RT-PCR

RNA was isolated from $5x10^6$ cells of each cell line grown to confluence in 75 cm^2 flasks by using the RNeasy Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with 1 μg of isolated RNA from each cell line by using Omniscript Reverse Transciptase (Qiagen) in a 20 μl reaction volume for 1 h at 37 °C. Oligo-dT-Primer and RNasin were purchased from Promega (Madison, USA). After inactivation of the Reverse Transcriptase for 5 min at 95 °C, 1.5 μl of each reaction product were used for a PCR reaction (final volume 100 μl). As a control, the expression of the house keeping gene GAPDH was examined. The primer sets used were: OATP8_for (5′-TCA TAA ACT CTT TGT TCT CTG CAA-3′) and OATP8_rev (5′-GTT GGC AGC AGC ATT GTC TTG-3′) for human OATP1B3 generating a 471 bp product [9] and GAPDH_for (5′-ACC ACA GTC CAT GCC ATC AC-3′) and GAPDH_rev (5′-TCC ACC ACC CTG TTG CTG TA-3′) for human GAPDH generating a 452 bp product [10]. cDNA was amplified by 35 amplification cycles with the following settings: 94 °C for 30 s, 58 °C for 60 s, 72 °C for 30 sec. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

2.4. Uptake studies

For uptake assays cells were seeded in 24-well plates coated with Poly-D-lysine at a density of 4×10^5 cells/well. Expression of recombinant OATP1B3 was further enhanced by culturing cells with 10 mM sodium butyrate [5].

Before the uptake experiments cells were washed once with uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose and 12.5 mM HEPES, pH 7.4). After incubation for 1 h in uptake buffer the transport assay was started by adding 500 μl uptake buffer containing Fluo-3. After incubation at 37 °C for different times the cells were washed three times with icecold uptake buffer and lysed with 0.05% SDS in a 1 mM $CaCl_2$ solution. Uptake of Fluo-3 was determined by measuring fluorescence in a Cytofluor II microplate fluorescence reader (PerSeptive Biosystems, Wiesbaden, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. K_m value for the Fluo-3 uptake was determined using Sigma Plot for Windows, Version 8.0 (Systat Software, Erkrath, Germany).

For screening of other potential substrates and inhibitors by competitive inhibition of Fluo-3 uptake, test substances were included in the uptake buffer for preincubation and in the Fluo-3 solution at indicated concentrations.

2.5. Determination of IC_{50}values

Cells were seeded in 24-well plates as described above. Inhibition studies were conducted as described with different inhibitor concentrations ranging from 0.01 to 400 μM. Uptake was stopped after 1 h by washing the cells three times with icecold uptake buffer. IC_{50} values were determined by using Sigma Plot for Windows, Version 8.0 (Systat Software, Erkrath, Germany).

3. Results

3.1. Fluo-3 is a substrate for OATP1B3 in HEK-OATP8 cells

Cui *et al.* [1] showed that the fluorescent Ca^{2+} indicator Fluo-3 is also a substrate for OATP1B3 in MDCK cells double transfected with OATP1B3 and MRP2. Since it is easily detectable, we examined its transport in HEK293 cells transfected with OATP1B3 (HEK-OATP8). Presence of OATP1B3 expression was shown by RT-PCR in comparison to Caco-2 cells reported not to express OATP1B3 [2] (Fig. 1). Cells were seeded on 24-well plates in a density of 400,000 cells/well and grown with sodium butyrate for 24 h to increase expression of OATP1B3 [11].

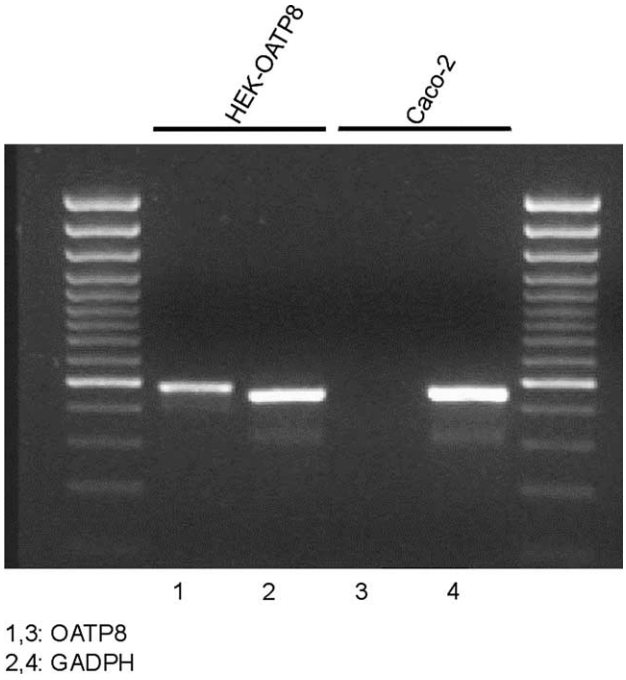

Fig. 1. Expression of OATP1B3 in HEK-OATP8 cells compared to Caco-2 cells. GAPDH was used as internal standard. The bands for OATP1B3 and GAPDH are located on the gel at a position consistent with the expected size of 471 bp and 452 bp respectively.

A saturable uptake of Fluo-3 by the HEK-OATP8 cells with a K_m value of 6.8 ± 1.3 μM could be shown during a time period of 2 h (Fig. 2).

Rifampicin has been reported to inhibit the transport of estradiol-17-β-glucuronide and BSP by OATP1B1 and OATP1B3 competitively [12,13]. We tested its ability to inhibit Fluo-3 transport in the HEK-OATP8 cell system by adding it at various concentrations simultaneously with 2 μM Fluo-3 to the cells. In a concentration of 5 μM

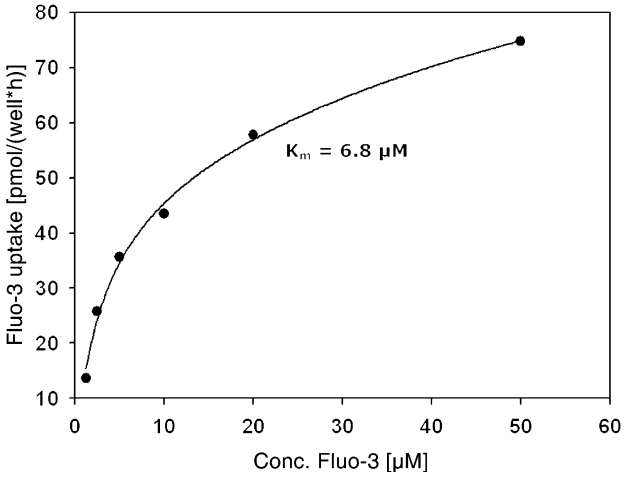

Fig. 2. Concentration dependence of Fluo-3 uptake by HEK-OATP1B3 cells. Fluo-3 uptake into HEK293 cells transfected with OATP1B3 (HEK-OATP8) was measured at concentrations between 0.125 and 50 μM. Data are means from a duplicate experiment. The K_m value was determined by using Sigma Plot for Windows, Version 8.0 (Systat Software, Erkrath, Germany).

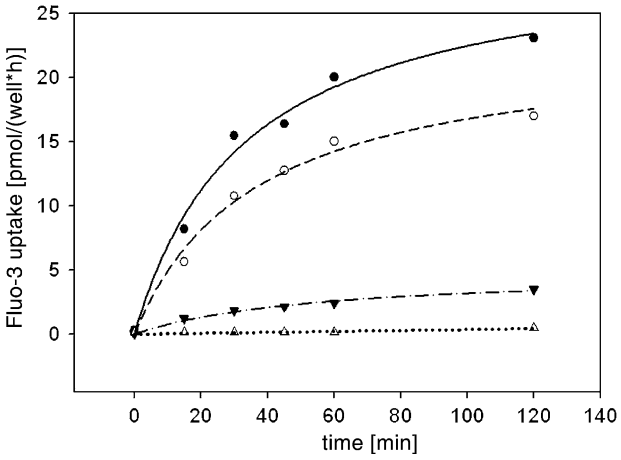

Fig. 3. Inhibition of Fluo-3 uptake by rifampicin. Inhibition of Fluo-3 uptake by rifampicin at a Fluo-3 concentration of 2 μM was measured for 120 minutes. Concentrations of rifampicin were as indicated. Data are means from a duplicate experiment. ——w/o rifampicin; – – – – 0.5 μM rifampicin; – · – · – 5 μM rifampicin; ····· 50 μM rifampicin.

rifampicin inhibits the transport of Fluo-3 into HEK-OATP8 cells almost completely confirming the transport of Fluo-3 by OATP1B3 (Fig. 3). Although rifampicin itself is reported to be a substrate of OATP1B3 we could not detect its uptake into HEK-OATP8 cells by measuring absorption at 475 nm. This is probably because the amount transported is below the detection limit.

3.2. Uptake of Fluo-3 is inhibited by known substrates of OATP1B3

To verify the suitability of the system to screen for potential new substrates, we tested known substrates for

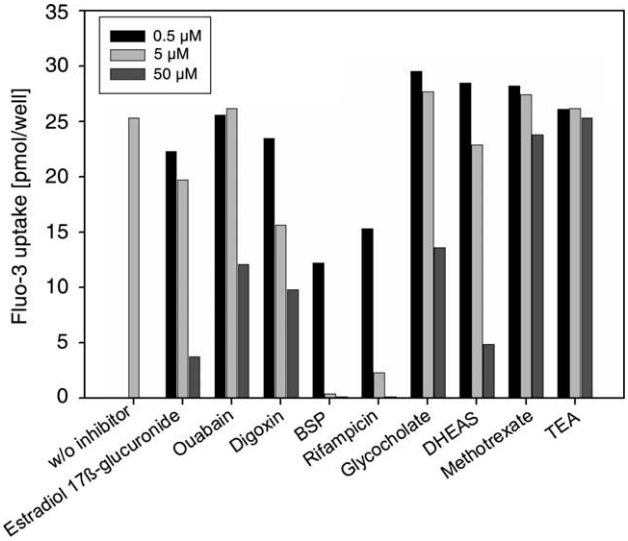

Fig. 4. Competitive inhibition of Fluo-3 transport by substrates of OATP1B3. Uptake of Fluo-3 from a 2 μM solution without inhibitor and with inhibitor in a concentration between 0.5 and 50 μM were examined after 30 and 60 minutes. Bars show the amount of Fluo-3 uptaken by the cells in [pmol/(well*h)].

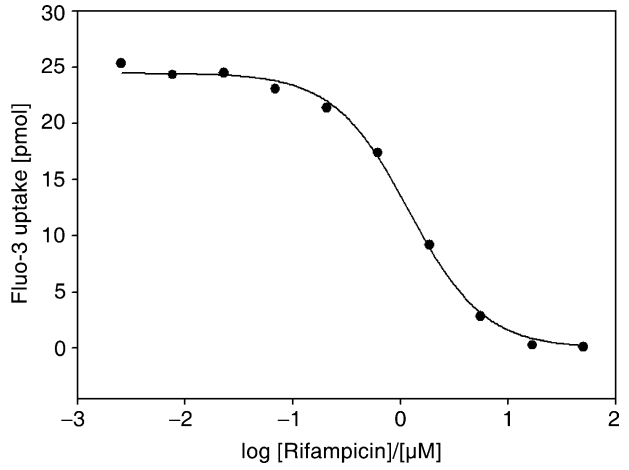

Fig. 5. Determination of IC_{50} value for rifampicin. Uptake of Fluo-3 from a 2 μM solution was determined with different rifampicin concentrations ranging from 0.01 to 200 μM. Uptake was stopped after 1 h by washing the cells three times with icecold uptake buffer. The IC_{50} value was determined by using Sigma Plot for Windows, Version 8.0 (Systat Software, Erkrath, Germany).

competitively decreasing the Fluo-3 transport by giving them simultaneously with a 2 μM Fluo-3-solution to the cells. As test substrates we chose the organic anion BSP [5,6,8], estradiol-17-β-glucuronide [5,6,8], the cardiac glycosides ouabain and digoxin [6], dehydroepiandrosterone sulfate (DHEAS) [5,6,8,14], methotrexate [2], sodium glycocholate [5,6] and ketoconazole [4]. The transport of Fluo-3 was decreased by all substrates chosen in a concentration dependent manner. The cationic substance triethylammoniumchloride (TEA) was used as a negative control and showed no effect on Fluo-3 transport (Fig. 4).

In order to rank the transporter affinity of the OATP1B3 substrates, IC_{50} values for the inhibition of Fluo-3 transport by these substrates were determined (Fig. 5, Table 1). The eight tested substrates have IC_{50} values between 0.57 and 435 μM. Ouabain and BSP possess the lowest values and the highest inhibitory effect on Fluo-3 transport respectively, while methotrexate showed the weakest effect.

Table 1
IC_{50} values of the examined inhibitors

Inhibitor	IC_{50} value [μM]	K_m [μM]	Reference
Ouabain	0.57 ± 0.1		
BSP	0.65 ± 0.03	0.4–6	[5,6,8,14]
rifampicin	1.5 ± 0.13	2	[12]
ketoconazole	14.1 ± 1.07		
DHEAS	20.6 ± 1.05		
estradiol-17-β-glucuronide	21.0 ± 0.6	5	[5,14]
digoxin	41.8 ± 6.2		
glycocholate	42.3 ± 1.1		
methotrexate	124.6 ± 1.26	25	[2]

IC_{50} values are given as means ± SD ($n = 2$)

4. Discussion

In this work, we established a fluorescent-based assay to easily determine if a substance is a modulator (substrate or inhibitor) of OATP1B3 or not. We used HEK293 cells transfected with OATP1B3 as a model system. The expression of OATP1B3 was confirmed by RT-PCR. It could be shown that the fluorescent Ca^{2+} indicator Fluo-3 is a substrate of OATP1B3 in the HEK cell system as already reported by Cui *et al.* [1] for MDCKII cells double-transfected with OATP1B3 and MRP2. Uptake of Fluo-3 can be inhibited by rifampicin a competitive inhibitor of OATP1B3. This result together with our observation that unselected cells lose both the OATP1B3 gene and the ability to transport Fluo-3, confirms that Fluo-3 is indeed transported by OATP1B3. The easy detection by fluorescence measurement makes Fluo-3 an interesting alternative to the radioactively labeled substrates used so far in such inhibition studies.

For further validation of our assay-system we tested eight substances already reported to be substrates of OATP1B3 for inhibiting Fluo-3 uptake. The substances, namely bromosulfophtalein (BSP), estradiol-17-β-glucuronide, ouabain, digoxin, DHEAS, methotrexate, glycocholate and ketoconazole were given simultaneously with Fluo-3 to the cells. All eight tested substrates showed an inhibition of Fluo-3 transport confirming the suitability of our system for screening of OATP1B3 modulators. We could further rank the substrates concerning transporter affinity by determining IC_{50} values for the inhibition of Fluo-3 transport.

5. Conclusion

The described fluorescence-based assay allows a quick and easy prescreening of potential substrates for OATP1B3. This assay will allow us to test a high number of potential substrates without using radioactive substances. Such a tool is elementary for the development and verification of a computational model to predict the transport by OATP1B3.

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References

[1] Y. Cui, J. Konig, D. Keppler, Vectorial transport by double-transfected cells expressing the human uptake transporter SLC21A8 and the apical export pump ABCC2, Mol. Pharmacol. 60 (2001) 934–943.

[2] T. Abe, M. Unno, T. Onogawa, T. Tokui, T.N. Kondo, R. Nakagomi, H. Adachi, K. Fujiwara, M. Okabe, T. Suzuki, K. Nunoki, E. Sato, M. Kakyo, T. Nishio, J. Sugita, N. Asano, M. Tanemoto, M. Seki, F. Date, K. Ono, Y. Kondo, K. Shiiba, M. Suzuki, H. Ohtani, T. Shimosegawa, K. Iinuma, H. Nagura, S. Ito, S. Matsuno, LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers, Gastroenterology 120 (2001) 1689–1699.

[3] B. Hsiang, Y. Zhu, Z. Wang, Y. Wu, V. Sasseville, W.P. Yang, T.G. Kirchgessner, A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters, J. Biol. Chem. 274 (1999) 37161–37168.

[4] J. Konig, Y. Cui, A.T. Nies, D. Keppler, A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane, Am. J. Physiol. Gastrointest. Liver Physiol. 278 (2000) G156–G164.

[5] J. Konig, Y. Cui, A.T. Nies, D. Keppler, Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide, J. Biol. Chem. 275 (2000) 23161–23168.

[6] G.A. Kullak-Ublick, M.G. Ismair, B. Stieger, L. Landmann, R. Huber, F. Pizzagalli, K. Fattinger, P.J. Meier, B. Hagenbuch, Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver, Gastroenterology 120 (2001) 525–533.

[7] M.G. Ismair, B. Stieger, V. Cattori, B. Hagenbuch, M. Fried, P.J. Meier, G.A. Kullak-Ublick, Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver, Gastroenterology 121 (2001) 1185–1190.

[8] K. Letschert, D. Keppler, J. Konig, Mutations in the SLCO1B3 gene affecting the substrate specificity of the hepatocellular uptake transporter OATP1B3 (OATP8), Pharmacogenetics 14 (2004) 441–452.

[9] A.F. Hurlstone, G. Reid, J.R. Reeves, J. Fraser, G. Strathdee, M. Rahilly, E.K. Parkinson, D.M. Black, Analysis of the CAVEOLIN-1 gene at human chromosome 7q31.1 in primary tumours and tumour-derived cell lines, Oncogene 18 (1999) 1881–1890.

[10] H. Kojima, A.T. Nies, J. Konig, W. Hagmann, H. Spring, M. Uemura, H. Fukui, D. Keppler, Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis, J. Hepatol. 39 (2003) 693–702.

[11] Y. Cui, J. Konig, J.K. Buchholz, H. Spring, I. Leier, D. Keppler, Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells, Mol. Pharmacol. 55 (1999) 929–937.

[12] S.R. Vavricka, J. Van Montfoort, H.R. Ha, P.J. Meier, K. Fattinger, Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver, Hepatology 36 (2002) 164–172.

[13] K. Fattinger, V. Cattori, B. Hagenbuch, P.J. Meier, B. Stieger, Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2, Hepatology 32 (2000) 82–86.

[14] Y. Cui, J. Konig, I. Leier, U. Buchholz, D. Keppler, Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6, J. Biol. Chem. 276 (2001) 9626–9630.