Development of a Cell-Based High-Throughput Assay to Screen for Inhibitors of Organic Anion Transporting Polypeptides 1B1 and 1B3

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Abstract: The two organic anion transporting polypeptides (OATPs) 1B1 and 1B3 are expressed at the sinusoidal membrane of hepatocytes. They have a broad and overlapping substrate specificity and transport many endobiotics and drugs. Specific inhibitors are required to determine the contribution of each OATP to the hepatocellular uptake of common substrates. We have developed a cell-based high-throughput assay to screen chemical libraries in order to identify such inhibitors for OATP1B1 and OATP1B3. We have used OATP1B1- or OATP1B3-expressing Chinese Hamster Ovary cells on 96-well plates and determined uptake of fluorescein-methotrexate (FMTX). We validated the assay with known inhibitors and screened the well characterized Prestwick library of 1120 drugs. Along with several known OATP inhibitors including rifampicin, cyclosporine A and mifepristone we identified some new inhibitors. For inhibitors that seemed to be able to distinguish between OATP1B1- and OATP1B3-mediated FMTX uptake IC_{50} values were determined. Estropipate (estrone-3-sulfate stabilized with piperazine) was the most selective OATP1B1 inhibitor (IC_{50} = 0.06 μM vs. 19.3 μM for OATP1B3). Ursolic acid was the most selective OATP1B3 inhibitor (IC_{50} = 2.3 μM vs. 12.5 μM for OATP1B1). In conclusion, this cell-based assay should allow us to identify even more specific inhibitors by screening larger chemical libraries.

Keywords: OATP1B1, OATP1B3, fluorescein-methotrexate, cell based assay, high-throughput screening.

INTRODUCTION

Organic anion transporting polypeptides (humans: OATPs; rodents: Oatps) form a large gene superfamily of transport proteins expressed in multiple organs [1, 2]. Many of their members are multispecific transporters that mediate the uptake of numerous endo- and xeno-biotics. Human OATP1B1 and OATP1B3 are expressed in the sinusoidal membrane of hepatocytes and are thought to be liver-specific under normal physiological conditions. They are responsible for the uptake of numerous drugs, including statins, endothelin receptor antagonists, anticancer drugs like methotrexate and paclitaxel, as well as the antibiotic rifampicin [1]. Besides specific substrates like prostaglandin E_2 for OATP1B1 and CCK-8 for OATP1B3 [3, 4], OATP1B1 and OATP1B3 have a number of common drug substrates including bosentan [5], fluvastatin [6], methotrexate [7, 8], olmesartan [9], pitavastatin [10], rifampicin [11], rosuvastatin [12], and valsartan [13]. Given that both OATPs are expressed in human hepatocytes, it will be almost impossible without specific inhibitors to elucidate which of the two proteins is more important for the transport of a certain drug. Furthermore, these transporters can affect the bioavailability of drugs. In 1996 we demonstrated that the peptide-based thrombin inhibitor CRC 220 is a substrate of rat Oatp1a1 [14]. This explained the very high hepatic first pass elimination of CRC 220 which was partially responsible for its low bioavailability. Specific OATP1B1 or OATP1B3 inhibitors could be co-applied with a drug which is efficiently cleared by the liver and this could increase its bioavailability.

In order to identify potentially specific inhibitors for OATP1B1 and OATP1B3, we decided to develop an assay for high-throughput screening. Fluorescent substrates are preferred for high-throughput screening because of the sensitivity and dynamic range that the fluorescence signal affords. So far only fluorescent bile acids [15] and Fluo-3, a fluorescent calcium indicator [16, 17] have been demonstrated to be substrates for OATP1B3 while fluorescein methotrexate (FMTX) has been reported as a substrate for OATP1B1 [18]. However, methotrexate has been shown to be a substrate for both OATP1B1 and OATP1B3 [8] and is commercially available as fluorescein methotrexate (FMTX) and Alexa Fluor® 488 methotrexate (AMTX). Preliminary data from our laboratory also demonstrated that the anticancer drug paclitaxel is a substrate for both OATPs. A fluorescent version of paclitaxel is also available commercially as Oregon Green® 488 Taxol (Flutax-2). Therefore, we compared these different fluorescent compounds along with fluorescein as substrates for OATP1B1 and OATP1B3. We further characterized FMTX transport by both OATPs and established an assay on 96-well plates for high-throughput screening. We

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Table 1. Statistics of Prestwick Library Screening

OATP1B1														
Plate number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mean (neg control)	5,170	5,906	5,428	7,400	6,166	4,984	4,866	6,113	5,670	6,713	6,390	6,054	6,042	9,159
STDEV (neg control)	390	1,107	807	924	579	645	451	721	634	1,150	425	605	444	921
Mean (pos control)	9,411	10,044	9,499	14,100	10,904	9,080	8,950	11,267	10,641	11,879	12,990	12,183	14,967	16,572
STDEV (pos control)	871	715	753	1,520	1,267	803	537	1,042	935	1,663	954	1,226	1,249	1,760
Sample mean	9,654	10,381	9,101	13,350	9,857	8,448	8,967	11,136	10,397	11,423	12,343	11,735	12,785	16,997
Z' factor	0.11	-0.32	-0.15	-0.09	-0.17	-0.06	0.27	-0.03	0.05	-0.63	0.37	0.10	0.43	-0.08
OATP1B3														
Plate number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mean (neg control)	7,161	8,034	7,766	10,591	7,901	7,506	7,734	9,639	8,273	8,231	8,418	9,093	9,047	9,856
STDEV (neg control)	760	1,315	1,037	1,814	523	834	1,194	1,495	761	332	370	472	570	1,026
Mean (pos control)	26,768	31,694	27,097	31,987	28,351	25,272	30,369	26,319	26,882	30,781	31,653	34,132	34,603	32,986
STDEV (pos control)	1,704	2,889	1,778	1,212	1,789	2,271	2,257	1,792	1,171	4,063	1,281	3,003	3,917	3,014
Sample mean	26,308	26,850	23,819	33,715	26,838	25,368	25,738	29,565	26,661	29,799	29,365	33,188	31,714	36,503
Z' factor	0.62	0.47	0.56	0.58	0.66	0.48	0.54	0.41	0.69	0.42	0.79	0.58	0.47	0.48

Screening of Prestwick Library

To screen the 1120 compounds in the Prestwick library, we included 8 wells of negative (DMSO controls) and 8 wells of positive (10 μM rifampicin in DMSO) controls in each plate. As can be seen from the screening statistics shown in Table **1**, we have developed a useful assay to screen for OATP1B3 inhibitors but the assay needs to be improved to be used with OATP1B1. Z' values for the OATP1B1 part were close to zero or even negative, while the values for the OATP1B3 part were between 0.41 and 0.79. Nevertheless, we identify inhibitors for both OATP1B1 and OATP1B3 mediated uptake and a stimulator for OATP1B3. Among the strongest inhibitors found were known OATP substrates and/or inhibitors like cyclosporine A, rifampicin, saquinavir and several statins. In addition to these compounds, several new substances were found to inhibit FMTX transport mediated by either OATP1B1 or OATP1B3 or by both.

To confirm these hits of the screening results, we determined IC_{50} values for several compounds that preferentially inhibited either OATP1B1 or OATP1B3 or seemed to stimulate one or the other OATP. Among the tested compounds we identified five compounds that inhibited OATP1B1 more potently than OATP1B3 and three that preferentially inhibited OATP1B3 mediated FMTX transport (Table **2**, Fig. **5**). Estropipate, which is estrone-sulfate stabilized with piperazine, was the strongest OATP1B1 inhibitor with an IC_{50} of 0.06 ± 0.01 μM, while it hardly inhibited OATP1B3 at concentrations up to 1 μM (IC_{50} of 19.3 ± 6.3 μM) (Table **2**). In addition to estropipate, we found that bromocryptine, ergocryptine, fipexide, niflumic acid and repaglinide preferentially inhibited OATP1B1 (Table **2**, Fig. **5**), while beclamethasone, moricizine and ursolic acid preferentially inhibited OATP1B3 (Table **2**, Fig. **5**). Among the potential stimulators we could confirm that progesterone stimulated OATP1B3-mediated FMTX uptake with an EC_{50} of 0.2 μM, while it inhibited OATP1B1-mediated FMTX uptake with an IC_{50} of 5.4 ± 1.9 μM (Fig. **6**).

DISCUSSION

In this study, we established a good high-throughput assay for OATP1B3 (Z' factor > 0.4) that allows screening for inhibitors and stimulators of OATP1B3. The assay established for OATP1B1 needs more optimization perhaps using a different substrate to be as valuable as the one for OATP1B3. Initially we tested several fluorescent compounds as potential substrates for OATP1B1 and OATP1B3 and compared them to the known OATP1B1 and OATP1B3 substrate Fluo-3 [16, 17, 19]. Because fluorescein-methotrexate (FMTX) resulted in the highest signal for both OATP1B1 and OATP1B3 and because radiolabeled

Table 2. Inhibition of OATP1B1- and OATP1B3-Mediated Fluorescein-Methotrexate (FMTX) Uptake by Prestwick Library Compounds

Compound	IC_{50} on OATP1B1 (μM)	IC_{50} on OATP1B3 (μM)
2-aminobenzenesulfonamide	> 100	> 100
Aconitine	> 100	No inhibition
Beclomethasone	6.7 ± 1.2	1.4 ± 0.4
Bromocryptine	0.7 ± 0.08	1.8 ± 0.3
Captopril	No inhibition	No inhibition
Ergocryptine	0.8 ± 0.2	2.2 ± 1.2
Estropipate	0.06 ± 0.01	19.3 ± 6.3
Etomidate	> 100	> 100
Fipexide	34.5 ± 6.8	> 100
Metronidazole	> 100	> 100
Moricizine	8.1 ± 1.9	2.7 ± 0.4
Niflumic acid	3.7 ± 1.4	22.0 ± 6.8
Ramipril	4.0 ± 0.5	3.3 ± 0.3
Repaglinide	1.1 ± 0.2	4.8 ± 0.7
Resveratrol	11.2 ± 2.0	23.7 ± 2.5
Rifampicin	1.3 ± 0.2	1.5 ± 0.5
Trifluridine	No inhibition	No inhibition
Ursolic acid	12.5 ± 1.9	2.3 ± 0.03

Results are means ± SE of at least three independent experiments.

Fig. (5). Inhibition of OATP-mediated FMTX uptake by selected Prestwick compounds. Concentration dependent inhibition of 5 μM FMTX by indicated compounds was measured for 30 min at room temperature on 96-well plates with wild-type and OATP-expressing CHO cells. Values obtained with wild-type CHO cells were subtracted from values obtained with OATP-expressing cells and are given as percent of control. Means ± SE of the means of three independent experiments are shown and were used to calculate IC_{50} values.

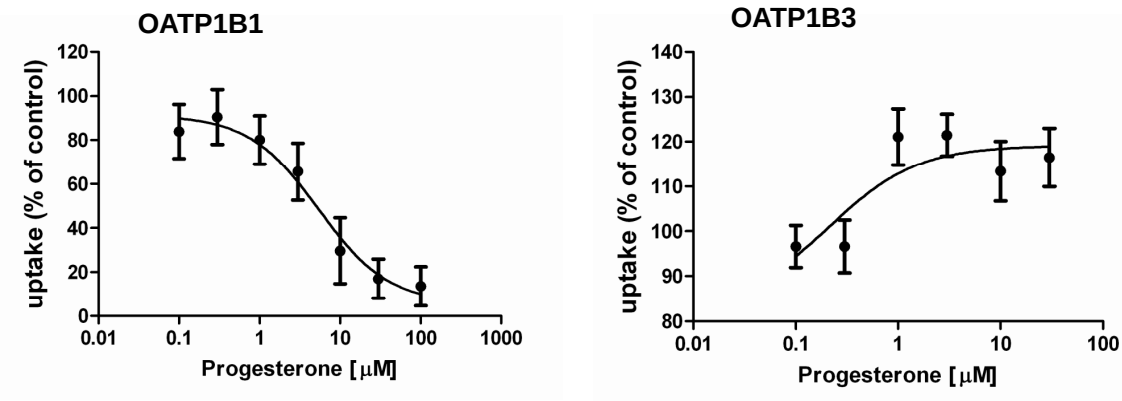

Fig. (6). Effect of progesterone on OATP1B1- and OATP1B3-mediated FMTX uptake. Concentration dependent inhibition of OATP1B1-mediated and stimulation of OATP1B3-mediated 5 μM FMTX uptake was measured for 30 min at room temperature on 96-well plates with wild-type and OATP-expressing CHO cells. Values obtained with wild-type CHO cells were subtracted from values obtained with OATP-expressing cells and are given as percent of control. Means ± SE of two combined independent experiments each performed in quadruplicates are shown and were used to calculate IC_{50} and EC_{50} values, respectively.

methotrexate has previously been identified as a substrate for both OATP1B1 and OATP1B3 [8], we decided to continue with this substrate and further characterize its transport. Our results show that FMTX is transported by both OATPs (Figs. **1**, **2** and **3**) and the kinetic parameters suggest that OATP1B3 has a 2–3 fold higher efficiency (V_{max}/K_m = 604) than OATP1B1 (V_{max}/K_m = 252) to transport FMTX. This is similar to the transport of Fluo-3 which is also transported much better by OATP1B3 than by OATP1B1 [19], suggesting that the fluorescent moiety might be more important for recognition of FMTX by these two OATPs than the methotrexate moiety. This hypothesis is further supported by the finding that inhibition of OATP1B1-mediated pitavastatin uptake by methotrexate was very weak (K_i > 300 μM) [22] and by our own observations that methotrexate at 100 μM only inhibited OATP-mediated FMTX uptake by 30% (data not shown). Therefore, it is likely that additional fluorescein-conjugates are transported by OATP1B1 and OATP1B3. Along the same lines, we also tested fluorescein itself and found that it is indeed a substrate for both transporters. However, the apparent affinity constants were about 10 fold higher for fluorescein (K_m OATP1B1 38 ± 5 μM; K_m OATP1B3 82 ± 10 μM) than for FMTX (K_m OATP1B1 3.8 ± 0.7 μM; K_m OATP1B3 7.9 ± 2.0 μM), suggesting that FMTX is the better substrate and that more than just the fluorescein moiety of the FMTX molecule is recognized by the transporters.

After having established that FMTX is a substrate for both OATP1B1 and OATP1B3, we tested the transport assay on 96-well plates at room temperature which would allow us to perform high-throughput screening. There was clear uptake signal for OATP1B1 and OATP1B3 (3.5 fold for OATP1B1 and 7.7 fold for OATP1B3) which could be inhibited by several common OATP substrates (Fig. **4**).

Although the Z' factors were not very high (0.26 for OATP1B1 and 0.46 for OATP1B3), we screened the Prestwick library and observed several hits for both OATPs. The screening statistics (Table **1**) confirmed that the assay is very useful with respect to OATP1B3 but needs improvement for OATP1B1. We are currently testing additional fluorescent compounds as substrates, in particular for OATP1B1, in order to increase the signal and establish a reliable assay also for OATP1B1. In the follow-up experiments, we determined IC_{50} values for several inhibitors identified by the primary screening for the inhibition of 5 μM FMTX transported by either OATP. Some of the compounds that seemed to be stimulators during the primary screening could not be confirmed as stimulators, but inhibited slightly at higher concentrations and are listed with IC_{50} values larger than 100 μM (2-aminobenxenesulfonamide, aconitine, etomidate, metronidazole and trifluridine). The only stimulator that could be confirmed was progesterone which stimulated OATP1B3-mediated transport but inhibited OATP1B1-mediated transport. Progesterone has previously been shown to also stimulate OATP2B1- mediated estrone-3-sulfate uptake [23].

Rifampicin which inhibited FMTX uptake of both OATPs with an IC_{50} of 1.3 to 1.5 μM (Table **2**) has previously been shown to be an inhibitor and substrate for both OATPs [11] and has since been used to characterize OATP mediated transport by many groups. However, inhibition by rifampicin is dependent on the transported substrate. Fexofenadine has been inhibited by rifampicin with K_i values of 0.5 μM for OATP1B1 and of 1.5 μM for OATP1B3 [24] while BSP was inhibited with K_i values of 17 μM for OATP1B1 and 5 μM for OATP1B3 [11]. Treiber *et al.* [5] reported inhibition of bosentan transport with IC_{50} values of 3.2 μM for OATP1B1 and 1.6 μM for OATP1B3 while uptake of the bosentan metabolite Ro 48-5033 was inhibited with IC_{50} values of about 50 μM for OATP1B1 and 0.8 μM for OATP1B3. These data clearly demonstrate substrate dependent inhibition patterns for OATP1B1 and OATP1B3-mediated transport. Previously, IC_{50} values between 0.9 and 1.5 μM for inhibition of OATP1B1 and 2.6 μM for OATP1B3 mediated estradiol-17β-glucuronide have been reported [19, 25]. These values are in good agreement with the values obtained in this study with FMTX and could indicate that estradiol-17β-glucuronide and FMTX are handled by the transporters in a very similar way while bosentan and its metabolite are probably transported *via* a different substrate binding site or translocation pathway given that the inhibition affinities are reversed. Based on the same assumptions it can be concluded that BSP and FMTX are probably

handled in a very similar way given that IC_{50} values of repaglinide inhibition for OATP1B1 and OATP1B3 obtained in this study are very similar to the ones obtained with BSP as a substrate [26]. Thus, the results of this study confirm that it is important to keep in mind that multispecific transporters like OATP1B1 and OATP1B3 seem to have multiple overlapping substrate binding/transport sites that are also affected by inhibitors in a substrate dependent way.

Estropipate (estrone-sulfate stabilized with piperazine) was the only compound among the real inhibitors that can be considered selective for OATP1B1-mediated FMTX transport. It inhibited OATP1B1 with an IC_{50} value of 0.06 μM, while the IC_{50} value for OATP1B3-mediated FMTX transport was approximately 320-fold higher (Table **2**). Besides indocyanine green which has been shown to be a specific inhibitor for OATP1B1-mediated estradiol-17β-glucuronide transport while not affecting OATP1B3-mediated estradiol-17β-glucuronide transport at concentrations up to 10 μM [27], estropipate is as far as we know the only other selective inhibitor that inhibits OATP1B1 with hardly any effect on OATP1B3 at concentrations up to 1 μM at which concentration it completely inhibits OATP1B1 (Fig. **5**). The most selective OATP1B3 inhibitors we identified were ursolic acid and beclomethasone that were about 5-fold more selective for OATP1B3 than for OATP1B1, but they were not as selective as the two OATP1B3 selective inhibitors T-3095 and T-3157 [28].

In conclusion, we have established that FMTX is a substrate for OATP1B1 and OATP1B3 and that it can be used for high-throughput screening with OATP1B3. Using this assay we have identified estropipate as a selective inhibitor for OATP1B1-mediated FMTX transport, and ursolic acid and beclomethasone as preferential inhibitors for OATP1B3-mediated FMTX transport. To identify additional more specific OATP1B1 and OATP1B3 inhibitors, we are currently improving the assay especially for OATP1B1 and then plan to extend the screening to larger libraries that are available at the High Throughput Laboratory of the University of Kansas.

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