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Ligand-dependent modulation of hOCT1 transport reveals discrete ligand binding sites within the substrate translocation channel

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Abstract

The human hepatic organic cation transporter 1 (hOCT1) is a well-known transporter of both xenobiotic and endogenous cations. The substrates and inhibitors of hOCT1 are structurally and physiochemically diverse and include some widely prescribed drugs (metformin and imatinib), vitamins (thiamine), and neurotransmitters (serotonin). It has been demonstrated that the closely related renal isoform, hOCT2, is subject to ligand-dependent modulation, wherein one ligand may enhance or inhibit transport of a second, chemically unrelated, ligand. This phenomenon has important implications for drug-drug interactions due to the ubiquity of polypharmacy and the large number of drugs that are present as cations under physiological conditions. Therefore, the objective of this study was to determine if hOCT1 is subject to the same ligand-dependent modulation as hOCT2, and to identify unique putative ligand binding sites in the translocation channel for a sub-set of ligands using computational modeling. The competitive counter flow (CCF) assay was employed to examine ligand-dependent effects by utilizing four different radiolabeled probe substrates: MPP⁺, serotonin, metformin, and TEA. We identified 20 ligands that modulated the transport of the four test substrates examined. One of the putative ligands identified, BSP, is an anion at physiological pH. Direct uptake studies of radiolabeled BSP suggested that it is an hOCT1 substrate with a K_m of $13.6 \pm 2.6 \mu\text{M}$ and V_{max} of 55.1 ± 4.1 pmol/mg protein/min. Each ligand identified was computationally docked into a homology model of hOCT1 using the UCSF DOCK software package. The docking study revealed three separate ligand binding pockets within the hOCT1 translocation pathway, defined by their interactions with three prototypical substrates: MPP⁺, TEA, and acyclovir. Our results suggest that hOCT1 is not

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only subject to ligand-dependent modulation, but individual ligand binding occurs at discrete sites within the hOCT1 translocation pathway which may influence ligand binding at the other sites.

Graphical abstract

Keywords

OCT1; drug-drug interactions; substrate-dependent modulation; translocation channel; ligand-binding site; anion transport

1. Introduction

The human organic cation transporter 1 (hOCT1) is a liver-specific drug transporter known to mediate the uptake of numerous chemically diverse cationic drugs and endogenous compounds into the liver for metabolism and/or excretion. These include both widely prescribed drugs [1–3], as well as endogenous compounds, such as nutrients [4,5], and neurotransmitters [6]. Since many drugs exist as cations at physiological pH, OCT1's location and function make it a prime candidate for drug-drug interactions [7–10]. As such, certain regulatory agencies, including the European Medicines Agency (EMA) [11] and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) [12] recommend including hOCT1 inhibition studies in their recommended preclinical testing of new drug candidates.

Recently, substrate-dependent interactions have been described for several drug transporters, including close relatives of hOCT1. For example, the antifungal drug clotrimazole strongly inhibited OATP1B3 mediated-uptake of the substrate Fluo-3, but stimulated the uptake of estradiol- β 17-glucuronide, and exhibited no effect on estrone-3-sulfate uptake [13]. Similar substrate-dependent effects were described for green tea catechins interacting with OATP1B3 [14]. Additionally, several studies have identified substrate-dependent modulation for hOCT2, a member of the same SLC22 subfamily which shares 70% sequence identity with hOCT1 [3, 15, 16]. In Zolk, et al. [16] demonstrated that several structurally diverse compounds were markedly more potent inhibitors of metformin uptake by hOCT2 than the prototypical substrate MPP⁺, as determined by IC₅₀ values. In addition, widely-varied IC₅₀ values were observed for hOCT2 inhibitors when screened against MPP⁺ versus the novel cationic substrate NBD-MTMA [15]. Furthermore, Hacker, et al. [17] compared the inhibitory effect of a number of hOCT2 ligands on transport of MPP⁺, ASP⁺, and metformin, and observed that several of the tested compounds inhibited only one or two substrates. Similar substrate-dependent interactions were also reported for MATE1 [18]. These findings point to a more generalized phenomenon, where transport may be modulated through interactions with either a substrate, inhibitor, or ligand that serves as neither a

substrate nor an inhibitor for the transporter. Therefore, here we will refer to the phenomena using the preferable term “ligand-dependent modulation” that encompasses all forms of small molecule-transporter interaction.

More recent publications also suggest the possibility of ligand-dependent effects for hOCT1. A previous publication from our lab demonstrated that acyclovir, cimetidine, and metformin, all reported substrates or inhibitors of hOCT1, did not inhibit serotonin uptake [6]. Also, in our hands, imatinib elicited a 10-fold higher IC₅₀ value with serotonin employed as probe substrate versus that published using metformin as probe substrate [10]. Additionally, IC₅₀ values determined for MPP⁺, TEA, and cimetidine were much higher when ethidium was used as probe substrate than previously reported values utilizing other probe substrates [19]. Furthermore, quercetin and lamivudine did not competitively inhibit hOCT1-mediated transport of MPP⁺ [20] and TEA [21], respectively, despite being substrates of hOCT1 [22–24]. While it is difficult to directly compare IC₅₀ values between laboratories due to inter-assay variability, all of these studies taken together in consideration with reports that describe multiple binding sites and/or translocation pathways within hOCT1 [25–28] strongly suggest the potential for ligand-dependent modulation of hOCT1.

If ligand-dependent interactions occur with hOCT1, guidelines recommending inhibition studies utilizing one or two model probe substrates may not be sufficient to identify potentially dangerous drug-drug interactions. Therefore, concrete identification of ligand-dependent effects of hOCT1 is crucial for developing informed preclinical testing recommendations. In the present study, we have identified and characterized ligand-dependent interactions of hOCT1 with multiple hOCT1 probe substrates and numerous other known ligands utilizing the competitive counterflow (CCF) assay and explored the observed interactions through homology modeling and docking studies.

Materials and Methods

2.1 Reagents

[³H]-1-methyl-4-phenylpyridinium iodide (MPP⁺, 85.0 Ci/mmol), and [¹⁴C]-metformin (0.1 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [³H]-hydroxytryptamine creatinine sulfate (serotonin, 28.3 Ci/mmol), and [¹⁴C]-tetraethylammonium bromide (3.5 mCi/mmol) were purchased from Perkin Elmer (Boston, MA). [³H]-bromosulphothalein (BSP, 11.5 Ci/mmol) was purchased from International Isotopes Clearing House, Inc. (Shawnee Mission, KS). Sodium chloride, potassium chloride, and ethidium bromide were obtained from Fisher Scientific (Waltham, MA). Dolutegravir was purchased from Advanced ChemBlocks (Burlingame, CA). Prostaglandin E2 and imatinib were purchased through Toronto Research Chemicals (Toronto, ON, Canada). Erlotinib, gefitinib, and oxaliplatin were obtained from the NCI/DTP Open Chemical Repository (<http://dtp.cancer.gov>) as part of the approved oncology drug set II library. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Generation and Culture of Stable Cell Lines

Flp-InTM-293 cells (Invitrogen, Carlsbad, CA), derived from Human Embryonic Kidney (HEK) cells, were cultured in Dulbecco's Modified Eagle's medium (ATCC, Manassas, MA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich), and 100 µg/mL Zeocin (Invitrogen) at 37°C under humidified 5% CO₂. Per manufacturer's protocol, to generate HEK-EV and -hOCT1 cells, Flp-InTM-293 cells were co-transfected with pcDNA5/FRT plasmid (empty vector, EV) or pcDNA5/FRT-hOCT1 (hOCT1) plasmid, as discussed in Boxberger, et al. [6]. The Flp-InTM pOG44 plasmid was transfected using the FuGENE HD® transfection reagent (Promega, Madison, WI). After transfection, cells were grown in medium, using conditions described above, with hygromycin (100 µg/mL, Invitrogen) replacing Zeocin to select for stable transfectants. Colonies were isolated and propagated through clonal expansion. Clones were assayed for hOCT1 transport activity. HEK-hOCT1 clones, which demonstrated transport of [³H]-MPP⁺, and HEK-EV clones, which did not show significant uptake of [³H]-MPP⁺ were selected, expanded, frozen, and stored per manufacturer protocol for future use. Cells were seeded on 48-well plates coated with 0.1 mg/mL poly-D-lysine (Invitrogen) at a density of 160,000 cells/well, and incubated at 37°C for approximately 48 hours until confluency was reached. For BSP transport assays, cells were seeded on 24-well plates coated with 0.1 mg/mL poly-D-lysine (Invitrogen) at a density of 250,000 cells/well, and incubated as described above.

2.3 Transport Assays

Transport assays were performed approximately 48 hours after cells were plated. All assays (excluding BSP transport assays) were performed at room temperature, using room-temperature uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM D-glucose, and 12.5 mM HEPES, adjusted to pH 7.4 with Tris base) to prewash cells, and ice-cold uptake buffer to stop transport. BSP transport assays were performed at 37°C, using pre-warmed uptake buffer to prewash the cells. Buffer components (with the addition of 1% bovine serum albumin dissolved in final wash buffer to limit the non-specific binding of BSP) and stop conditions were as described above.

Time-dependent uptake was measured by incubating HEK-EV and -hOCT1 cells with 150 µL of uptake buffer containing radiolabeled [³H]-MPP⁺ (0.4 µCi/mL), [³H]-serotonin (0.4 µCi/mL), [¹⁴C]-metformin (0.2 µCi/mL), or [¹⁴C]-TEA (0.2 µCi/mL) for 30 seconds to 180 minutes. Net uptake was calculated by subtracting uptake into HEK-EV cells from that of HEK-hOCT1 cells.

Competitive counterflow (CCF) experiments involved pre-loading HEK-hOCT1 cells with radiolabeled probe substrates (as described above and Harper and Wright, 2012). Briefly, the HEK-hOCT1 or control cells were allowed to accumulate substrate for 90 minutes until steady-state saturation was reached. This was the optimal incubation time where the transporter-mediated flux of the substrate was found to be independent of the incubation time. At this point, the cell content of radiolabeled substrate does not change because the cells have already achieved steady-state distribution due to an equal amount of influx and efflux of radiolabeled substrate. Following preload incubation, 1.5 µL of concentrated efflux

solution containing water (control) or unlabeled test compound and an equivalent labeled substrate concentration as used in the pre-load conditions was added to the wells and plates were vortexed briefly to initiate efflux. In the case of hOCT1 ligands that function as substrates, the labeled intercellular substrate is exchanged with the unlabeled test ligand and the amount of measured radioactivity in the cell decreases. Compounds used to induce CCF were ideally employed at concentrations 10-fold higher than published K_m values (see Table 1 for concentrations of test compounds employed). For time-dependent efflux, cells were incubated with efflux solutions for 15 seconds to 10 minutes. For CCF assays, cells were incubated with efflux solutions for 30 seconds (MPP⁺) or 5 minutes (serotonin, TEA).

Once the assays were complete, all cells were lysed with 150 μ L/well for 48-well plates or 300 μ L/well for 24-well plates of 1% Triton X-100 in PBS. Then, 100 or 200 μ L of each lysate was transferred to a 96- or 24-well scintillation plate (Perkin Elmer, Waltham, MA), and mixed with 100 or 300 μ L Optiphase HiSafe 3 scintillation cocktail (Perkin Elmer). Radioactivity was determined by liquid scintillation counting using a MicroBeta Trilux liquid scintillation counter (Perkin Elmer). Where protein concentration was determined, protein was measured in duplicate for each condition on 96-well plates using the Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA). Each data point represents the average of three independent experiments, wherein each condition was completed in triplicate.

2.4 Determination of Kinetic Parameters

To determine kinetic parameters, net uptake values, obtained by subtracting uptake into HEK-EV cells from that of HEK-hOCT1 cells from each of three independent experiments, were averaged and plotted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Subsequently, the data were analyzed by nonlinear regression and, for kinetics analysis, fit to the Michaelis-Menten equation.

2.5 Generation of a Human hOCT1 Homology Model

A homology model of human hOCT1 (GenPept accession #: O15245) was prepared using the BIOVIA Discovery Studio's version 4.5 (<http://accelrys.com/>) BHM (Build Homology Models) protocol, which supports a software plugin for UCSF Modeller automodel, ver. 9.14 (<https://salilab.org/modeller/contact.html>). Chain A of the crystal structure of the bacterial glycerol-3-phosphate transporter from *E.coli*, GlpT (PDB ID # 1PW4_A) [29], was used as an input template. The GlpT transporter was chosen due to the fact that it belongs to the MFS class of proteins, shares approximately 18% sequence identity (~50% sequence similarity) to hOCT1, and functionally parallels hOCT1 in regard to its propensity for substrate promiscuity.

In addition to the 12 TMDs predicted from computational modeling, a large extracellular loop domain is predicted to exist between helices 1 and 2. Unfortunately, due to the limitations of the modeling software used in this study, we were not able to incorporate in this putative extracellular loop region in our structure. It should be noted that loop, or unstructured regions more generally, are notoriously difficult to predict computationally with any accuracy, and at over 100 residues long, any computationally derived model from this

loop region would be highly speculative at best. Therefore, to facilitate efficient modeling of the hOCT1 transporter, the large extracellular loop region between helical TMDs 1 and 2, and the intracellular loop region between helical TMDs 6 and 7, were both removed from the structure prior to model construction. Parameters in the Parameters Explorer box of the BHM menu in Discovery Studio were set to the following: Cut Overhangs was set to True in order to cut the terminal residues of the input model sequence that did not align properly with the input templates, Number of Models was set to 5 in order to define the number of models to create from an initial structure, and the Optimization Level was set to Low in order to specify the amount of molecular dynamics to perform with simulated annealing. In order to build refinement models on the detected loop regions, i.e., the model sequence segments of at least 5 residues length which are not specifically aligned with the templates, the LOOPER function was used to systematically search loop conformations and rank them using CHARMM, with Refine Loops set to True. The BHM protocol uses the DOPE (Discrete Optimized Protein Energy) [30] method to refine loops, which results in a more energetically accurate portrait of the loop structure. Refine Loops, Number of Models was set to 5 to specify the number of models to be created by loop optimization, and the Refine Loops Optimization Level was set to Low. Refine Loops with the DOPE method was set to High Resolution. After running the BHM protocol, the Best Model Structure Superimposed to Templates was chosen from the generated output models for the final three-dimensional model structure of the human hOCT1 transporter.

2.6 Docking of Ligands into the hOCT1 Homology Model

In order to identify the potential binding modes and protein interaction sites of various ligands with hOCT1, an in silico molecular docking strategy was employed using the UCSF DOCK 6.7 software suite, under academic license (University of California, San Francisco: http://dock.compbio.ucsf.edu/DOCK_6/index.htm, [31–33]. The 3D conformer for each ligand was downloaded directly from the PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>), parameterized for partial charges, and used for all docking studies described below. Extraneous information not required for the docking simulation, including the headers, connect records, and waters, were deleted from the PDB text file. Additionally, all hydrogens were removed from the receptor using the SELECT function from the UCSF Chimera 1.11.2 software suite [34].

The hOCT1 transporter was then prepared for docking by using the UCSF Chimera 1.11.2 DOCK PREP function. Once preparation of the protein had been completed, it was saved in the MOL2 format. Sphere generation was accomplished by inputting a dot molecular surface (DMS) parameterization of the transporter surface into the SPHGEN module of the DOCK software suite. The DMS parameterization is accomplished by rolling a ball the size of a water molecule over the Van der Waal's surface of the receptor. The surface normal vector at each surface point is computed and used to calculate the size of each sphere generated. After initial sphere generation, the total number of sphere clusters was pruned to only the clusters representing the active site of the protein using the DOCK SHOWSPHERE module. This resulted in the largest sphere cluster being contained within the putative translocation channel. Next, a molecular docking grid was generated by enclosing the spheres in a

rectangular box and using the GRID utility to obtain files representing both the electrostatic and Van der Waals forces according to the following equation:

$$E = \sum_{i=1}^{lig} \times \sum_{j=1}^{rec} \times \left(\frac{A_{ij}}{r_{ij}^a} - \frac{B_{ij}}{r_{ij}^b} + 332 \frac{q_i q_j}{D r_{ij}} \right)$$

where each term is a double sum over ligand atoms i and transporter atoms j , A_{ij} and B_{ij} are the attraction and repulsion parameters, respectively; r is the distance between the Van der Waals radii of particles i and j , in the case of repulsion a or attraction b , q is the electrostatic surface potential of i or j , and D is the coefficient of the well depth of the interaction energy between the ligand and receptor atoms, as defined in Kuntz, et al. [35]. The GRID utility then generated files representing the contact score and the energy score which are utilized in the docking routine. The final size of the docking grid box was 16.7 Å x 25.9 Å x 16.7 Å. The docking parameters were as follows: maximum number of orientations was set to between 50 and 1000, Van der Waals energy component from between 20 to 25, and maximum number of iterations to 500 (except for berberine, which was set to 1,000). The VIEWDOCK utility of UCSF Chimera 1.11.2 was used for visualization of the docking poses and measuring distances and angles between atoms of interest.

3.2.7 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was used to elucidate the relationships between individual ligands and specific ligand binding modes. In PCA, the covariance matrix of properties is diagonalized to determine its eigenvalues and corresponding eigenvectors. To carry out PCA, an inventory of the individual ligands physiochemical properties was conducted. These properties included: molecular weight, K_m , docking grid score, ClogP, H-bonds (donor), H-bonds (acceptor), rotatable bonds, pK_a , and pK_b . These distinct ligand properties served as the input parameters for the PCA. Projection of the data onto the first two principal components with the largest eigenvalues makes it suitable for visualization of distinct clusters of ligand physiochemical properties. A Pearson (n) PCA was run using the above defined variables for each ligand examined, with the maximum number of factors set to five. The PCA was conducted using the XLSTAT plugin (Addinsoft, Inc., New York, NY) for MicroSoft Excel (MicroSoft, Inc., Redmond, WA).

3. Results

3.1 Functional Validation of Generated hOCT1 Stable Transfectants

HEK293 cells were used to generate stable cells lines containing pcDNA5/FRT empty vector (control) and pcDNA5/FRT-hOCT1 vectors using the Flp-In™ system. Clones stably-expressing hOCT1 were assayed for transport of model cation MPP⁺ and probe substrate serotonin (Figure 1). Significant uptake of both substrates, compared with control cells, was observed in the selected hOCT1-expressing cells at multiple time points, demonstrating adequate functionality of the stably-expressed transporters.

3.2 Ligand-dependent Interactions with hOCT1 as Determined by Competitive Counterflow Assay

To characterize the previously observed putative ligand-dependent interactions of hOCT1, the competitive counterflow (CCF) assay, as originally developed by Harper and Wright [36], was employed. CCF capitalizes on the observation that OCTs transport substrates bilaterally, and therefore can function as exchangers by transporting one substrate into the cell and subsequently effluxing another from the cytoplasm. In this study, CCF was employed to examine ligand-dependent effects by utilizing four different radiolabeled probe substrates: MPP⁺, serotonin, metformin, and TEA.

Before CCF could be performed, the time point where substrate transport reached steady-state equilibrium first needed to be determined. To accomplish this, time-dependent uptake assays were performed for all four substrates (Figure 2, A-D). MPP⁺, metformin, and TEA uptake reached steady-state by 60 minutes, while serotonin uptake plateaued at between approximately 90 and 120 minutes. Therefore, pre-load incubations prior to CCF were conducted for 90 minutes. To determine the time point with the best efflux signal, time-dependent efflux was performed with all four substrates, using unlabeled MPP⁺, serotonin, metformin, and TEA as the efflux solution for their respective pre-load substrates. As can be seen in Figure 2, E-H, maximum efflux was reached by 30 seconds for MPP⁺, and 5 minutes for serotonin and TEA. Interestingly, unlabeled metformin did not induce efflux of radiolabeled metformin at any time point measured (Figure 2, G). This may suggest a metformin “sink” within the cell that functions to sequester the ligand and thereby reduce its interaction with OCT1. In any case, because of this, metformin was excluded as a probe substrate for further CCF studies, and 30 seconds (MPP⁺) and 5 minutes (serotonin and TEA) were used as CCF efflux time points.

For CCF, several known hOCT1 substrates and inhibitors were employed as test compounds, as well as some compounds which have not been tested for interactions with hOCT1. MPP⁺, serotonin, metformin, and TEA were used as positive controls for efflux, while glucose and bromosulfophthalein (BSP) were selected as negative controls, as glucose does not interact with hOCT1 and, as a negatively-charged molecule, BSP was not expected to interact. A “cut-off” value for efflux was set at 80% substrate content to distinguish substrates from non-substrates.

Because the maximum efflux varied for each of the pre-load substrates, as seen in Figures 2, E-G, and 3A, a correction factor for CCF efflux values was necessary to compare the efficiency of efflux elucidated by the test compounds for each probe substrate. Initially, an 80% of control “cut-off” value was determined mathematically as a function of the maximal amount of control substrate (MPP⁺) uptake signal compared with vehicle (empty vector HEK293 cells) obtained under steady-state conditions (Figure 3, A). The value of 80% was chosen to ensure that any signals detected in the CCF assay resulted from a direct interaction between the transporter and the test ligand, and not due to background from the assay itself. The data shown in Figure 3, B are corrected for the observed differences in maximum efflux between probe substrates by setting the observed value of substrate content when TEA and thiamine were employed as test compounds as the maximum efflux for each respective

probe substrate. This factor effectively changed the previously assigned “cut-off” value from 80% (uncorrected data, Figure 3, A) to 65% (corrected data, Figure 3, B).

As expected, MPP⁺, serotonin, metformin, and TEA induced efflux of all three probe substrates, as did thiamine (Figure 3). Interestingly and unexpectedly, BSP also induced efflux of all three substrates, suggesting that BSP may be a substrate of hOCT1. Lamivudine, ranitidine, famotidine, diphenhydramine, fluoxetine, and berberine all induced efflux of radiolabeled MPP⁺ and serotonin, but not TEA (Figure 3). The remaining test compounds did not induce efflux of any of the probe substrates.

3.3 Characterization of hOCT1-mediated Bromosulfophthalein Uptake

To confirm the results of the CCF assay which suggested that BSP is a substrate of hOCT1, direct uptake studies using [³H]-BSP were performed. Time-dependent uptake studies using 0.1 μM and 50 μM BSP demonstrated an increase of uptake into HEK-hOCT1 cells over HEK-EV cells by five minutes (Figure 4, A-B), indicating that BSP is indeed a substrate for hOCT1. Kinetics analysis was then performed to further characterize BSP transport by hOCT1 (Figure 4, C). Michaelis-Menten analysis yielded a K_m of 13.6 ± 2.6 μM and a V_{max} of 55.1 ± 4.1 pmol/mg protein/min for BSP.

3.4 Computational Modeling of Ligand Interactions

To further investigate the observed interactions in the CCF assay, we constructed a homology model of hOCT1 and performed in silico docking of several of the employed test compounds. The hOCT1 homology model generated by BIOVIA Discovery Studio's UCSF Modeller automodel plug-in produced 9 helical TMD regions with an inward facing conformation (Figure 5), similar to both the original GlpT structure upon which it was based and the PiPT model [25]. Previous homology models have been constructed using both the *E. coli* lactose permease, LacY [37], and more recently, the high affinity phosphate transporter from *Piriformospora indica*, PiPT [25]. Our rationale in choosing the GlpT protein was that it, and PiPT, are functionally similar to hOCT1 in that they both transport molecules that are charged at physiological pH (cations in the case of hOCT1, and anions in the cases of GlpT and PiPT), in contrast to LacY, which is known to transport lactose, a neutral compound, and its closely related analogues. The structural model of hOCT1 was well resolved and overall closely resembled the tertiary structure of GlpT. In particular, residues W218, Y222, and T226 are all located in TMD4, with their respective side chains pointed toward the interior of the translocation channel (Figure 5). These residues have been previously implicated in substrate translocation via site-directed mutagenesis experiments [38]. Similarly, I443, I447, and Q475, which have also been implicated in substrate translocation [39], are located near the putative active site cavity. These results increased our confidence in the accuracy of the homology model for use as a docking template.

To understand the structural implications of multiple ligand modulation of hOCT1 transport activity, we used our constructed hOCT1 homology model as a template to dock several of the drugs that demonstrated significant differences in the CCF assay. It should be noted that both the large extracellular and small intracellular loop were omitted from our model for simplicity sake, despite some reports [40, 41] indicating that these regions may be important

in the initial step in ligand recognition. Most compounds tested in the CCF assay (MPP⁺, serotonin, TEA, metformin, BSP, acyclovir, ritonavir, lamivudine, cimetidine, famotidine, imatinib, fluoxetine, lamotrigine, thiamine, prostaglandin E2, quercetin, berberine, and ethidium) were docked into the homology model using UCSF DOCK 6.7 (ranitidine was not docked due to issues with improper ligand parameterization). Figure 5, A illustrates all the ligand structures docked into the homology model, exhibited as a mesh of the solvent accessible surface of the protein. This can be viewed more clearly in Figure 5, B, which reveals putative binding pockets within the transporter (grey ribbon) shown as the van der Waals surfaces of all the ligand structures docked. In order to more dramatically demonstrate this, the helix closest toward the viewer has been cut away in Figure 5, B so that the ligand binding site in the translocation pore may be more readily visible. All ligands, with the exception of BSP, docked within the putative translocation pore, in close proximity to residues that have been identified to be crucial for transporter-ligand interactions (e.g., W218, Y222, T226, I443, I447, and Q475), further enhancing our confidence in the structural validity of the hOCT1 homology model.

Interestingly, all docked ligands, apart from BSP, appeared to fall into one of three categories: 1) those that docked in a binding site overlapping with MPP⁺, 2) those that docked in a binding site overlapping with TEA, and 3) those that overlapped with the binding site of acyclovir (Figure 6). BSP was an outlier from all the other ligands in that it bound to hOCT1 outside the confines of the translocation channel (Figure 5), likely due to its inherent negative charge, and, therefore, was not considered further. These observations led us to identify three distinct, but not mutually exclusive, ligand binding sites within the substrate translocation channel: the “MPP⁺ binding pocket”, the “TEA binding pocket”, and the “acyclovir binding pocket”. These separate binding pockets are illustrated in Figure 6, as both (A) the chemical structures of ligands docked within the ribbon structure, and (B) the Van der Waal’s electrostatic surface of residues lining the binding regions. These pockets were defined by residues Y240, Q241, F244, E386, I446, S470, and C473 (“MPP⁺ pocket”), A33, C36, V359, L360, C473, D474, G477, and I478 (“TEA pocket”) (Figure 5, B), and Q362, G363, N374, L377, D378, Y381, and A383 (“acyclovir pocket”), all located within 3 Å of the ligand (Figure 6, D).

3.5 Principal Component Analysis

In an effort to correlate trends in ligand physiochemical properties with specific ligand binding sites, principal component analysis (PCA) was performed comparing the ligands docked within each binding pocket with various ligand-specific physiochemical properties. As illustrated in Figure 7A, correlations were observed between certain individual physiochemical properties and specific ligand binding sites, and several general trends could be identified. The GRID docking score, pK_a , pK_b , and K_m were all predictors of ligand-transporter interaction based on their cumulative proportion of variance, using 64.9% as cutoff point (Table 2). Additionally, the Eigen values for principal components F1 through F4 were all greater than 1, satisfying the Kaiser criterion for these four components (Table 2, Figure 7, B). The first principal component (F1) was most positively correlated with the GRID score and K_m and negatively correlated with H-bond acceptor status and the total number of rotatable bonds, suggesting that affinity and binding energy are the most

important determinants of ligand-protein interaction, with overall conformational mobility and hydrogen bonding capacity being negative predictors of interaction. The second principal component (F2), was positively correlated with pK_a and K_m , indicating the importance of both affinity and ionization potential. However, this component was less correlated with GRID score than F1. When analyzed in the biplot (Figure 8), smaller, more positively charged ligands such as metformin, cimetidine, thiamine, serotonin, and lamivudine were most strongly associated with components F1 and F2 and the physical parameters of K_m and pK_a . It is notable that both thiamine and serotonin both belong to this group, as they were the only endogenous ligands included in this study. Also of note, the larger ligands lamotrigine, acyclovir, berberine, were associated as a group and more strongly correlated with the overall GRID score. The very large ligands: ritonavir, imatinib, fluoxetine, famotidine, and prostaglandin E2 were also grouped together and shared a stronger correlation with the number of rotatable bonds, H-bond donors, and the ClogP, than the other ligands examined. Finally, BSP, quercetin, and ethidium, which are non-drug substrates for OCT1, were all outliers and had negative loadings for both F1 and F2 components in the biplot (Figure 8).

4. Discussion

Ligand-dependent interactions have been confirmed for several drug transporters, including close relatives of hOCT1 [13–15, 18, 42], and as discussed in the introduction, a number of studies suggest ligand-dependent effects for hOCT1. This study identified ligand-dependent interactions for several hOCT1 ligands and employed computer modeling simulations to predict the structural basis underlying potential mechanisms of ligand-dependent modulation.

The CCF assay employed in this study was originally developed to identify novel substrates [36]. As discussed previously, for our study, a “cut-off” value of 80% remaining substrate for the uncorrected data (Figure 3, A) or 65% for corrected values (Figure 3, B) was used to distinguish substrates from non-substrates. Based on the CCF results, MPP⁺, serotonin, metformin, TEA, BSP, lamivudine, ranitidine, famotidine, diphenhydramine, fluoxetine, thiamine, and berberine were all identified as substrates, given that they induced efflux of at least one radiolabeled probe substrate. MPP⁺, serotonin, metformin, TEA, lamivudine, ranitidine, famotidine, thiamine, and berberine were all previously reported as substrates [3, 4, 6, 23, 43–45]. This study confirms their status as such. However, the CCF assay also identified diphenhydramine and fluoxetine as novel substrates of hOCT1. Diphenhydramine and fluoxetine have been reported to interact with hOCT1 in the past, and our data presented here establishes that they are indeed substrates [6, 46, 47].

Acyclovir, ritonavir, cimetidine, imatinib, gefitinib, erlotinib, oxaliplatin, verapamil, midazolam, lamotrigine, acebutolol, salbutamol, prostaglandin E2, EGCG, quercetin, and ethidium did not elicit significant efflux (Figure 3). Interestingly, acyclovir, imatinib, oxaliplatin, lamotrigine, salbutamol, prostaglandin E2, quercetin, and ethidium have all been reported to be substrates [19, 22, 48–53]. The fact that they did not induce efflux of any of the radiolabeled probe substrates employed in the assay suggests that their interaction with hOCT1 is ligand-dependent.

Ligand-dependent interactions were observed with those test compounds which were determined to be substrates, as well. Several substrates induced efflux of radiolabeled MPP⁺ and serotonin, but not TEA (Figure 3), indicating an interaction at the MPP⁺ binding site but not the TEA pocket. And, even within this group of compounds, there appears to be variation in the magnitude of efflux between probe substrates. However, these differences cannot necessarily be attributed to differences in substrate affinities. Some of these interactions are corroborated by previous reports in the literature. For example, berberine, which induced significant efflux of radiolabeled MPP⁺ and serotonin, but not TEA, was a more potent inhibitor of MPP⁺ than of TEA in a study characterizing berberine transport by hOCT1 conducted by Nies, et al. [45].

In our study, the CCF assay proved useful in identifying ligand-dependent modulation, though limitations exist. Due to the nature of the assay, wherein an extracellular substrate is exchanged with a cytoplasmic substrate, ligand-dependent effects cannot be observed for test compounds which are exclusively inhibitors, as they would not induce efflux of probe substrates. To determine ligand-dependent interactions with hOCT1 inhibitors, inhibition studies must be completed analyzing inhibitors versus multiple substrates. However, in this study, test compounds which had previously only been described as inhibitors (diphenhydramine and fluoxetine) and compounds which had never been reported and were not expected to interact with hOCT1 (BSP) were identified as putative novel substrates (Figures 3, 4).

Another limitation of this particular methodology is that it cannot distinguish between ligand interaction with different conformational forms of the transporter, such as the “inward facing” and the “outward facing” form. It is certainly true that different forms, or conformational intermediates, of the OCT1 transporter are likely to occur during the substrate translocation cycle and that the inward facing conformation, upon which our homology model is based, represents only one “snapshot” in time of the transporter, based on a homologous X-ray crystal structure. While the initial interaction between ligand and the transporter is likely to take place with the outward facing conformer of the transporter, the ligands must certainly pass through the translocation pathway before exiting on the other side of the cellular membrane. In this regard, our model represents the interactions that are likely to take place within this translocation channel. Since the core of the helix bundle is present in both the inward and outward facing conformers, we believe that the transporter-ligand interactions that we observed in our study would be captured in both models. However, it is possible that additional transporter-ligand interactions that we did not observe may also be captured with a docking study with a homology model based on the outward facing forms. If the transporter is indeed asymmetrical, then it is possible that an internal binding site may exhibit altered ligand specificity and affinity in comparison to an external binding site. This is one possible interpretation of the observed data that cannot be dismissed. However, only carefully resolved crystal structures with the transporter in the ligand bound state would be able to definitively confirm this.

The observation that BSP induced efflux of any of the probe substrates, let alone all three (Figure 4), was particularly unexpected. BSP was selected as a negative control for hOCT1 CCF assays because it possesses a net negative charge (−2). Human OCT1 is generally

thought to transport only positively-charged or neutral compounds [54, 55], but not negatively-charged compounds. Some examples include the aforementioned lamivudine [24, 56] and prostaglandin E₂ [49, 57], with the latter species being highly controversial [57]. Despite this, it was believed that the negative charge associated with BSP would prevent its interaction with hOCT1. However, as is evident in Figure 4, BSP induced efflux of each of the probe substrates, which suggests that it is a hOCT1 substrate. To further explore the possibility that BSP is a substrate, direct uptake was measured using [³H]-bromosulfophthalein. HEK-hOCT1 cells demonstrated significant uptake of BSP compared to HEK-EV cells, and time-dependent uptake was linear over two minutes, both distinctive hallmarks of hOCT1 substrates (Figure 4, A-B). In order to characterize BSP uptake, kinetics analysis was completed. The K_m and V_{max} were determined to be $13.6 \pm 2.6 \mu M$ and 55.1 ± 4.1 pmol/mg protein/min, respectively (Figure 4, C), indicating that hOCT1 is a high affinity, but low capacity transporter of BSP. This appears to be the first negatively-charged substrate characterized for hOCT1. However, it should be noted that, due to the limitations of our experimental approach, there are alternative possible explanations for the observed results. Notably, even though substrate translocation with the radiolabeled BSP was observed, the effect was modest when compared with typical hOCT1 substrates, suggesting at best that it is a very low affinity substrate for hOCT1, or may possibly be a substrate for a yet uncharacterized transporter expressed in the HEK293 cells. Indeed, transport of BSP, which is used as the prototype of bilirubin, has been described in HEK293 cells and in some cases has been attributed to hOAT transporters [58]. Alternatively, it might be that the induction of OCT1 expression modifies some cell features such as expression of other transporters or, more simply, the membrane potential. This, in turn, may change the BSP out/in equilibrium that is mediated by transporters other than OCT1. Clearly, more work must be done to elucidate the potential mechanism of transport of a negatively-charged substrate for hOCT1.

Interestingly, several test compounds previously reported as hOCT1 substrates, including imatinib, lamotrigine, and prostaglandin E₂, did not induce efflux of any probe substrate in the CCF assay (Figure 3). Controversy exists regarding the status of imatinib and prostaglandin E₂ as OCT substrates [49, 52, 57, 59–61]. Our data may add further support for the argument that imatinib and prostaglandins are not substrates for hOCT1.

The OCT1 based homology model was useful to provide a structural interpretation of the results from the uptake experiments (Figure 5). Certain structural features of the ligand-free homology model, including an edge-to-face pi-electron charge pairing interaction between W218 and Y222, which had previously been implicated in substrate translocation, increased our confidence in the model. However, a limitation to our model was the absence of the putative large extracellular and helices 1 and 2. This reflects a shortcoming of the computational modeling of unstructured regions of proteins.

In comparison to the PiPT model [25], which was also based on a structure representing the inward facing conformation, Chen et al. did not report the number of TMDs from their resulting structure(s). However, given that they were using a homology model built with the same software (UCSF MODELLER), it is highly likely that the putative extracellular loop was absent from their structure as well, although, again, this is not reported in their

manuscript. If that is indeed the case, then we would expect similar results between the two models. Indeed, based on the reported binding sites for the ligands that were docked in their study, this appears to be the case with several of the ligands interacting with similar residues (e.g., W217, F244, I449, D357, Q447, and G476) as we report here. Therefore, despite the absence of this region of the protein, we believe that our model preserves the core interactions that are likely to occur between the transporter and ligand.

Furthermore, in our model lamotrigine docked closest to the “MPP⁺ binding site,” (see below). However, it did not appear to overlap with any of the docked probe substrates (Figure 5). Therefore, it is possible that the lack of overlap causes a change in conformation within the translocation pathway that prevents exchange of probe substrates for CCF.

The observed interactions in the CCF assay could be attributed to differential binding regions for specific ligands. It has been proposed that hOCT1 contains multiple binding sites within the translocation pathway of the transporter [25–27]. As indicated above, our homology model appears to align closely with previously published models and contains a large translocation pore that supports the multiple binding site theory. Additionally, amino acids reported to interact with ligands in previous reports, including C36, F244, S358, I446, C473, D474, also outlined the binding regions identified in our docking studies (Figure 5, B). As discussed previously, MPP⁺, TEA, and acyclovir docked within the translocation pore, but in disparate locations (Figure 5). Accordingly, we termed these pockets the “MPP⁺ binding pocket,” the “TEA binding pocket,” and the “acyclovir binding pocket.” A majority of the test compounds seemed to dock within one of these pockets, with some overlap. Many of the docked substrates docked in or around the MPP⁺ binding pocket, a handful docked within the TEA and acyclovir pockets, and two ligands, imatinib and ritonavir, spanned the entire translocation pore. However, the model produced by Chen, et al., (2017) [25] appeared to contain two distinct binding sites, while our model suggests a third binding site. This discrepancy may be due, in part, to the fact that Chen, et al., (2017) [25] used two putative pre-determined binding sites and subsequently docked ligands into both sites, selecting the site which yielded the most favorable score as the binding site for that ligand. However, our docking studies permitted docking ligands anywhere within the transporter, and therefore allowed for the distinction of three binding pockets. Since our model excluded the large extracellular loop region that has previously been implicated in transporter-ligand interactions, it cannot be ruled out that this region may play some role in the ligand-dependent effects that we observed in this study. However, our docking efforts demonstrate that discrete ligand binding environments exist within the substrate translocation channel, giving credence to the idea that ligand-dependent interactions may occur independently within the translocation core.

Principal component analysis (Figures 7, 8) suggested that the MPP⁺ pocket allows for greater flexibility in ligand binding, with ligands that tended to be smaller in size and ionizable, trends that held true for both endogenous ligands and drugs examined in this study. Conversely, ligands docked in the acyclovir pocket tended to be larger, more hydrophobic and flexible (based on the number of rotatable bonds). No trends emerged when relating ligand properties directly to the TEA pocket. However, interestingly, all the ligands which docked in or near the TEA binding pocket: berberine, cimetidine, and

lamivudine, did not induce efflux of radiolabeled TEA in the CCF assay, even though they have all been determined to be substrates of hOCT1 [24, 45, 62]. This may suggest an occlusion of this site, either by TEA or the test compounds, which prevents substrate exchange. Ligands that spanned both the TEA and MPP⁺ binding sites (imatinib and ritonavir) tended to be larger ligands dominated by H-bond donors, with a high cLogP and a large number of rotatable bonds. Notably, a similar GRID docking score was associated with ligands that occupied both the MPP⁺ and TEA pockets, which suggests approximately equivalent binding energy for interaction at either site. The fact that these molecules effectively spanned the entire translocation pore may explain why they function more as inhibitors of hOCT1 rather than substrates, as recent reports suggest is the case for imatinib [59, 60]. BSP was an outlier in all cases, largely due to the nature and location of its unique binding pocket lying outside of the translocation channel.

Despite the small sample size, clear ligand-dependent interactions were observed in our CCF assay, confirming ligand-dependent effects for hOCT1. While the number of test compounds employed in this study is small, it can be considered a proof of principle study, and as such, it has uncovered certain trends which will be further investigated in planned larger future studies. Moreover, the observed ligand-dependent effects strengthen the argument for investigating multiple probe substrates in preclinical transport studies to improve identification of potentially dangerous drug-drug interactions.

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Abbreviations:

OCT1	Organic Cation Transporter 1
hOCT1	human OCT1
HEK	Human Embryonic Kidney
EV	empty vector
MPP 1	methyl-4-phenylpyridinium
TEA	tetraethylammonium
CCF	competitive counter flow
TMD	transmembrane domains
CHARMm	Chemistry at Harvard Macromolecular Mechanics
PCA	Principal Component Analysis

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Figure 1. Uptake of MPP⁺ and serotonin by HEK-EV and-hOCT1 stable transfectants.

To functionally validate the generated HEK-EV (empty vector) and -hOCT1 stable cell lines, cells from each pool were incubated with radiolabeled (a) MPP⁺ (0.9 μ Ci/mL, 11 nM) and (b) serotonin (1.2 μ Ci/mL, 42 nM) at 37°C for the indicated amount of time. Each condition was completed in triplicate and the mean $\pm$ SD is shown.

Figure 2. Time-dependent uptake and efflux of MPP^+ , serotonin, metformin, and TEA by hOCT1.

To establish the time when hOCT1 transport reaches steady-state, HEK-EV and -hOCT1 cells were incubated with radiolabeled (a) MPP^+ ($0.4 \mu\text{Ci/mL}$, 4.7 nM), (b) serotonin ($0.4 \mu\text{Ci/mL}$, $14 \mu\text{M}$), (c) metformin ($0.2 \mu\text{Ci/mL}$, $2 \mu\text{M}$), or (d) TEA ($0.2 \mu\text{Ci/mL}$, $54 \mu\text{M}$) at room temperature for 15 seconds to 180 minutes. Once steady-state was determined, HEK-hOCT1 cells were pre-loaded with (e) MPP^+ ($0.4 \mu\text{Ci/mL}$, 4.7 nM), (f) serotonin ($0.4 \mu\text{Ci/mL}$, $14 \mu\text{M}$), (g) metformin ($0.2 \mu\text{Ci/mL}$, $2 \mu\text{M}$), or (h) TEA ($0.2 \mu\text{Ci/mL}$, $54 \mu\text{M}$), for 90 minutes to reach equilibrium, at which point CCF (efflux) was initiated by adding

unlabeled MPP⁺ (100 μ M), serotonin (1 mM), metformin (10 mM), or TEA (1 mM) to each well and incubating them for 15 seconds to 10 minutes (2 minutes for MPP⁺). Each condition was done in triplicate and the mean $\pm$ SD of three independent experiments is shown.

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Figure 3. Substrate-dependent ligand interactions as determined by competitive counterflow. HEK-hOCT1 cells were pre-loaded with radiolabeled MPP⁺ (0.4 μ Ci/mL; black bars), serotonin (0.4 μ Ci/mL; hatched bars), or TEA (0.2 μ Ci/mL; grey bars) for 90 minutes to reach equilibrium. CCF was initiated by adding unlabeled test compounds at concentrations approximately equal 10-times the published K_m values for hOCT1 (see Table 1 for concentrations used) to the respective wells. CCF was measured for 30s when MPP⁺ was employed as the pre-load substrate, and 5 minutes when serotonin and TEA were used. Raw data are shown in panel (A). The dashed line corresponds to 80% of uptake activity. The data shown in (B) were corrected for the observed differences in maximum efflux between pre-load substrates. The dashed line corresponds to 65% of uptake activity. Each condition was done in triplicate and the mean $\pm$ SD of three independent experiments is shown.

Figure 4. Bromosulfophthalein is a substrate of hOCT1.

HEK-EV and -hOCT1 cells were incubated in the presence of (A) 0.1 μM (0.3 $\mu\text{Ci/mL}$) or (B) 50 μM (0.9 $\mu\text{Ci/mL}$) [^3H]-BSP for increasing amounts of time to demonstrate time-dependent transport. To evaluate the kinetic parameters of BSP transport, (C) HEK-EV and -hOCT1 cells were incubated with increasing concentrations of BSP, ranging from 0.1 μM (0.3 $\mu\text{Ci/mL}$) to 50 μM (0.9 $\mu\text{Ci/mL}$), for 2 minutes at 37°C. Net uptake, obtained by subtracting uptake in empty vector cells from that of hOCT1-expressing cells, was fit to the Michaelis-Menten equation to obtain a K_m of $13.6 \pm 2.6 \mu\text{M}$ and V_{max} of $55.1 \pm 4.1 \text{ pmol/mg protein/min}$. Each condition was completed in triplicate and the plotted values $\pm$ SD are the result of three independent experiments.

Figure 5. Ligand structures docked into the hOCT1 homology model.

The chemical structures of several of the ligands yielding results in the CCF assay were docked into an hOCT1 homology model using the UCSF DOCK software package. These ligands included: MPP⁺, serotonin, metformin, TEA, BSP, acyclovir, ritonavir, lamivudine, cimetidine, famotidine, imatinib, fluoxetine, lamotrigine, thiamine, PGE2, quercetin, berberine, and ethidium. (A) The chemical structures are shown in multiple colors within the pore of the transporter, which is shown as a grey mesh of the van der Waals surface of the transporter. (B) The van der Waals surfaces of the docked ligands are illustrated in magenta (BSP) and blue (remaining ligands docked), and the backbone structure of the protein is shown in grey. The equivalent of one helix was removed from these graphics to better visualize the ligand structures. Additionally, the transporter is represented in the side view, with the viewer being parallel with the edge of the plasma membrane. With the exception of BSP, all other ligands included in the docking study docked within a central pore of the transporter.

Figure 6. Ligands docked in disparate pockets within hOCT1 homology model.

In general, all ligands docked within one of three separate “binding pockets”. These pockets are demonstrated here by (A,C) the chemical structures and (B,D) the van der Waal’s surface of amino acid residues lining the binding pockets of MPP⁺ (yellow), TEA (red), and acyclovir (green). A portion of one helix was removed from (A) to better visualize the MPP⁺ chemical structure.

Figure 7. Principal Component Analysis (PCA) of OCT1 ligand independent variables and PCA scree plot.

Biplot of the PCA demonstrating correlations observed between individual physiochemical properties and specific ligand binding sites (A). Scree plot detailing the number of significant principal components (B).

Figure 8. PCA biplot for F1 and F2 of OCT1 ligand independent variables.

Biplot scatterplot demonstrating the relationships between the first two principal components, F1 and F2.

Table 1.Published K_m/IC_{50} values and concentrations of test compounds employed in CCF assays.

Drug List	K_m or $[K_i/IC_{50}]$ (μM)	References	Desired Concentration (mM)	Actual Concentration (mM) ^a
MPP ⁺	15, 32, 35	Boxberger, et al., 2014, Grundemann, et al., 2003, Zhang, et al., 1997b	0.1	0.1
Serotonin	197	Boxberger, et al., 2014	1	1
Metformin	1470	Kimura, et al., 2005	10	10
TEA	168, 229	Bednarczyk, et al., 2003, Zhang, et al., 1998	1	1
BSP	--- ^b		1	0.59
Glucose	--- ^b		1	1
Acyclovir	151	Takeda, et al., 2002	1	1
Dolutegravir	--- ^b		1	1
Ritonavir	[5]	Zhang, et al., 2000	0.1	0.1
Lamivudine	249, 1250	Jung, et al., 2008, Minuesa, et al., 2009	1	1
Cimetidine	[95], [166]	Ciarimboli, et al., 2004, Zhang, et al., 1998	10	10
Ranitidine	[22], 70	Bednarczyk, et al., 2003, Bourdet, et al., 2005	1	1
Famotidine	[28]	Bourdet, et al., 2005	1	1
Diphenhydramine	[4]	Boxberger, et al., 2014, Muller, et al., 2005	0.1	0.1
Imatinib	[0.1], [1.5], [10]	Boxberger, et al., 2014, Minematsu and Giacomini, 2011, Nies, et al., 2014	0.1	0.1
Gefitinib	[1]	Minematsu and Giacomini, 2011	0.1	0.1
Erlotinib	[0.35]	Minematsu and Giacomini, 2011	0.1	0.1
Oxaliplatin	[4] ^c	Zhang, et al., 2006	0.1	0.1
Verapamil	[1.5], [1.8], [3]	Boxberger, et al., 2014, Dickens, et al., 2012, Zhang, et al., 1998	0.1	0.1
Midazolam	[3.7]	Zhang, et al., 1998	0.1	0.1
Fluoxetine	[3], [6]	Boxberger, et al., 2014, Haenisch, et al., 2012	0.1	0.1
Lamotrigine	62	Dickens, et al., 2012	1	1
Acebutolol HCl	[96]	Zhang, et al., 1998	1	1
Salbutamol	[277]	Salomon, et al., 2015	1	1
Thiamine	[434], 780	Bednarczyk, et al.,	10	10

Drug List	K _m or [K _i /IC ₅₀] (μM)	References	Desired Concentration (mM)	Actual Concentration (mM) ^a
		2003, Chen, et al., 2014		
Prostaglandin E ₂	[0.7]	Kimura, et al., 2002	0.1	0.1
EGCG	[140] ^d	Knop, et al., 2015	1	0.93
Quercitin	2.2	Glaeser, et al., 2014	0.1	0.1
Berberine	15	Nies, et al., 2008	0.1	0.09
Ethidium	0.8	Lee, et al., 2009	0.1	0.1

^a In some instances, actual concentration varied from desired concentration due to solubility issues when preparing stock solutions

^b Affinity has not been determined for hOCT1.

^c IC₅₀ determined by cytotoxicity.

^d Value calculated from data presented in Knop, et al., 2015.

PCA analysis summary statistics

[illegible]