Organic anion transporter 1 is inhibited by multiple mechanisms and shows a transport mode independent of exchange

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Running Title: OAT1 is inhibited by multiple mechanisms

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ABBREVIATIONS: Chinese hamster ovary, CHO; para-aminohippurate, PAH; waymouth buffer, WB;
Abstract

The mechanism by which drugs inhibit OAT1 was examined. OAT1 was stably expressed in Chinese hamster ovary (CHO) cells and para-aminohippurate (PAH) and 6-carboxyfluorescein were the substrates. Most compounds (10 of 14) inhibited competitively, increasing the Michaelis constant ($K_m$) without affecting maximal transport rate ($J_{max}$). Others were mixed-type (lowering $J_{max}$ and increasing $K_m$) or non-competitive (lowering $J_{max}$ only) inhibitors. The interaction of a non-competitive inhibitor (telmisartan) with OAT1 was examined further. Binding of telmisartan to OAT1 was observed, but translocation was not. Telmisartan did not alter plasma membrane expression of OAT1, indicating that it lowers $J_{max}$ by reducing turnover number. PAH transport following telmisartan treatment and its washout recovered faster in the presence of 10% fetal bovine serum in the washout buffer, indicating that binding of telmisartan to OAT1 and its inhibitory effect are reversible. Together, these data suggest that telmisartan binds reversibly to a site distinct from substrate and stabilizes the transporter in a conformation unfavorable for translocation. In the absence of an exchangeable extracellular substrate, PAH efflux from CHO-OAT1 cells was relatively rapid. Telmisartan slowed PAH efflux, suggesting that some transporter-mediated efflux occurs independent of exchange. Although drug-drug interaction predictions at OAT1 assume competitive inhibition, these data show that OAT1 can be inhibited by other mechanisms, which could influence the accuracy of drug-drug interaction predictions at the transporter. Telmisartan was useful for examining how a non-competitive inhibitor can alter OAT1 transport activity, and for uncovering a transport mode independent of exchange.
Introduction

The kidney is important for the urinary elimination of a variety of organic anions of physiological and pharmacological importance (Rizwan and Burckhardt, 2007). Renal excretion is accomplished by glomerular filtration, tubular secretion and tubular reabsorption. For many hydrophilic organic anions transporter-mediated tubular secretion is a major determinant of their overall urinary elimination. The organic anion transporter 1 (OAT1; SLC22A6) contributes to organic anion uptake across the peritubular membrane of proximal tubule cells, and a variety of transporters are implicated in apical efflux into the glomerular filtrate (Pelis and Wright, 2011). In a retrospective study that examined elimination pathways of 200 of the top-prescribed therapeutic drugs, 32% are predominately eliminated in the urine, with 92% of these actively secreted by renal tubules (Morrissey, et al., 2012). Of the drugs actively secreted, 28% are substrates of OAT1, highlighting the potential importance of OAT1 to pharmacokinetics (Morrissey, et al., 2012).

OAT1 interacts with a wide variety of structurally diverse organic anions, making it a potential site of pharmacokinetic drug-drug interactions (Vanwert, et al., 2010; Burckhardt, 2012). Given OAT1’s potential involvement in drug-drug interactions, the United States Food and Drug Administration and the European Medicines Agency recommend that pharmaceutical companies investigate whether investigational drugs inhibit OAT1 (US Food Drug Admin, 2012; European Medicines Agency, 2012). The studies are typically done in vitro using cell lines, such as human embryonic kidney or Chinese hamster ovary (CHO) cells expressing OAT1 (Giacomini, et al., 2010). Depending on the potency with which investigational drugs inhibit OAT1, and the anticipated maximal therapeutic unbound plasma concentration of the investigational drug, a clinical drug-drug interaction study may be warranted. Thus, it is important to accurately assess the potency with which drugs inhibit OAT1, but to also investigate...
the mechanism of inhibition, as it could influence drug-drug interaction potential. Although competitive inhibition has been well described in the literature, and is often presumed, it is not clear if OAT1 is inhibited by other mechanisms.

We previously examined the effect of a physiologic plasma concentration of α-ketoglutarate on the interaction of ligands with OAT1 expressed in CHO cells (Ingraham, et al., 2014). For some compounds, their IC$_{50}$ value against OAT1 increased when α-ketoglutarate was present, but this was not the case for all compounds. From these data we speculated that for inhibitors where IC$_{50}$ values shifted when α-ketoglutarate was present, they act in a competitive manner to reduce transport activity, whereas for inhibitors where a shift was not observed, they are non-competitive inhibitors. Given our previous results, and recent data showing that a related transporter, organic cation transporter 2, is inhibited by multiple mechanisms (Harper and Wright, 2013), we hypothesized that OAT1 can be inhibited by multiple mechanisms as well. The purpose of this study was to examine the mechanism by which drugs in diverse classes inhibit OAT1. We examined twelve drugs in seven different classes (uricosuric, antiviral, non-steroidal anti-inflammatory, loop diuretic, angiotensin II receptor antagonist, proton pump inhibitor and statin), morin, a flavonoid, and estrone-3-sulfate, a metabolite of the endogenous hormone estrone. These compounds were chosen since they inhibit OAT1, they are structurally diverse, and a previous study suggested that some may inhibit OAT1 via mechanisms other than simple competition (Ingraham, et al., 2014). Consistent with our hypothesis, we observed other types of inhibition than just competition. The interaction of telmisartan (a non-competitive inhibitor) with OAT1 was further examined as it was useful for examining how a non-competitive inhibitor can alter OAT1 transport activity, and for uncovering a transport mode independent of exchange.
Materials and Methods

Reagents & Chemicals – \(^{3}\text{H}\)-para-aminohippurate (60 Ci/mmol) and \(^{3}\text{H}\)-telmisartan (15 Ci/mmol) were from American Radiochemicals (St. Louis, MO, USA). F12 Kaighn’s modification medium, fetal bovine serum (Certified, US Origin), 1% penicillin-streptomycin solution, zeocin, hygromycin B, 4-12% Tris-glycine gels and 6-carboxyfluorescein were purchased from Life Technologies (Burlington, ON). The bicinchoninic acid protein assay kit, Sulfo-NHS-SS-biotin reagent, Streptavidin UltraLink resin and the SuperSignal® West Pico chemiluminescent substrate were from Thermo Scientific (Rockford, IL). Olmesartan was obtained from Tocris Biosciences (Bristol, UK). All other chemicals were of the highest purity possible and were obtained from Sigma-Aldrich (St. Louis, MO). Waymouth buffer (WB) used for transport experiments contained (in mM): 135 NaCl, 28 D-glucose, 5 KCl, 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), 0.8 MgSO\(_4\) and 13 HEPES-NaOH, pH 7.4.

Cell Culture – Cloning and stable expression of the human ortholog of OAT1 in CHO Flp-In cells (CHO-OAT1 cells) has been described (Ingraham, et al., 2014). The CHO cells were grown in complete medium containing F12 Kaighn’s modification medium, 10% fetal bovine serum and 1% penicillin-streptomycin. Medium used for culturing the CHO-OAT1 cells was supplemented with hygromycin B (200 \(\mu\)g/ml, final concentration) whereas medium used for culturing the parental CHO cells (CHO parental cells) was supplemented with zeocin (100 \(\mu\)g/ml, final concentration). Cells were grown at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air.

Cell Surface Biotinylation and Western Blotting – Cell surface biotinylation was done to determine if telmisartan exposure results in loss of OAT1 at the cell surface. CHO-OAT1 cells were treated without or with telmisartan (5 \(\mu\)M) diluted in WB for 10 seconds and the
telmisartan was removed by rinsing the cells rapidly three times (0.5 ml each) with room temperature WB. The cells were then placed in room temperature WB for 2, 5, 20, 40 or 60 minutes (recovery periods), and rinsed once with WB prior to performing the cell surface biotinylation. All solutions were kept ice-cold for cell surface biotinylation, and long incubations were conducted on ice with gentle shaking. One well of a 12-well plate containing CHO-OAT1 cells grown to confluence was used for each biotinylation reaction. Cell surface biotinylation was performed using Sulfo-NHS-SS-biotin (0.5 mg/ml) using procedures identical to that described previously (Astorga, et al., 2011). The biotinylated proteins were separated on 4-12% tris-glycine gels. The protocol for detection of OAT1 protein by immunoblotting is essentially the same as described previously (Astorga, et al., 2011).

**Cellular Accumulation Studies with Radiolabeled compounds** – All transport experiments using [3H]para-aminomhippurate ([3H]PAH) and [3H]telmisartan were conducted using cells grown to confluence in 24-well flat bottom plates. The transport solution consisted of WB containing radiolabeled compound, and in some cases, inhibitor and/or unlabeled compound. Prior to the uptake period the medium was aspirated and the cells were rinsed once with room temperature WB. In some cases, cells were permeabilized for five minutes in phosphate buffered saline containing 0.1% saponin, followed by two rapid rinses with WB prior to measuring the cellular accumulation of radiolabeled compounds. After the uptake period the cells were rinsed three times with ice-cold WB. The cells were lysed with 0.5 N NaOH/1% SDS (0.4 ml) for ~30 min on an orbital shaker after which the NaOH was neutralized with 1 N HCl (0.2 ml). Cell lysates (0.5 ml) were transferred to scintillation vials and liquid scintillation cocktail (CytoScint ES, MP Biomedicals) was added (7 ml). Cellular radioactivity content was
determined with a Beckman LS6500 liquid scintillation counter. Protein content in the wells was determined using the bicinchoninic acid method.

**Cellular Accumulation Studies with 6-Caboxyfluorescein** – Cells grown to confluence in 96-well black-walled flat-bottom plates were used for transport experiments with 6-carboxyfluorescein. The transport solution consisted of WB containing 6-carboxyfluorescein, and in some cases, inhibitor. Briefly, cells were rinsed once with WB after which the uptake of 6-carboxyfluorescein was determined. After the uptake period the cells were rinsed three times with ice-cold WB. After the last rinse the WB was aspirated and 6-carboxyfluorescein in the cells was determined at excitation and emission wavelengths of 485 and 525 nm, respectively, using a Cytation 3 multimodal plate reader (BioTek, Winooski, VT, USA). 6-carboxyfluorescein concentration was determined from a standard curve and was expressed relative to the surface area of the bottom of an individual well.

**Analysis** – Data are reported as mean ± standard error of the mean. All experiments were performed in triplicate using cells of the same passage, with the number of observations based on the number of experiments done using cells of a different passage. Comparison of sample means was done using a two-tailed unpaired Student’s t-test or One-Way Analysis of Variance followed by the Newman-Keuls post hoc test. Inhibitory concentration 50 values (IC50) were determined by non-linear regression as described previously (Ingraham, et al., 2014). The maximal transport rate (Jmax) and Michaelis constant (Km) of PAH and 6-carboxyfluorescein transport were determined by non-linear regression analysis using the Michaelis-Menten equation as described previously (Ingraham, et al., 2014)). All graphing, non-linear regression analysis and statistical analysis were performed with GraphPad Prism (version 5.04).

**Results**
The inhibitory effect of ibuprofen, telmisartan and omeprazole on PAH and 6-carboxyfluorescein transport is shown in Figure 1 – these compounds were chosen as examples given their different inhibition mechanisms (see below). We chose to examine two different substrates to determine the potential for inhibition mechanism to be substrate dependent. In the examples in Figure 1, ibuprofen, telmisartan and omeprazole inhibited [3H]PAH uptake by CHO-OAT1 cells with IC50 values of 2.7 μM, 0.36 μM and 10.4 μM, respectively, and the drugs were near equipotent inhibitors of 6-carboxyfluorescein transport as PAH transport (Figure 1). Under control conditions, and when the individual inhibitors were present at a fixed concentration, PAH uptake (Figure 2A, 2C and 2E) and 6-carboxyfluorescein uptake (Figure 2B, 2D and 2F) by CHO-OAT1 cells was a saturable process. Ibuprofen caused a significant increase in the K_m value, without affecting the J_max value for both PAH transport (Figure 2A and Table 1) and 6-carboxyfluorescein transport (Figure 2B and Table 2), an effect consistent with competitive inhibition. The J_max value for both PAH transport and 6-carboxyfluorescein transport was significantly reduced by telmisartan, but the K_m value was unchanged, indicating that telmisartan is a non-competitive inhibitor of OAT1 (Figure 2C, 2D and Table 1 and Table 2). Omeprazole showed mixed inhibition of both PAH transport (Figure 2E and Table 1) and 6-carboxyfluorescein transport (Figure 2F and Table 2), causing changes in both K_m (an increase) and J_max (a decrease) values. Linear transformations of the Michaelis-Menten curves highlight the kinetic effect the inhibitors had on OAT1-mediated PAH (Figure 3A, 3C and 3E) and 6-carboxyfluorescein (Figure 3B, 3D and 3F) transport. Table 1 and Table 2 summarizes the kinetic effect all of the compounds tested had on PAH transport and 6-carboxyfluorescein transport, respectively. Most of the compounds tested were competitive inhibitors, except
telmisartan, azilsartan, irbesartan and omeprazole, which were either characterized as non-competitive or mixed inhibitors.

We further characterized the interaction of telmisartan with OAT1 since it is a non-competitive inhibitor, it is a potent OAT1 inhibitor (IC$_{50} < 0.5$ μM, (Ingraham, et al., 2014; Sato, et al., 2008)) relative to many others (Vanwert, et al., 2010), and it is extremely hydrophobic (experimental LogP value of 7.73 CSID:59391, http://www.chemspider.com/). It is important to note that telmisartan, based on its C$_{max, \text{unbound}}$/IC$_{50}$ (US Food Drug Admin, 2012), is not expected to cause a drug-drug interaction at OAT1, as the unbound plasma concentration of telmisartan at a therapeutic dose (<10 nM at an 80 mg dose; (Taylor, et al., 2011)) is ~30-50 times lower than its IC$_{50}$ value. Although telmisartan is not a clinical safety concern with respect to OAT1 inhibition, we felt it important to further characterize its interaction with OAT1, as other drugs on the market or in development may be non-competitive inhibitors and interact similarly with the transporter. Two types of experiments were conducted to assess the possibility of active transport of telmisartan by OAT1 – room temperature versus ice-cold conditions to slow active transport or cells were permeabilized with saponin prior to performing transport assays. Saponin removes cholesterol from membranes leaving small pores of ~50 Å (Jamur and Oliver, 2010). The cellular accumulation of [$^3$H]telmisartan was significantly higher in CHO-OAT1 cells compared to CHO parental cells without or with membrane permeabilization with saponin, which was effective at completely inhibiting PAH transport (Figure 4A). The cellular content of [$^3$H]telmisartan was also significantly higher in CHO-OAT1 cells compared to parental cells when transport was conducted either at room temperature or when using ice-cold transport solution (Figure 4B). Expectedly, the cellular accumulation of [$^3$H]PAH was reduced 90% when using ice-cold transport solutions, indicating that low temperature was effective at almost
completely inhibiting OAT1 substrate translocation (Figure 4B). The time course of 
$[^3H]$telmisartan accumulation into CHO-OAT1 cells and parental cells conducted using ice-cold 
transport buffer is shown in Figure 4C – with the difference in cellular accumulation between 
the two cell lines plotted as specific binding to OAT1. At each time point examined the cellular 
accumulation of telmisartan was higher in CHO-OAT1 cells compared to parental cells, with 
specific binding reaching equilibrium at ~20 minutes. Attempts to determine the equilibrium 
dissociation constant and maximal binding were unsuccessful since the passive component of 
cellular telmisartan accumulation masked the specific binding, especially at higher telmisartan 
concentrations (data not shown).

Previous work with OATP1B1 suggests that the transport protein can be inhibited by 
more complex mechanisms than by simple competition, perhaps even by an irreversible 
mechanism, i.e., mechanism-based inhibition (Amundsen, et al., 2010; Shitara, et al., 2013). To 
examine the possibility that a non-competitive inhibitor can irreversibly inhibit OAT1 we 
examined the ability of transport activity to recover following telmisartan treatment and its 
removal (Figure 5A). In these experiments $[^3H]$PAH uptake was examined either in the absence 
or presence of telmisartan (5 μM) added to the transport solution, or, the cells were treated 
briefly with telmisartan (5 μM), the telmisartan was removed by extensive washing with WB, 
and the cells were allowed to recover for 2, 5, 10, 20, 40 or 60 minutes in WB prior to another 
rapid washing with WB and measurement of $[^3H]$PAH uptake. The concentration of telmisartan 
used (5 μM) was ~15-fold higher than its IC$_{50}$ value against OAT1-mediated PAH transport 
(Ingraham, et al., 2014). Telmisartan added to the transport solution reduced $[^3H]$PAH uptake to 
7% of the control value, indicating that the drug was effective at inhibiting OAT1 activity 
(Figure 5A). $[^3H]$PAH uptake was only 20% of the control value two minutes after removal of
telmisartan (Figure 5A). [3H]PAH uptake was reduced 47% compared to control 60 minutes after telmisartan washout. A possible explanation is that telmisartan is slow to leave the cells and that a residual amount of telmisartan is available to inhibit activity following its removal. Indeed, following brief telmisartan treatment, ~8% of the [3H]telmisartan dose was present in the cells after one hour (Figure 6). In a separate set of recovery experiments we used the same protocol as described above, except that the cells were allowed to recover for 30 or 60 minutes in WB containing 10% fetal bovine serum (Figure 5B). [3H]PAH uptake was not significantly different from control following a 30 or 60 minute washout in the presence of 10% fetal bovine serum. Accordingly, the removal of cellular [3H]telmisartan was relatively rapid when the cells were incubated in WB containing 10% fetal bovine serum (Figure 6).

The maximal transport rate is a product of the number of transporters at the plasma membrane and their turnover number. To determine if telmisartan non-competitively inhibited OAT1 by reducing the number of OAT1 transport proteins in the plasma membrane cell surface biotinylation was performed. The protocol for treating the cells with telmisartan and allowing them to recover in WB was identical to that outlined for the transport experiments in Figure 5A. Cells were treated with telmisartan, it was removed, and the cells were allowed to recover for 2, 5, 20, 40 or 60 minutes, after which the cell surface biotinylation was performed. The apparent differences in cell surface expression of OAT1 were small (Supplemental Figure 1), and cannot explain the reduction in J_{max} caused by telmisartan.

Since telmisartan was an effective inhibitor of OAT1-mediated substrate uptake and has a relatively high apparent passive permeability, we speculated that it might inhibit efflux as well. Thus, we examined the time course of [3H]PAH efflux from CHO-OAT1 cells in the absence or presence of telmisartan (5 μM) added to the extracellular buffer (Figure 7). Under control
conditions nearly all of the intracellular [³H]PAH was effluxed from the cells by 20 minutes (Figure 7A-C). As anticipated, including a saturating concentration of unlabeled PAH (250 μM) in the extracellular buffer trans-stimulated [³H]PAH efflux (Figure 7A). In contrast, efflux slowed considerably when telmisartan (5 μM) was included in the extracellular buffer (Figure 7B). The inhibitory effect of telmisartan on [³H]PAH efflux was similar whether in the absence (Figure 7B) or presence (Figure 7C) of PAH (250 μM) added to the extracellular buffer.

Discussion

In the present study we used the OAT1 substrates PAH and 6-carboxyfluorescein to examine the mechanism by which compounds inhibit OAT1. Regardless of the substrate used, the individual compounds tested had similar effects on their kinetics, perhaps suggesting that inhibition mechanism at OAT1 is substrate independent. Most of the compounds inhibited transport in a competitive manner (10 out of 14), but several showed mixed or non-competitive inhibition. Importantly, competitive, mixed and non-competitive inhibitors could differentially impact the rate of tubular organic anion secretion mediated by OAT1 in vivo, and hence, overall renal organic anion elimination and drug-drug interaction magnitude. That is, inhibition potential of a reversible competitive inhibitor (perpetrator drug) is dependent on the concentration of itself as well as the concentration of the drug substrate (victim drug) at the OAT1 ligand binding surface, both of which are changing with time following administration, i.e., high concentrations of victim can outcompete the perpetrator for binding to OAT1, and vice versa. In contrast, the inhibition potential of a non-competitive perpetrator is only dependent on the concentration of itself at OAT1, and independent of the victim concentration. Consequently, a non-competitive inhibitor has greater potential to cause a drug-drug interaction than a competitive inhibitor – assuming both inhibit OAT1 with the same potency and have similar
plasma concentration-time profiles. Both static and mechanistic models, such as $C_{\text{max, unbound/IC}_{50}}$ (US Food Drug Admin, 2012) and physiologically based pharmacokinetic models, respectively, are used to predict drug-drug interactions at OAT1. However, these models assume reversible competitive inhibition, and by doing so, could underestimate drug-drug interaction magnitude with compounds that inhibit by other mechanisms.

Experiments further examining the interaction of telmisartan with OAT1 were useful for investigating the possible mechanism by which a non-competitive inhibitor can reduce OAT1 transport activity. Experiments conducted at room temperature versus ice-cold conditions, or following permeabilization of the plasma membrane with saponin, showed that telmisartan binds to OAT1, but does not appear to be a translocated substrate. That is, the cellular accumulation of telmisartan was significantly higher in CHO-OAT1 cells compared to parental cells despite using procedures that completely inhibit OAT1 translocation. Although reducing bath temperature appeared to lower the cellular accumulation of telmisartan by CHO-OAT1 cells (as well as CHO parental cells), we attribute this effect to a reduction in passive membrane permeability at low temperatures, as opposed to an effect on active transport. Indeed, Poirier et al. (Poirier, et al., 2008) showed that permeability of organic solutes, including organic anions, across CHO cell as well as artificial membranes is highly sensitive to temperature. The apparent modest reduction in [${}^{3}\text{H}$]telmisartan accumulation by CHO-OAT1 cells in the presence versus absence of saponin pre-treatment is consistent with the observed modest reduction in OAT1 expression at the cell surface as determined by cell surface biotinylation (data not shown). Although our data is consistent with binding of telmisartan to OAT1 without translocation, we cannot rule out the possibility that the compound is actively transported. It is possible that the high passive membrane permeability of telmisartan masks any active transport mediated by OAT1.
Regardless, less than one percent of a telmisartan dose is recovered in the urine of humans (Michel, et al., 2013), suggesting that if transported by OAT1, the activity is not important for its elimination.

Telmisartan reduced the maximal transport rate, which is a function of the number of transporters expressed at the plasma membrane and their turnover number. The level of OAT1 expressed at the plasma membrane was unaffected by telmisartan, indicating that it inhibits by reducing OAT1 turnover number. Based on these results, we speculate that both ligands (substrate and non-competitive inhibitor) interact at distinct sites in the OAT1 ligand binding pocket, and the presence of telmisartan stabilizes the transporter in an unfavorable conformation for substrate translocation.

A previous study showed that OATP1B1 can be inhibited for long periods following drug exposure and its removal, perhaps suggesting a mechanism-based inhibition (Shitara, et al., 2013). Thus, we performed experiments where the cells were treated with telmisartan (5 μM), it was removed by extensive washing, the cells were allowed to recover for various time periods, and the cells were washed again prior to measuring PAH uptake. This protocol is somewhat similar to one used by Shitara et al (Shitara, et al., 2013) with OATP1B1. After one hour only ~50% of the PAH transport activity had recovered. Given telmisartan’s high apparent passive permeability, we hypothesized that it gets into the cells and stays there for long periods, despite washing. To examine this possibility the cells were treated with [³H]telmisartan and put through identical washing steps as in the functional studies. After one hour, 8% of the [³H]telmisartan dose remained. If we assume that 8% of the telmisartan dose remained in the functional studies, where cells were treated initially with 5 μM telmisartan, then ~0.4 μM telmisartan would have remained after the one hour washout period. This is enough telmisartan to inhibit ~50% of
OAT1 activity based on its IC₅₀ value – almost exactly what was observed. The recovery of PAH transport following telmisartan treatment and its removal was much faster when including 10% serum in the washout buffer. Accordingly, [³H]telmisartan that had accumulated in CHO-OAT1 cells following a brief incubation was more rapidly removed when 10% serum was included in the washout buffer. Given telmisartan’s high degree of plasma protein binding (~99.5%; (Stangier, et al., 2000)), the plasma protein likely acted as a sink to more quickly remove telmisartan from the cells. These data are consistent with those of Bow et al. (Bow, et al., 2006), who showed that plasma protein can profoundly influence the interaction of ligands with renal organic anion transporters, including OAT1. Together, these data indicate that telmisartan binding to OAT1 and its inhibitory effect are reversible with time and that the relatively long-lasting inhibitory effect is due to the slow rate at which telmisartan leaves the cells.

Given the apparent high passive permeability of telmisartan we speculated that, in addition to uptake, it could inhibit OAT1-mediated efflux as well. Accordingly, we examined the time-course of [³H]PAH efflux from CHO-OAT1 cells under a variety of conditions. The rate of [³H]PAH efflux from control cells was relatively rapid, with nearly all of the intracellular [³H]PAH eliminated by 20 minutes. This was unexpected given that the extracellular buffer did not contain an ionic species considered exchangeable on OAT1 – the predominant anions were chloride and HEPES. Replacement of chloride with gluconate or HEPES with Tris-HCl had no appreciable effect on the rate of [³H]PAH efflux from CHO-OAT1 cells (Supplemental Figure 2). This is consistent with previous work examining PAH transport by renal basolateral membrane vesicles, which showed that neither chloride nor gluconate are exchangeable with PAH (Schmitt and Burckhardt, 1993), and HEPES does not cis-inhibit PAH uptake mediated by
OAT1 (personal observation), suggesting that it is not an exchangeable substrate either.

Although a study using basolateral membrane vesicles suggests that OATs can operate via PAH/OH⁻ exchange (Eveloff, 1987), lowering extracellular pH from 7.4 to 5.5 had no influence on [³H]PAH efflux (Supplemental Figure 2). As anticipated, [³H]PAH efflux was stimulated in the presence of a saturating concentration of unlabeled PAH in the extracellular buffer, indicating PAH/[³H]PAH self-exchange. In the absence of an exchangeable substrate in the extracellular buffer, one may speculate that efflux under control conditions is driven by intracellular [³H]PAH moving down its concentration gradient across the plasma membrane by passive diffusion. Yet, telmisartan either alone or in the presence of a saturating concentration of unlabelled PAH slowed [³H]PAH efflux compared to control, indicating that a large fraction of flux occurred through the transporter. Despite the fact that OAT1 is considered an obligatory exchanger, an explanation for the efflux activity observed is that some PAH flux through OAT1 occurs independent of exchange, i.e., via molecular slips in the OAT1 transport protein. One mechanism by which this could be explained is that following translocation of intracellular [³H]PAH to the outside of the cell, the unloaded OAT1 protein can return to its inward facing conformation. An alternative explanation is that substrate binding causes a conformational change that favors channel formation and organic anion conductance. The phenomenon of molecular slippage in membrane transporters and the possible mechanisms noted above has been reviewed in the literature (Nelson, et al., 2002).

In summary, we have shown that OAT1 is susceptible to multiple mechanisms of inhibition, including competitive, mixed and non-competitive. The mechanism of OAT1 inhibition caused by a perpetrator drug should be considered when modeling drug-drug interactions at OAT1 in order to more accurately predict the perpetrator’s influence on renal...
elimination of victim drugs. The non-competitive inhibitor telmisartan most likely binds reversibly to a site distinct from substrate and stabilizes the transporter in a conformation unsuitable for substrate translocation – in either the uptake or efflux direction. Future work is required to determine if this is a common mechanism by which non-competitive inhibitors reduce OAT1 activity. Telmisartan was also useful for uncovering a transport mode for OAT1 that appears to occur independent of exchange.
Authorship Contributions

Participated in research design: RMP, AH and TG

Conducted experiments: ML, LI, AH, TG, UK and LB

Performed data analysis: ML, AH, TG, UK and LB

Wrote or contributed to the writing of the manuscript: RMP and AH
References


Footnotes

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Figure Legends

**Figure 1.** Representative experiments (n = 1) showing the inhibitory effect of increasing concentrations of ibuprofen, telmisartan or omeprazole on either [³H]PAH (A, C and E) or 6-carboxyfluorescein (B, D and F) uptake by CHO-OAT1 cells. The concentrations of [³H]PAH and 6-carboxyfluorescein in the transport solution (room temperature) were 20 nM and 5 μM, respectively. [³H]PAH and 6-carboxyfluorescein uptake experiments were conducted for one and five minutes, respectively. These were determined to be initial rate time points in preliminary experiments (data not shown).

**Figure 2.** Michaelis menten curves showing the kinetics of PAH (A, C and E) or 6-carboxyfluorescein (B, D and F) uptake by CHO-OAT1 cells done in the absence (Control) or presence of a fixed concentration of ibuprofen (5 μM, A and B), telmisartan (0.5 μM, C and D) or omeprazole (20 μM, E and F). The transport solutions were equilibrated to room temperature. PAH and 6-carboxyfluorescein uptake experiments were conducted for one and five minutes, respectively. These were determined to be initial rate time points in preliminary experiments (data not shown). The data are the mean ± standard error of at least four preparations (n ≥ 4). The kinetic constants obtained (J_max and K_m) and the type of inhibition caused by each inhibitor is shown in Table 1 (PAH) and Table 2 (6-carboxyfluorescein).

**Figure 3.** Linear transformations of the kinetic data shown in Figure 2 highlighting the effect on PAH (A, C and E) or 6-carboxyfluorescein (B, D and F) kinetics of a fixed concentration of ibuprofen (5 μM, A and B), telmisartan (0.5 μM, C and D) or omeprazole (20 μM, E and F).

**Figure 4.** (A) The cellular accumulation of either [³H]telmisartan (20 nM) or [³H]PAH (20 nM) into CHO parental or CHO-OAT1 cells at room temperature without or with a 5 minute pre-
treatment of the cells with 0.1% saponin. Uptake was conducted for five minutes. The data are the mean ± standard error of four preparations (n=4). *P<0.05, **P<0.01, significantly different from uptake by CHO parental cells, unpaired student’s t-test. (B) The cellular accumulation of either [³H]telmisartan (7 nM) or [³H]PAH (10 nM) into CHO parental or CHO-OAT1 cells using either room temperature or ice-cold transport solution. Uptake was conducted for five minutes. The data are the mean ± standard error of four preparations for telmisartan (n=4) and three preparations for PAH (n=3). *P<0.05, **P<0.01, significantly different from uptake by CHO parental cells, unpaired student’s t-test. (C) The time course of [³H]telmisartan (7 nM) accumulation into CHO parental or CHO-OAT1 cells using ice-cold transport solution. The data are from a single experiment (n=1). The difference in cellular [³H]telmisartan accumulation in CHO parental vs. CHO-OAT1 cells represents specific binding of telmisartan to OAT1 (Binding).

Figure 5. Time course of recovery of OAT1-mediated [³H]PAH uptake by CHO-OAT1 cells following treatment with telmisartan and its removal (recovery time after washout) using standard buffer (A) or buffer containing 10% fetal bovine serum (B). The cells were not treated with telmisartan (Control), telmisartan (5 μM) was included in the uptake buffer (Telmisartan), or the cells were treated with telmisartan (5 μM) for ~10 seconds, the telmisartan was removed by repeated washing with WB (3 rapid rinses), and the cells were put into WB without (panel A) or with (panel B) 10% fetal bovine serum for the time points indicated (Recovery time after telmisartan washout). Following the washout period, the cells were rinsed once with WB (no fetal bovine serum) prior to measuring one minute uptakes of [³H]PAH (10 nM). Data are mean ± standard error of four preparations (n = 4). **P<0.01, ***P<0.001, significantly different from
control, One-Way Analysis of Variance followed by the Newman-Keuls test for pairwise comparisons.

**Figure 6.** The time course of $[^3\text{H}]$telmisartan washout from CHO-OAT1 cells done either in standard buffer (Buffer alone) or buffer containing 10% fetal bovine serum (Buffer + 10% FBS). CHO-OAT1 cells were treated with $[^3\text{H}]$telmisartan diluted in WB (no fetal bovine serum) for ~10 s, the cells were then washed repeatedly with WB (three rapid rinses) and incubated in WB or WB containing 10% fetal bovine serum (WB + 10% FBS) for 2, 10, 20 or 60 minutes, after which the cells were rinsed once with WB and the cellular accumulation of $[^3\text{H}]$telmisartan determined. Data are mean ± standard error of three preparations ($n = 3$).

**Figure 7.** Efflux of $[^3\text{H}]$PAH from CHO-OAT1 cells. Cells were loaded with $[^3\text{H}]$PAH (~20 nM) for 20 minutes at room temperature, and the cells were washed rapidly three times with ice-cold WB prior to the efflux period. Efflux was conducted for 30 sec, 2 min, 5 min or 20 min in room temperature WB in the absence (Control) or presence of unlabeled PAH (250 µM, A), telmisartan (5 µM, B) or both in combination (C) added to the extracellular buffer. Cells were rinsed rapidly three times with ice-cold WB prior to measuring intracellular $[^3\text{H}]$PAH. Data are presented as a percentage of the intracellular $[^3\text{H}]$PAH level immediately prior to performing efflux ($t=0$ min). Data are the mean ± standard error of four preparations ($n = 4$). *P<0.05, **P<0.01, ***P<0.0001, significantly different than control, unpaired student’s t-test.
### Tables

Table 1. The kinetics of PAH transport by OAT1 in the absence versus presence of inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>$J_{\text{max}}$ (pmol $\cdot$ mg protein$^{-1}$ $\cdot$ min$^{-1}$)</th>
<th>$K_{\text{m}}$ (μM)</th>
<th>Inhibition Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1095 ± 269</td>
<td>22.3 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Probenecid (15 μM)</td>
<td>897 ± 233</td>
<td>42.0 ± 4.8**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>1095 ± 172</td>
<td>14.8 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Cidofovir (100 μM)</td>
<td>1388 ± 373</td>
<td>50.1 ± 17.6**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>759 ± 88.0</td>
<td>14.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen (5 μM)</td>
<td>1057 ± 93.8</td>
<td>49.3 ± 5.4***</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>632 ± 80.4</td>
<td>15.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Estrone-3-sulfate (250 μM)</td>
<td>620 ± 78.6</td>
<td>57.9 ± 9.5**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>939 ± 145</td>
<td>21.9 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (5 μM)</td>
<td>921 ± 174</td>
<td>51.3 ± 15**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>733 ± 133</td>
<td>19.0 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Furosemide (30 μM)</td>
<td>710 ± 138</td>
<td>38.5 ± 5.0**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>762 ± 147</td>
<td>19.2 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Morin (15 μM)</td>
<td>684 ± 113</td>
<td>72.5 ± 6.8***</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>945 ± 20.9</td>
<td>17.6 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>Pravastatin (1000 μM)</td>
<td>1043 ± 130</td>
<td>48.8 ± 13.8*</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>828 ± 81.2</td>
<td>14.8 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Valsartan (15 μM)</td>
<td>637 ± 138</td>
<td>39.7 ± 6.8**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>721 ± 113</td>
<td>13.3 ± 0.95</td>
<td></td>
</tr>
<tr>
<td>Olmesartan (15 μM)</td>
<td>801 ± 114</td>
<td>77.6 ± 12***</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>518 ± 42.1</td>
<td>29.6 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>Telmisartan (0.5 μM)</td>
<td>121 ± 9.1***</td>
<td>37.3 ± 6.9</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>Control</td>
<td>1083 ± 71.7</td>
<td>17.3 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Azilsartan (15 μM)</td>
<td>833 ± 68.2*</td>
<td>27 ± 5.3</td>
<td>Non-competitive</td>
</tr>
</tbody>
</table>
Table 1. The kinetics of PAH transport by OAT1 in the absence versus presence of inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Irbesartan (10 μM)</th>
<th>Mixed-type</th>
<th>Omeprazole (20 μM)</th>
<th>Mixed-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>933 ± 76.9</td>
<td>574 ± 65.8*</td>
<td>31.8 ± 5.3*</td>
<td>698 ± 47.7*</td>
<td>37.0 ± 3.8***</td>
</tr>
<tr>
<td></td>
<td>16.8 ± 2.7</td>
<td>31.8 ± 5.3*</td>
<td>15.5 ± 1.5</td>
<td>37.0 ± 3.8***</td>
<td>Mixed-type</td>
</tr>
</tbody>
</table>

The concentration of inhibitor drug used is indicated in the parentheses. The inhibitor concentrations used were fixed at a concentration expected to inhibit ~50% - 75% of transport activity. The concentrations were based on previously determined inhibitory concentration 50 (IC50) values (Ingraham, et al., 2014), or were determined in preliminary experiments. Data are mean ± standard error of the mean of four to five experiments. Significantly different than control, *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed student’s t-test.
Table 2. The kinetics of 6-carboxyfluorescein transport by OAT1 in the absence versus presence of inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>$J_{max}$ (pmol cm$^{-2} \cdot$ 5 min$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>Inhibition Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.48 ± 0.52</td>
<td>6.05 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>Probenecid (15 μM)</td>
<td>4.13 ± 0.50</td>
<td>25.3 ± 2.9***</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>5.48 ± 0.52</td>
<td>6.05 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>Cidofovir (100 μM)</td>
<td>4.29 ± 0.36</td>
<td>33.3 ± 6.9**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>5.22 ± 0.73</td>
<td>10.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>PAH (20 μM)</td>
<td>5.63 ± 1.3</td>
<td>45.2 ± 3.8***</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>5.22 ± 0.73</td>
<td>10.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen (5 μM)</td>
<td>4.43 ± 0.38</td>
<td>39.8 ± 4.8**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>4.33 ± 0.79</td>
<td>7.58 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (5 μM)</td>
<td>2.54 ± 0.29</td>
<td>14.5 ± 0.90**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>4.33 ± 0.79</td>
<td>7.58 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>Furosemide (30 μM)</td>
<td>3.96 ± 0.41</td>
<td>45.1 ± 7.2**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>4.33 ± 0.79</td>
<td>7.58 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>Valsartan (15 μM)</td>
<td>3.57 ± 0.38</td>
<td>29.4 ± 3.95**</td>
<td>Competitive</td>
</tr>
<tr>
<td>Control</td>
<td>5.37 ± 0.75</td>
<td>6.58 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>Telmisartan (0.5 μM)</td>
<td>0.89 ± 0.26**</td>
<td>9.29 ± 1.5</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>Control</td>
<td>5.22 ± 0.73</td>
<td>10.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Omeprazole (20 μM)</td>
<td>3.16 ± 0.24*</td>
<td>30.1 ± 4.0**</td>
<td>Mixed-type</td>
</tr>
</tbody>
</table>

The concentration of inhibitor drug used is indicated in the parentheses. The inhibitor concentrations used were fixed at a concentration expected to inhibit ~50% - 75% of transport activity. The concentrations were based on previously determined inhibitory concentration 50 (IC$_{50}$) values (Ingraham, et al., 2014), or were determined in preliminary experiments. Data are mean ± standard error of the mean of four experiments. Significantly different than control, *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001, two-tailed student’s t-test.
Figure 1

**A**  
![Graph A](image1)  
IC$_{50} = 2.7$ µM

**B**  
![Graph B](image2)  
IC$_{50} = 2.7$ µM

**C**  
![Graph C](image3)  
IC$_{50} = 0.36$ µM

**D**  
![Graph D](image4)  
IC$_{50} = 0.27$ µM

**E**  
![Graph E](image5)  
IC$_{50} = 10.4$ µM

**F**  
![Graph F](image6)  
IC$_{50} = 10.2$ µM

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Figure 2

A

PAH Uptake (pmol·mg protein⁻¹·min⁻¹)

[PAH], μM

Control
Ibuprofen

B

6-CF Uptake (pmol·cm⁻²·5 min⁻¹)

[6-CF], μM

Control
Telmisartan

C

PAH Uptake (pmol·mg protein⁻¹·min⁻¹)

[PAH], μM

Control
Omeprazole

D

6-CF Uptake (pmol·cm⁻²·5 min⁻¹)

[6-CF], μM

Control
Telmisartan

E

PAH Uptake (pmol·mg protein⁻¹·min⁻¹)

[PAH], μM

Control
Omeprazole

F

6-CF Uptake (pmol·cm⁻²·5 min⁻¹)

[6-CF], μM

Control
Omeprazole
Figure 3

**A**
- PAH Uptake (pmol mg protein⁻¹ min⁻¹)
- J/S
- Control
- Ibuprofen

**B**
- 6-CF Uptake (pmol cm⁻² 5 min⁻¹)
- J/S
- Control
- Ibuprofen

**C**
- PAH Uptake (pmol mg protein⁻¹ min⁻¹)
- J/S
- Control
- Telmisartan

**D**
- 6-CF Uptake (pmol cm⁻² 5 min⁻¹)
- J/S
- Control
- Telmisartan

**E**
- PAH Uptake (pmol mg protein⁻¹ min⁻¹)
- J/S
- Control
- Omeprazole

**F**
- 6-CF Uptake (pmol cm⁻² 5 min⁻¹)
- J/S
- Control
- Omeprazole

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Figure 4

A. Cellular [3H]Telmisartan Accumulation (fmol mg protein⁻¹ min⁻¹)

B. Cellular [3H]PAH Accumulation (fmol mg protein⁻¹)

C. Cellular [3H]Telmisartan Accumulation (fmol mg protein⁻¹) over Time (min)

* 90% inhibition

CHO parental

CHO-OAT1
Figure 5

A

[\textsuperscript{3}H]PAH Uptake (fmol \textperiodcentered mg protein\textsuperscript{-1} \textperiodcentered min\textsuperscript{-1})

Control
Telmisartan
Recovery time after telmisartan washout (min)

0 50 100 150 200 250

*** ****

B

[\textsuperscript{3}H]PAH Uptake (fmol \textperiodcentered mg protein\textsuperscript{-1} \textperiodcentered min\textsuperscript{-1})

Control
Telmisartan
Recovery time after telmisartan washout (min)

0 50 100 150 200 250

***
Figure 7

A  

B  

C  

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