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Organic anion transporting polypeptide 2B1 (OATP2B1), an expanded substrate profile, does it align with OATP2B1's hypothesized function?

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Organic anion transporting polypeptide 2B1 (OATP2B1), an expanded substrate profile, does it align with OATP2B1's hypothesized function?

1. An expanded view of the substrate landscape of organic anion transporting polypeptide (OATP) 2B1 was pursued with the goal of understanding if the identification of novel *in vitro* substrates could shed additional light on the impact of OATP2B1 on intestinal absorption and brain penetration.

2. To examine this hypothesis, a series of experiments measured the cellular accumulation of a diverse array of compounds. Representative angiotensin II receptor blockers (ARBs) and other compounds of interest were subsequently investigated for inhibition, time dependence, and kinetics.

3. The study identified ARBs as a class of OATP2B1 substrates and found balsalazide, olsalzine, and gavestinel to be novel substrates of OATP2B1 too. Some compounds previously reported to be OATP2B1 substrates in the literature, aliskiren, erlotinib, montelukast, fexofenadine, and taurocholate could not be confirmed as substrates.

4. Literature describing *in vivo* outcomes for OATP2B1 substrates, coproporphyrin III, ARBs, balsalazide, olsalzine, and gavestinel highlight the absence of a substantial impact of OATP2B1 on the oral absorption and/or brain penetration of OATP2B1 substrates. Suggestions of including OATP2B1 assessment as part of the drug approval process are likely premature and further mechanistic work with more robust OATP2B1 substrates, which may include some of those described here, is desirable.

Keywords: Organic anion transporting polypeptide, OATP2B1, angiotensin II receptor blocker, coproporphyrin, drug absorption, brain penetration

Introduction

OATP2B1 is a transporter of the organic anion transporting polypeptide (OATP) family that shows broad mRNA tissue distribution (Tamai, et al., 2000). However, the precise membrane localization of OAT2B1 protein has not achieved consensus in most cases. Furthermore, OATP2B1 has been suggested to demonstrate broad substrate specificity that in some cases may be unique relative to the other primary liver OATPs, OATP1B1 and OATP1B3 (McFeely, et al., 2019). The broad tissue distribution of OATP2B1 and the substrate poly-specificity of the transporter have led to much speculation surrounding its role in the liver uptake clearance of compounds, the absorption of drugs, the distribution of drugs to various tissues such as retina, lung and heart, and the blood-brain barrier (BBB) penetration of drugs. The impact of OATP2B1 on the oral absorption of drugs is of interest because the transporter may facilitate the absorption of poorly permeable compounds achieving a bioavailability suitable for therapy. The interest has fuelled a number of studies despite a lack of consensus regarding the intestinal OATP2B1 localization (Keiser, et al., 2017; Sai, et al., 2006). OATP2B1-mediated blood-brain barrier (BBB) penetration of drug is of interest for similar reasons, where it could facilitate the central nervous system entry of compounds that would normally not be brain penetrant. While there has been much speculation in this area, there is little data to support OATP2B1-mediated blood-brain barrier (BBB) penetration of drugs.

The pharmacokinetic impact of OATP2B1 has largely been derived from drug-drug and food-drug interactions studies (McFeely, et al., 2019). However, food/fruit juice interactions due to their “infinite” number of components are ill-defined, and the interaction may not be limited to actual drug interactions, but may reflect osmolality effects as recently demonstrated for the previously reported OATP2B1 substrate

atenolol (Funai, et al., 2019). Additionally, many of the intestinal drug interactions have demonstrated a modest effect (e.g. <2-fold; McFeely, et al., 2019) and frequently the liver drug interactions are not limited to OATP2B1 (e.g. Ely, et al., 2015a, Ely, et al., 2015b).

The functional evidence for intestinal OATP2B1-mediated uptake is limited and the magnitude of its impact on oral absorption is not well described in the literature. Most of the *in vivo* studies investigating OATP2B1-mediated intestinal uptake have been fruit juice interaction studies that are largely undefined in terms of what the perpetrating inhibitor actually is and if the interaction limiting drug absorption is limited to inhibition of OATP2B1. Moreover, described OATP2B1 substrates frequently have limited *in vitro* data and furthermore often demonstrate modest differences between transporter and control conditions (e.g. Bauer, et al., 2018; Mougey, et al., 2009). Additionally, a recent report shows no effect of knocking out mouse OATP2B1 (mOatp2b1) in mice on rosuvastatin pharmacokinetics, despite robust *in vitro* mOatp2b1-mediated transport of the compound (Medwid, et al., 2019). By contrast, the same study demonstrated a statistically significant pharmacokinetic absorptive impact of mOatp2b1 on what appeared to be a less-well transported compound *in vitro*, fexofenadine. The *in vitro – in vivo* discontinuity between the rosuvastatin and fexofenadine was attributed to "yet-to-be-determined, non-OATP transporters" of rosuvastatin, but the gap in understanding is concerning.

Here it was elected to take an expanded view of the substrate landscape of OATP2B1 to examine if the larger perspective could yield more insight into the absorption and distribution function of OATP2B1. The goal was to understand if *in vitro* data for additional novel substrates could shed further light on the impact of OATP2B1 in intestinal absorption and brain penetration when leveraging

existing published *in vivo* data for the broader set of substrates relative to previously reported substrates. Assessment of previously reported substrates provides context to newly identified substrates in the same experimental system creating value and enabling direct comparison not easily done between various publications and experimental systems. The work may provide additional tool compounds and insight into whether or not OATP2B1-mediated transport represents an adequate or common mechanism for the intestinal uptake and brain penetration of drugs. Additional drugs tested as OATP2B1 substrates were identified from reported inhibition data (Karlgren, et al., 2012; Unger, et al., 2020) that aligned with structure activity relationships of transported molecules (e.g. acidic molecules were favoured) and had potential for expansion within a therapeutic category (e.g. angiotensin II receptor blockers).

Materials and Methods

Chemicals

Coproporphryins I and III hydrochloride salts were purchased from Sigma-Aldrich (St. Louis, MO) and Frontier Scientific (Logan, UT), respectively. Atorvastatin, balsalazide, and montelukast were purchased from Tokyo Chemical Industry Co. (TCI America, Portland, OR); candesartan was purchased from Alfa Aesar (Haverhill, MA); pemetrexed was purchased from BioVision (Milpitas, CA); and irbesartan and losartan were purchased from Selleckchem (Houston, TX). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or were acquired through the Novartis compound hub at >95% purity. Cell culture media, buffer solutions, and supplements were from Corning (Tewksbury, MA). Chromatography reagents were obtained from JT Baker (Radnor, PA).

Cell culture

HEK cells (HEK-vector and HEK-OATP2B1) were maintained at 37°C in a humidified atmosphere with 5% CO_2 in DMEM (high glucose, 4.5 g/L; Corning Life Sciences, Acton, MA), supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin solution (100 μg/mL), alanyl-glutamine (2 mM), non-essential amino acids (1%), and 1mg/mL G418 (all supplements Corning Life Sciences). Cells were passaged twice per week at a density of 2×10^6 cells into an Omnitray (Nunc, Thermo Fisher Scientific, Rochester, NY)

Transport experiments

HEK cells were seeded at a density of approximately 100,000 cells/well into 96-well tissue culture plates coated with poly-d-lysine (Greiner Bio-One, Austria). Transport experiments were conducted approximately 48 hours post seeding.

Transport experiments, chromatography/mass spectroscopy, and data analysis were conducted in a manner similar to that previously described (Bednarczyk and Boiselle, 2016). The experiments were initiated by aspirating media and washing the cells three times with Hank's Balanced Salt Solution (HBSS; Corning Life Sciences) +10 mM HEPES (pH 7.4). The cells were maintained in the third HBSS wash solution for 15 minutes to equilibrate with the buffer; inhibitor was not present during this period. Following the 15-minute equilibration period, the third wash solution was aspirated and the experiment was initiated by the administration of a compound solution, or a compound solution containing inhibitor. Compound concentrations used are noted in the respective figure legends or corresponding text. The cells were incubated in the substrate solution for 10 minutes for preliminary cellular accumulation experiments, up to 30 minutes for the time course assessment, and 3 minutes for kinetic analysis.

Cellular accumulation experiments were terminated by aspirating the substrate solution and washing with HBSS + 10 mM HEPES (pH 7.4) three times. For mass spectrometer analysis, the cells were extracted in a 50:50 solution of methanol/water containing 250 nM internal standard (glibenclamide) for 10 minutes while shaking at approximately 200 rpm. The extracts were then transferred to a 96-well plate and centrifuged for 10 minutes at 4000g at 4ºC. The supernatants were transferred to a clean 384-well plate for LC/MS/MS analysis (see below).

Compound concentrations were determined from standard curves prepared in matrix. Total protein determinations were made using a bicinchoninic acid (BCA) assay kit with bovine serum albumin as the standard (Pierce Biotechnology, Rockford, lL).

Mass spectroscopy/chromatography

For most compounds the extracted samples were loaded onto a RapidFire C4 cartridge by means of a RapidFire autosampler (Agilent, Santa Clara, CA). Chromatography was performed at a flow rate of 1.25 mL/min, loading with 0.1% formic acid in water and eluting in 0.1% formic acid in methanol. Mass spectroscopy was performed using an AB Sciex API5000 (Sciex, Framingham, MA) equipped with a turbo ion spray source. Compound concentration was calculated from the chromatographic peak area ratio of analyte to internal standard (glibenclamide), using Multiquant software V3.0 (Sciex, Framingham, MA).

For compounds where poor retention or poor signal using the RapidFire instrumentation was observed, extracted samples (containing azilsartan, balsalzide, eprosartan, estradiol-3-sulfate, or estrone-3-sulfate) were loaded onto a Phenomenex Kinetex XB-C18 column (30×2 mm, 5 µm; Torrance, CA) by means of a Leap autosampler (Carrboro,

NC). Chromotography was also utilized for inhibition experiment samples. Chromatography was performed at a flow rate of 0.9 mL/min, using a biphasic gradient: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Chromatography was executed using the following gradient profile: 0-0.5 min, 5% B; 0.5-1.9 min, linear gradient to 100% B; 1.9-2.0 min, 100% B; 2.0-2.1 min, linear gradient to 5% B; and 2.1-2.2 min 5% B. Mass spectroscopy was performed using the AB Sciex API5000 noted above.

Data analysis

Cellular accumulation of the transporter transfectant was compared to the corresponding vector control. OATP2B1-mediated transport was determined by subtraction of the substrate accumulation in cells expressing vector from the substrate accumulation in cells expressing OATP2B1 (e.g. HEK-OATP2B1 – HEK-Vector = OATP2B1-mediated transport). Error of the OATP2B1-mediated transport was determined by means of propagation of errors where HEK-OATP2B1 and HEK-Vector have independent random error ($\delta z^2 = \delta x^2 + \delta y^2$). Michaelis–Menten kinetic parameters, K_m and V_{max}, were determined by nonlinear regression (GraphPad Prism) of the OATP2B1-mediated transport fit to the following equation: $V = (V_{max} \times [S])/(K_m + [S])$, where V is the measured rate of cellular accumulation, [S] is the substrate concentration, V_{max} and K_m are the maximal rate of transport, and substrate concentration at the half-maximal rate, respectively.

For substrate determination, the difference in cellular accumulation between a vector control and the corresponding transporter transfectant was tested for significance using an unpaired two-tailed t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Compound accumulation in HEK-Vector and HEK-OATP2B1 cells

The compound accumulation of a series of compounds previously tested against OATP2B1 were initially repeated here (figure 1A-B, table 1). Repeating previously reported substrates provides context to the transport of newly identified substrates. That is, a comparison of transport of a newly identified substrate to multiple established substrates can be made in the same experimental system to provide a relative rank order of activity, which is challenging to do between multiple labratories. Additionally, the exercise can further validate findings of others which has value to the field as a whole. Compounds classified as "Selective for OATP2B1", aliskiren, erlotinib, and pemetrexed (McFeely, et al., 2019) demonstrated no meaningful OATP2B1-mediated transport (figure 1B). The statins, atorvastatin, fluvastatin, pitavastatin, and rosuvastatin were mixed, where atorvastatin and rosuvastatin demonstrated a significant difference between the HEK-OATP2B1 and HEK-Vector cells, but the more highly permeable statins, fluvastatin and pitavastatin, were more variable and not significant (figure 1A, table 1). The coporpopophyrins both achieved statistical significance, however, the asymmetry previously reported (Bednarczyk and Boiselle, 2016) was strongly maintained here. The steroid sulfates, estradiol-3-sulfate and estrone-3-sulfate, being structurally similar demonstrated similarly strong OATP2B1-mediated uptake (figure 1B). The remaining compounds, fexofenadine, montelukast, and taurocholate did not demonstrate a significant difference between the HEK-OATP2B1 and HEK-Vector cells illustrating those molecules are unlikely to be OATP2B1 substrates (table 1). Sulfasalzine, had a p-value of 0.0502, just missing the cut-off of statistical significance, but did demonstrate at least a 3-fold difference between the HEK-OATP2B1 and HEK-Vector cells in each individual experiment indicating that it was likely a substrate of

OATP2B1.

Figure 2A-B illustrates the cellular accumulation of angiotensin II receptor blockers (ARBs) in HEK-OATP2B1 and HEK-Vector cells (table 1). The data clearly exhibit that all of the ARBs tested here demonstrate significantly greater accumulation in the HEK-OATP2B1 cells relative to the HEK-Vector cells (figure 2A) with azilsartan and losartan demonstrating the greatest OATP2B1-mediated transport and eprosartan and valsartan demonstrating the least (figure 2B).

Other novel compounds tested as potential OATP2B1 substrates are shown in figure 3A-B (table 1). Atatzanavir, ecabet, rapaglinide, and susalimod failed to achieve a significant degree of OATP2B1-mediated transport (figure 3B, table 1). By contrast, the OATP2B1-mediated transport of balsalzide, olsalazine, and gavestinel was robust and significant (figure 3B, table 1).

Inhibition

The ARBs, balsalazide, and gavestinel were further tested for inhibition of OATP2B1-mediated transport. All compounds displayed reduced cellular accumulation in the OATP2B1 cells in the presence of 30 μM bromosulfophthalein (BSP, figure 4). Most of the ARBs and balsalzide demonstrated inhibition of >85%. Two compounds that were comparatively more lipophilic and had among the highest transport rates, irbesartan and gavestinel, showed less inhibition relative to the other compounds.

Time course

Four representative compounds, two highly transported and two moderately transported, from the set of novel compounds positive for OATP2B1-mediated transport were further characterized in a time course assay and a kinetic assays (below).

Copropophyrin III, a previously characterized OATP2B1 substrate was included in the time course assessment. The time course data for azilsartan, gavestinel, valsartan, balsalzide, and coproporphyrin III all demonstrated divergence between the HEK-OATP2B1 and HEK-Vector cells with time (figure 5A-E). The HEK-OATP2B1 time course data for gavestinel exceeded the upper limit of quantification at the 15 and 30 minute time points. The data for the five compounds showed time linearity out to at least five minutes. A three-minute time point was selected for further kinetic studies.

Kinetics

The novel compounds previously subjected to the time course were further characterized in a kinetic assay. The kinetic data for azilsartan, gavestinel, valsartan, and balsalzide, each demonstrated saturable transport (figure 6A-D, table 2). Azilsartan and gavestinel displayed the highest affinity, 5.9 and 9.1 µM, and highest transport efficiency (V_{max}/K_m) mediated by OATP2B1, 91 and 87 µL/min/mg protein, respectively (table 2). Valsartan and balsalazide were lower in each respective comparison, K_m of 24.5 and 15.5 µM, and transport efficiency of 33 and 18 µL/min/mg protein (table 2).

Conclusions

By taking a comprehensive view of the substrate landscape in a single study that includes measurements of reported substrates compared and contrasted with novel substrates, a more logical comparison of compounds can be made. Additionally, the extended substrate identification presented here enables leveraging additional *in vivo* data from the literature, including literature where OATP2B1 substrates were administered without the explicit testing of an OATP2B1-based hypothesis surrounding absorption, brain penetration, etc.

OATP2B1-mediated transport of coproporphyrins I and III was previously demonstrated to be asymmetric, where transport of coproporphyrin III was strongly favoured as a substrate over coproporphyrin I by OATP2B1 (Bednarczyk and Boiselle, 2016, Shen, et al., 2017). The previous reports did not establish coproporphyrin I as an OATP2B1 substrate. However, the transport window here was much larger than previously reported and the OATP2B1-mediated transport asymmetry between coproporphyrin I and III was greater than 85-fold, further illustrating copropophyrin III is strongly favoured by OATP2B1 (figure 1, table 1). Coproporphyrin III as an established and robust OATP2B1 substrate would be anticipated to exhibit oral absorption, if OATP2B1 facilitated the intestinal absorption of compounds. The oral absorption of coproporphyrin III has previously been tested in canines and humans (Larson and Watson, 1949). The findings however did not support a transporter-mediated intestinal uptake of coproporphyrin III. The authors concluded that there was no evidence in dogs or humans of absorption of coproporphyrin III from the intestine. The lack of coproporphyrin III uptake in the gut is contrary to OATP2B1 facilitating intestinal absorption of substrate. Brain exposure of coproporphyrin III has also been investigated in dogs and rabbits (Chu and Watson, 1947; Watson and Larson, 1947). Similar to the lack of gut absorption of copropophyrin III, brain penetration of copropophyrin III was not observed when systemic concentrations were elevated through poisoning or arterial injection. The data for copropophyrin III suggests that OATP2B1 may not mediate blood-brain barrier penetration of substrates. Copropophyrin III appears to not only demonstrate a lack of oral bioavailability, but does not appear to show increased brain exposure when plasma concentrations are increased, despite being a robust OATP2B1 substrate; the existing *in vitro* and *in vivo* data does not appear to be aligned with OATP2B1 facilitating the oral absorption or

blood-brain barrier penetration of copropophyrin III.

Balsalzide and olsalazine are anti-inflammatory drugs used for the treatment of ulcerative colitis. Balsalzide is minimally absorbed and olsalzine absorption was approximately 2% (Prakash A and Spencer, 1998; Ryde and Ahnfelt, 1988) despite both demonstrating robust cellular accumulation in HEK-OATP2B1 cells relative to HEK-Vector cells at levels similar to or exceeding the established OATP2B1 substrate rosuvastatin (figure 3, 5, 6; table 1). Olsalzine also shows a non-linear increase in absorption with increased dose which is inconsistent with saturation of an uptake mechanism (Ryde and Ahnfelt, 1988) and more consistent with saturation of an efflux mechanism. The oral pharmacokinetics of balsalzide and olsalzine are not aligned with substantial OATP2B1-mediated transport from intestinal lumen to blood.

Gavestinel, an N-methyl-D-aspartate receptor antagonist once developed, and subsequently stopped, for the treatment of acute intracerebral haemorrhage, was among the best OATP2B1 substrates identified in this study (figure 3 versus 1, 2; figure 6; table 1). However, despite its robust OATP2B1-mediated transport, gavestinal appears to show little or no functional brain penetration. There was no reduction observed in infarct area when rodents were administered gavestinel, nor were there observed effects on motor coordination at gavestinel doses of up to 300 mg/kg (Dawson, et al., 2001). The lack of effect on motor coordination diverges from other N-methyl-D-aspartate receptor antagonists, where adverse motor coordination is an established adverse effect of the class. Similarly, human data has led to the brain penetration of gavestinel to be questioned (Lees, 1997). Related to that described for coproporphyrin III above, gavestinel, despite substantial OATP2B1-mediated transport, does not appear to achieve meaningful blood-brain barrier penetration as judged by the available literature.

ARB brain penetration has been documented, and as a class the ARBs elicit weak, minimal, or poor brain penetration, telmisartan excepted (Michel, et al., 2013). Similarly, as a class, the ARBs also appear to be quite good OATP2B1 substrates (figures 2, 5, 6; table 1). If OATP2B1 did mediate uptake into the brain, better brain exposure of these substrates may be anticipated. However, their poor or lack of brain penetration is not consistent with OATP2B1 mediating a substantial degree of ARB uptake into the brain. Additionally, the lack of ARB brain penetration is unlikely due to saturation of the transporter due to their low free concentrations in plasma.

Pemetrexed was statistically not found to be a substrate at pH 7.4 in this study (table 1), which was aligned with observations at pH 7.4 in a previous study (Visentin, et al., 2012). However, Visentin et al., did observe that pemetrexed was a substrate at pH 5.5 (Visentin, et al., 2012). The notable difference between the two studies was that pemetrexed experiments were carried out at pH 7.4 here versus pH 5.5 by Visentin, et al. (2012). The pH 5.5 condition was required for Visentin, et al. due to a lack of observable pemetrexed transport at pH 7.4. However, their control substrate, bromosulfophthalein, was not found to demonstrate pH dependent transport, and did show OATP2B1-mediated transport at pH 7.4. Moreover, the transport of pemetrexed at pH 5.5 in the Visentin, et al. (2012) study was less than 3% of the bromosulfophthalein transport at the same pH, indicating that pemetrexed, at best, only shows a modest amount of OATP2B1-mediated transport that may be difficult to detect in the presence of folate transporters that also contribute to its cellular flux. Furthermore, the cell line used to assess transport of pemetrexed lacked the reduced folate carrier and did not express the proton-coupled folate transporter which may have enabled a lower background control signal that that observed here, but also raises an issue of pemetrexed as an OATP2B1-specific substrate (McFeely, et al., 2019). Here it

was felt that the pH 7.4 condition, rather than pH 5.5 only, was more broadly applicable to translation to both OATP2B1-mediated absorption and distribution of compound. A similar result was also observed for taurocholate where at pH 7.4 the OATP2B1 transfectant accumulation was only ~9% greater than the corresponding control, but at pH 5.0 the difference was ~32% (Nozawa, et al., 2004). However, a 1.32-fold difference is well below the 2-fold threshold for substrate determination suggested by the International Transporter Consortium (Brouwer, et al., 2013).

Montelukast and erlotinib were not identified as substrates, as the difference between the HEK-OATP2B1 and HEK-Vector cells was not different (figure 1; table 1). The published literature regarding OATP2B1-mediated transport of montelukast varies, where a lack of OATP2B1-mediated montelukast uptake has been reported (Chu, et al., 2012; Brännström, et al., 2015), as has OATP2B1-mediated transport of montelukast (Mougey, et al., 2009; Varma, et al., 2017). The Varma, et al, (2017) *in vitro* data demonstrates inconsistencies between concentrations where there is significant OATP2B1-mediated transport at some concentrations and time points, but not others. Additionally, the time course in the presence of inhibitor is flat with respect to time (i.e. net difference between transport and control does not diverge with time). The Mougey, et al. (2009) data are not straightforward to interpret as OATP2B1-mediated cellular accumulation was not measured, and the authors relied on permeability measurements across monolayers. The published *in vitro* work of Brännström, et al., (2015) is probably the most robust study of OATP2B1-mediated transport to date and they concluded that OATP2B1 does not meaningfully transport montelukast; the data presented here are aligned with the Brännström, et al. work. *In vitro* OATP2B1-mediated transport of erlotinib is less well characterized in the literature and the reported magnitude of transport appears to be small (~20% greater than vector, Bauer,

et al., 2018). Furthermore, the passive cellular accumulation of erlotinib is approximately 4-fold greater than the reported OATP2B1-mediated uptake *in vitro* (Bauer, et al., 2018). The modest degree of reported transport to passive accumulation represents a difference that may be difficult to pick up in repeated *in vitro* studies, may be within differences observed between clonal cell lines, and is inconsistent with recommended substrate threshold criteria (Brouwer, et al., 2013). Moreover, due to the absence of a control compound in the Bauer, et al. (2018) work, it is challenging to assess the relative transport of erlotinib or the OATP function of A431 cell lines used. Based on the high passive accumulation observed here for erlotinib relative to other compounds (figure 1), the impact of OATP2B1 on the oral absorption erlotinib would be anticipated to be negligible, if detectable. Furthermore, the absence of a statistical difference between the HEK-OATP2B1 and HEK-Vector cells across repeated assays here supports the conclusion that erlotinib is not a meaningful substrate of OATP2B1.

Fexofenadine is another compound with mixed literature results, where it has been identified as a substrate (Shirasaka, et al., 2014, Medwid, et al., 2019), but was originally shown to be negative with respect to OATP2B1-mediated transport (Shimizu, et al., 2005; Glaeser, et al., 2007). Even within groups the OATP2B1-mediated transport of fexofenadine appear to be mixed, where the same group has produced contradictory *in vitro* findings of no OATP2B1-mediated transport of fexofenadine (Glaeser, et al., 2007) and a modest degree of *in vitro* mOatp2b1 transport (Medwid, et al., 2019). The findings here are more closely associated with the original findings that fexofenadine is not a substrate of OATP2B1.

The *in vitro* support for OATP2B1-mediated aliskiren uptake is comprised of a single kinetic experiment without a corresponding control condition (e.g. HEK-OATP2B1 without HEK-Vector, Vaidyanathan, et al., 2008). The finding of OATP2B1-mediated

transport of aliskiren was unable to be confirmed when control cells were made part of the experimental design in a subsequent publication (Rebello, et al., 2012). The findings here are aligned with Rebello, et al (2012) where aliskiren was not found to be a substrate of OATP2B1 across repeated experiments. Of additional note is a publication investigating nonsynonymous single nucleotide polymorphisms of OATP2B1 where the genotypes investigated demonstrated no effect on the pharmacokinetics of montelukast or aliskiren (Tapaninen, et al., 2013). The findings may be due to an absence of a single nucleotide polymorphism effect on the respective compounds, but the findings are also aligned with an absence of OATP2B1-mediated transport of either compound (i.e. neither are substrates). Further assessment of nonsynomymous single nucleotide polymorphisms associated with OATP2B1 may be of interest, but utilization of more robust OATP2B1 probes would be desirable.

To a large extent, the *in vitro* findings here, in the context reported *in vivo* literature, do not support a major role of OATP2B1 in either the oral absorption of drugs or penetration of the blood-brain barrier. The observed absence, or perhaps modest impact, of OATP2B1 on oral drug absorption may be due to the presence of efflux transporters localized to the apical membrane of the intestine and blood-brain barrier that simply outcompete OATP2B1 transport into the respective tissues. Another possibility of the lack of a substantial effect of OATP2B1 may be due to the absence of a complementary transporter on the opposing membrane to complete the transepithelial translocation of compound. For example, drug may able to enter cell across the apical membrane via OATP2B1, but there is an absence of a corresponding transporter with overlapping structural recognition to translocate it across the basolateral membrane into the blood or brain. Both scenarios would be consistent with expression of OATP2B1 in the respective tissues with no or only modest functional outcome. A further possibility

would be that OATP2B1 does not function to facilitate uptake from intestinal lumen to blood or from blood to brain, but perhaps is functionally oriented to facilitate transport from the respective tissues as may be the case for OATP2B1 at the blood-brain barrier, where it has been reported to be localized abluminally (basal, Roberts, et al., 2008) in rats. Similar outcomes have been reported for intestinal localization (Keiser, et al., 2017). The findings presented here are also aligned with recent publication suggesting that OATP2B1 is uncommonly the predominate determinant of drug disposition (Unger, et al., 2020). The conclusion is further supported by the commonly used ARBs that do not appear to have routine OATP2B1-related issues and that the role of OATP2B1 in the absorption and disposition of drugs to date may have been overestimated. Alternatively, high intestinal concentrations of drug may easily saturate intestinal OATP2B1 minimizing its observable impact on the oral absorption of a given compound.

In summary, the study identified ARBs as a class of OATP2B1 substrates and also found balsalazide, olsalzine, and gavestinel to be novel substrates of OATP2B1. Some compounds previously reported to be OATP2B1 substrates in the literature, including aliskiren, erlotinib, and montelukast, could not be confirmed and the *in vitro* results observed were simply too small to suggest that a meaningful *in vivo* OATP2B1-mediated transport effect could be achieved. *In vivo* literature describing the robust *in vitro* OATP2B1 substrates, coproporphyrin III, ARBs, balsalazide, olsalzine, and gavestinel highlight the absence of a substantial impact of OATP2B1 on the oral absorption and/or brain penetration of these OATP2B1 substrates. The *in vivo* studies with these substrates frequently pre-date the discovery and characterization of OATP2B1 and the conclusions surrounding the oral absorption or brain penetration were thus not subject to confirming or refuting an *in vitro* finding. Correspondingly,

suggestions of including OATP2B1 as part of the drug approval process (McFeely, et al., 2019) are likely premature and further mechanistic work with more robust OATP2B1 substrates, which may include some of those described here, is desirable.

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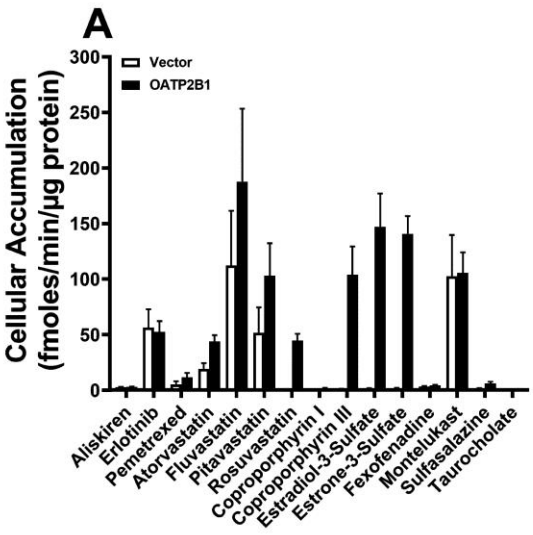

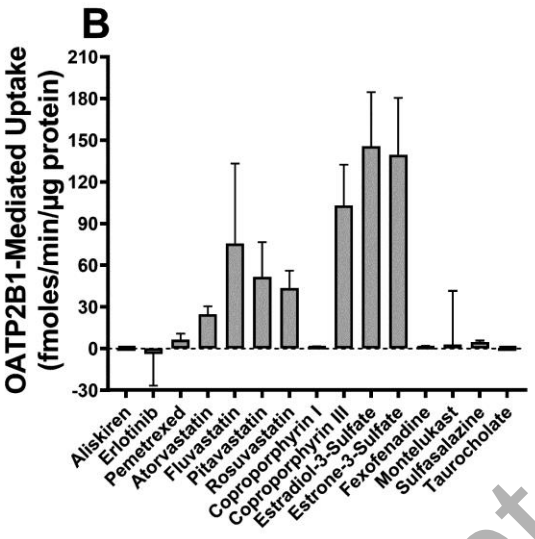

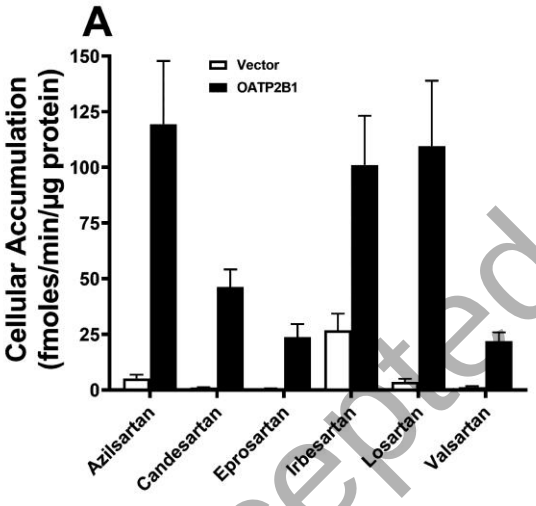

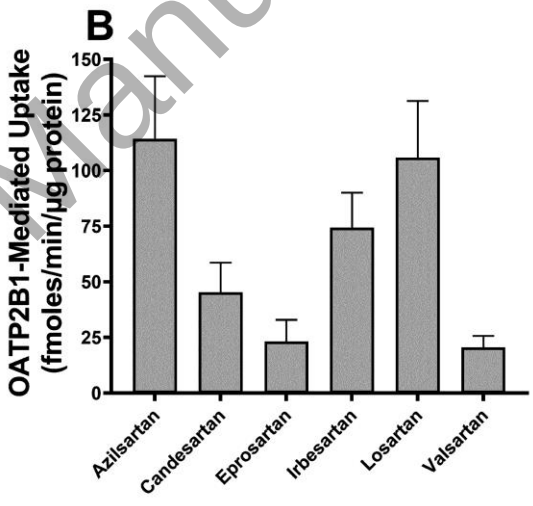

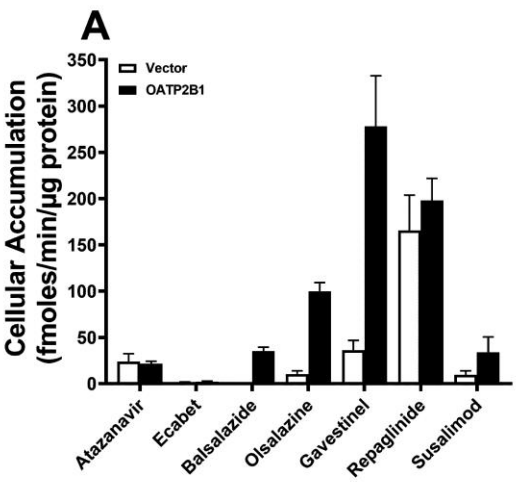

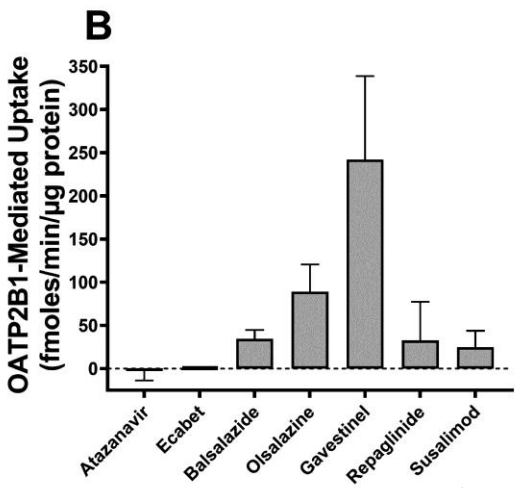

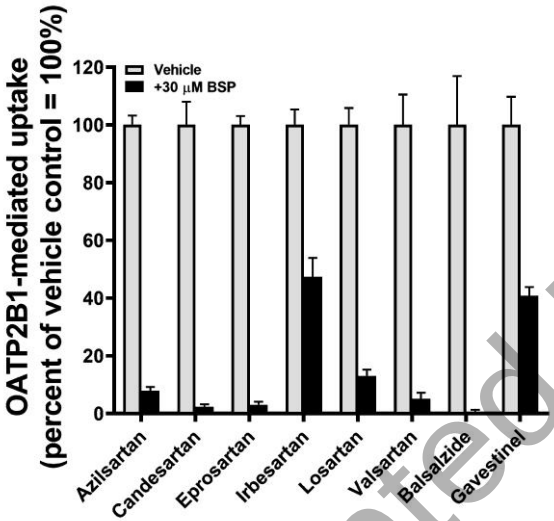

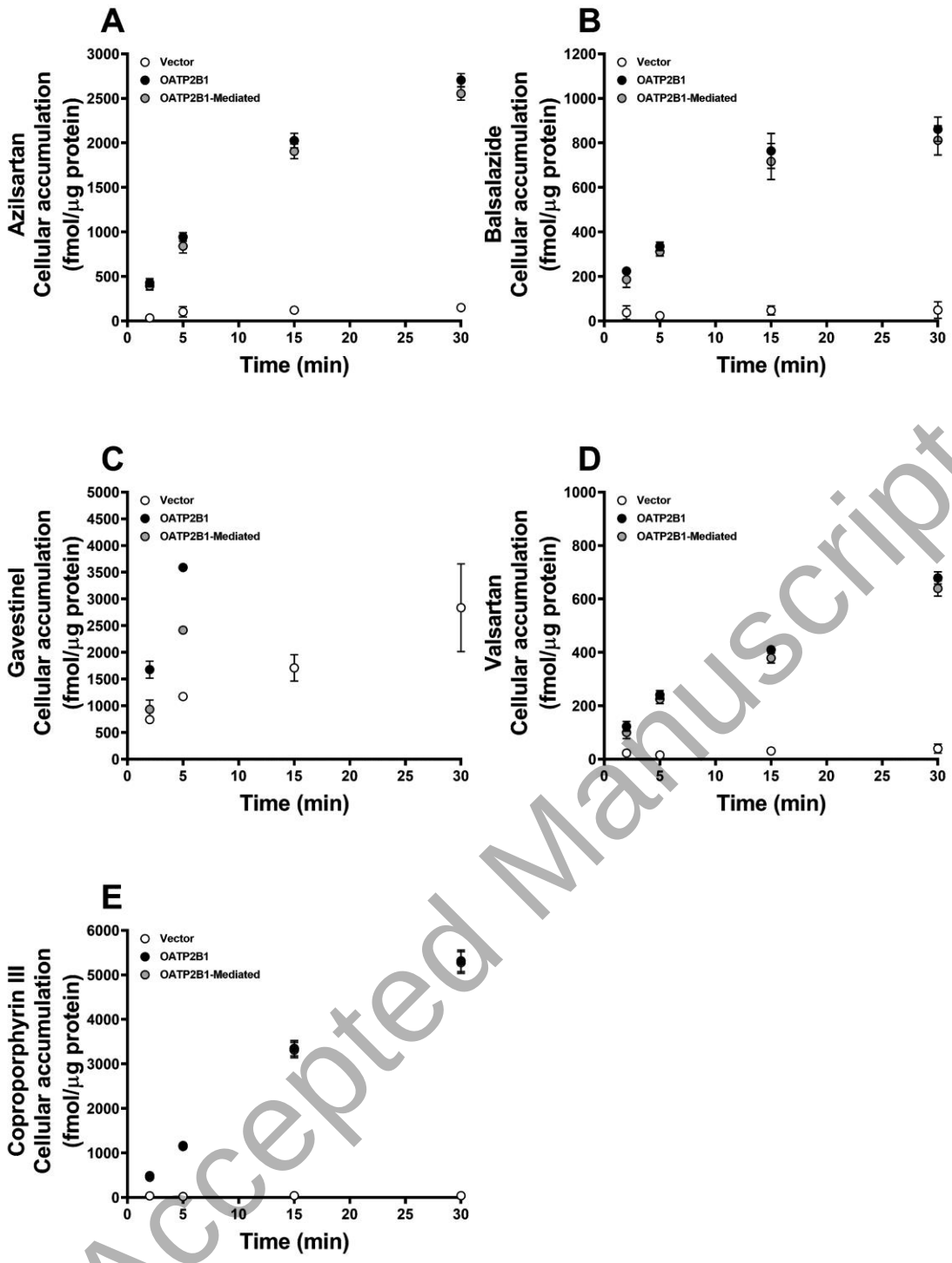

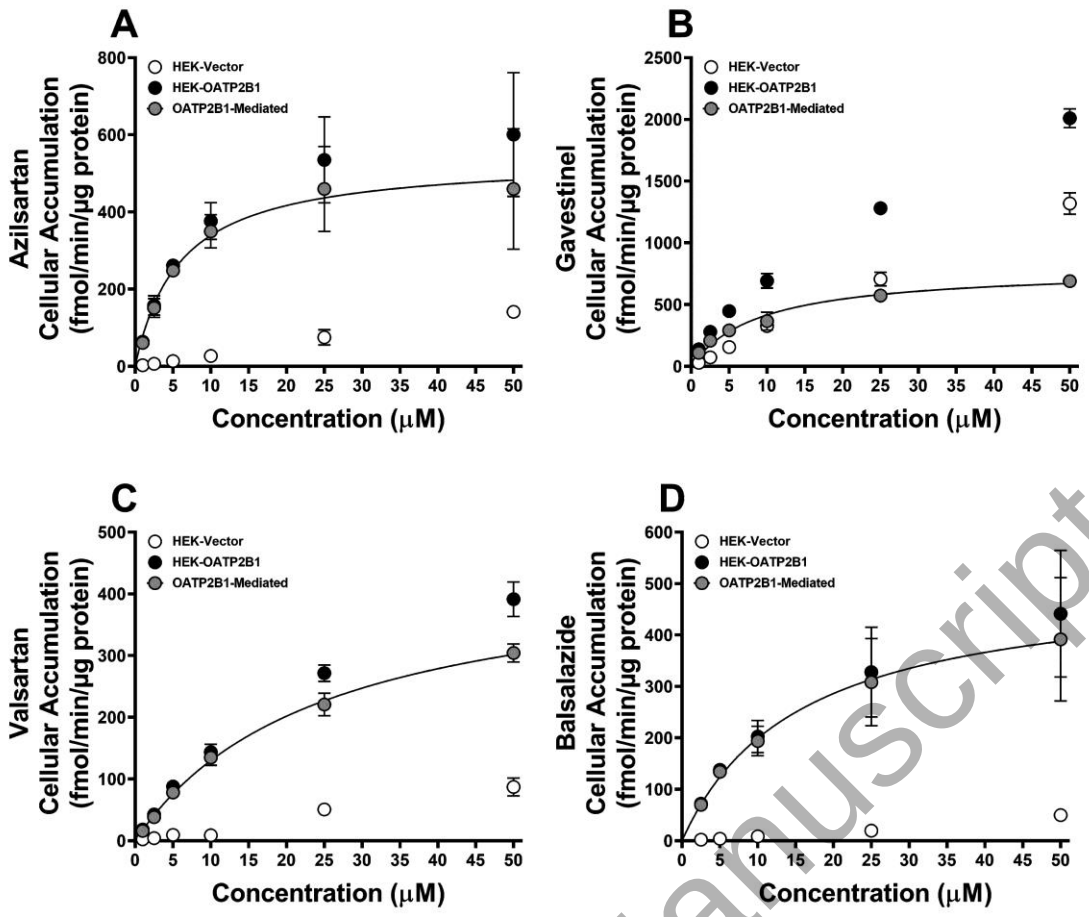

Table 1. Cellular accumulation of compounds in HEK-Vector and HEK-OATP2B1 cells. The net, or OATP2B1-mediated uptake (OATP2B1 – Vector), and the ratio (OATP2B1/Vector) are noted in the far right columns. Data represented is the mean and standard error of the means from three independent experiments (n = 3), each conducted in quadruplicate. * indicates p-value <0.05.

Compound	HEK-Vector (fmol/min/µg protein)		HEK-OATP2B1 (fmol/min/µg protein)		p-value	OATP2B1-Mediated (Net)		Ratio (OATP2B1/Vector)
	Mean	SEM	Mean	SEM		Mean	SEM	
Aliskiren	2.7	0.4	2.9	0.3	0.693	0.2	0.5	1.1
Atazanavir	23.8	8.5	21.2	7.4	0.792	-2.5	11.3	0.9
Atorvastatin	19.2	5.0	43.8	2.9	0.032*	24.6	5.8	2.3
Azilsartan	5.2	1.8	119.4	28.1	0.016*	114.2	28.2	23.1
Balsalazide	0.7	0.0	35.0	10.5	0.009*	34.3	10.5	50.0
Candesartan	1.1	0.2	46.3	13.4	0.004*	45.2	13.4	42.1
Coproporphyrin I	0.5	0.2	1.7	0.2	0.040*	1.2	0.3	3.3
Coproporphyrin III	1.0	0.5	103.9	29.4	0.015*	102.9	29.4	103.9
Ecabet	1.4	0.5	2.2	0.3	0.292	0.8	0.5	1.6
Eprosartan	0.7	0.1	23.8	9.8	0.017*	23.1	9.8	35.7
Erlotinib	56.3	16.7	52.4	15.7	0.851	-3.9	22.9	0.9
Estradiol-3-Sulfate	1.6	0.4	147.1	39.0	0.008*	145.5	39.0	93.9
Estrone-3-Sulfate	1.4	0.7	140.8	41.0	0.001*	139.4	41.0	98.3
Fexofenadine	3.2	0.7	4.4	0.4	0.216	1.2	0.8	1.4
Fluvastatin	112.1	49.3	187.5	30.2	0.411	75.4	57.8	1.7
Gavestinel	36.0	10.7	278.0	96.1	0.012*	242.0	96.7	7.7
Irbesartan	26.7	7.6	101.1	13.7	0.034*	74.3	15.7	3.8
Losartan	3.7	1.4	109.5	25.5	0.023*	105.8	25.5	29.9
Montelukast	102.5	37.3	105.3	10.4	0.949	2.9	38.7	1.0
Olsalazine	10.3	3.5	99.5	31.2	0.006*	89.2	31.4	9.7
Pemetrexed	5.2	2.8	11.6	3.4	0.263	6.4	4.4	2.2
Pitavastatin	51.6	23.0	103.1	9.9	0.237	51.5	25.1	2.0
Repaglinide	165.3	38.5	197.8	22.6	0.513	32.6	44.6	1.2
Rosuvastatin	1.0	0.2	44.6	12.4	0.002*	43.6	12.4	46.1
Sulfasalazine	1.5	0.5	6.1	0.8	0.050	4.7	1.0	4.2
Susalimod	9.3	4.5	34.0	18.7	0.220	24.7	19.2	3.7
Taurocholate	0.8	0.3	1.0	0.3	0.696	0.2	0.4	1.2
Valsartan	1.3	0.5	21.8	5.2	0.008*	20.5	5.2	16.8

Table 2. The kinetics of OATP2B1-mediated uptake. Affinity (K_m), maximal velocity (V_{max}), and transport efficiency (V_{max}/K_m) are reported. Data represented is the mean and standard error of the means from three independent experiments (n = 3), each conducted in duplicate.

Compound	K_m (µM)		V_{max} (fmol/min/µg protein)		V_{max}/K_m (µL/min/mg protein)
	Mean	SEM	Mean	SEM	
Azilsartan	5.9	3.0	539.8	83.7	91.3
Balsalzide	15.5	9.9	507.9	131.1	32.8
Gavestinel	9.1	1.9	792.1	58.1	87.3
Valsartan	24.7	4.0	450.7	34.0	18.3