Significance of organic anion transporter 2 and organic cation transporter 2 in creatinine clearance: Mechanistic evaluation using freshly-prepared human primary renal proximal tubule cells

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Running title: Transporter-mediated uptake in creatinine renal clearance

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ABBREVIATIONS: CrCL, creatinine clearance; DDI, drug-drug interactions; eGFR, estimated glomerular filtration rate; f, fraction transported; HEK, human embryonic kidney; hPTCs, human primary renal proximal tubule epithelial cells; IC₅₀, inhibition potency; Km, substrate affinity constant; MATE, multidrug & toxin extrusion pump; OAT, organic anion transporter; OCT, organic cation transporter; sCr, serum creatinine; Vmax, maximum transport velocity.
ABSTRACT

Creatinine, a clinical marker for kidney function, is predominantly cleared by glomerular filtration, with active tubular secretion contributing to about 30% of its renal clearance. Recent studies suggested the potential involvement of organic anion transporter (OAT)2, in addition to the previously known organic cation transporter (OCT)2-mediated basolateral uptake, in creatinine active secretion. Here, we characterized the transport mechanisms of creatinine using transfected human embryonic kidney (HEK)293 cells and freshly prepared human primary renal proximal tubule epithelial cells (hPTCs). Creatinine showed transport by OAT2 in transfected HEK293 cells. In addition, both creatinine and metformin showed transport by OCT2 and multidrug & toxin extrusion pump (MATE)1 and MATE2K, while penciclovir was selective for OAT2. Time-dependent cell accumulation was observed for creatinine and metformin in hPTCs. Their accumulation was increased by pyrimethamine, but inhibited by decynium-22, likely due to differential inhibition of OCT2 versus MATEs. Additionally, indomethacin (an OAT2 inhibitor) reduced penciclovir uptake (~75%) in hPTCs illustrating functional OAT2 activity. However, no modulation of creatinine and metformin cell accumulation was apparent with indomethacin. Creatinine transport characteristics in the presence of inhibitors approached those of metformin, an OCT2/MATE substrate, but were distinct from those of penciclovir, an OAT2-selective substrate. Moreover, indomethacin showed no significant effect on the basolateral-to-apical transport and net secretion of creatinine across hPTC monolayers. Collectively, the functional studies suggest OCT2 as the primary basolateral uptake mechanism, and that OAT2 has a minimal role, in creatinine renal secretion. Our results highlight the utility of hPTCs to enable the functional assessment of renal transport mechanisms.
SIGNIFICANCE STATEMENT

Our results obtained with primary hPTCs indicate that OCT2/MATE (versus OAT2) play a major role in the active renal secretion of creatinine. Quantitative pharmacokinetic models should therefore focus on OCT2/MATE when describing serum creatinine and creatinine clearance modulation by inhibitor drugs and genotype- or disease-related activity changes. The present study highlights the utility of freshly isolated hPTCs to support solute carrier phenotyping to enable the functional assessment of renal transport mechanisms.
INTRODUCTION

Organic cation transporter (OCT)2 along with multidrug and toxin extrusion pump (MATE)1/2K facilitate active renal secretion of many cationic drugs and are often associated with drug-drug interactions (DDIs). Inhibition of OCT2 and/or MATE1/2K can decrease overall renal clearance and elevate plasma exposure of substrate drugs (e.g. metformin) leading to potential safety issues (Zamek-Gliszczynski et al., 2018; Le and Lee, 2019; Mathialagan et al., 2021). Regulatory agencies recommend measurement of OCT2/MATEs in vitro inhibition potency (IC$_{50}$ or K$_{i}$) and employ agency decision trees to inform DDI risk and need for subsequent clinical study using a probe substrate drug such as metformin (EMA, 2012; US FDA, 2020).

Serum creatinine (SCr) is routinely measured and used widely as a biomarker for renal function in drug development and clinical practice. Transporter-mediated secretion contributes to about 30-40% of creatinine clearance (CrCL), while the remainder involves glomerular filtration (Chu et al., 2016; Mathialagan et al., 2021). When clinically relevant increases in SCr are noted, it is critical to distinguish the elevated SCr due to renal toxicity from the nonpathologic increase of SCr attributed to inhibition of renal transporters. Elevation in SCr or reduction in CrCL – in the absence of change in clearance of glomerular filtration markers such as iohexol and cystatin C – can be associated with inhibition of renal transporters (Chu et al., 2016; Mathialagan et al., 2017). However, while studies consistently demonstrated creatinine transport by OCT2 and MATEs, other in vitro studies suggested it is also transported by OAT2, an anionic transporter shown to be expressed in human kidney tissue (Ciarimboli et al., 2012; Lepist et al., 2014; Shen et al., 2015). Such studies have largely presented data obtained with transporter-transfected cell-based systems. In comparison, there are extremely limited transporter activity data using physiologically-relevant freshly isolated primary human renal proximal tubule cells (hPTCs).
(Brown et al., 2008). To date, direct evidence or clinical data supporting the significant contribution of OAT2 to CrCL are lacking. Understanding creatinine renal disposition is critical for reliable implementation of SCr concentration and CrCL data in the assessment of kidney function, and as a marker for OCT2/MATEs inhibition in vivo (Chu et al., 2016; Scotcher et al., 2020).

Here, we studied creatinine transport mechanisms in vitro using freshly isolated hPTCs in suspension and Transwell assay formats with several inhibitors of OAT2, OCT2 and MATEs. Additionally, transporter-specific activity was also evaluated using transfected Human Embryonic Kidney (HEK)293 cells. In these models, creatinine transport mechanisms were compared to those of metformin (OCT2/MATE selective versus OAT2) and penciclovir (OAT2 selective versus OCT2/MATE).
MATERIALS AND METHODS

Details on source of chemical and reagents can be found in Supplementary Material.

In vitro Transport Studies Using Transporter-transfected Cells

As previously described (Mathialagan et al., 2017; Mathialagan et al., 2021), un-transfected cells and transporter-transfected HEK293 cells (HEK-OAT2, HEK-OCT2, HEK-MATE1 and HEK-MATE2K) were seeded at densities of 0.5 - 0.7 × 10^5 cells/well in a volume of 0.1 mL/well, in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin, 1% NEAA, 1% glutamax and 1% sodium pyruvate in a 96-well poly-D-lysine coated plates for transporter substrate studies. Cells were maintained at 37°C, 5% CO2, and 90% relative humidity cultured for 2 days. Transport buffer was prepared at pH 7.4 using Hank’s balanced salt solution supplemented with 20 mM HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 5.55 mM dextrose. Cell plates were removed from incubation and rinsed three times with 0.15 mL of pre-warmed transport buffer, leaving the final rinse volume on the cells for at least 2 minutes to allow the cells to adjust to the buffer. HEK293-MATE1/2K cells were acidified by treating the cells for 20 minutes at 37°C with 0.1 mL of 30 mM ammonium chloride. Stock solutions of all compounds were made in 100% DMSO. Uptake assays were carried out using ^14^C-Creatinine (10 - 100 µM) for OCT2, OAT2 and MATEs, penciclovir (1 - 3 µM) for OATs and ^14^C-metformin (20 µM) for OCT2/MATE. In all cases, test compound concentrations used in the assays were well below their respective reported Km values. At different time points, the cellular uptake was terminated by washing the cells three times with 0.15 mL each of ice-cold transport buffer and for penciclovir lysed directly on the plate with 100% methanol containing internal standard, and samples were quantified by liquid chromatography tandem mass spectrometry methodology. For each compound, cellular uptake
was measured at six different time points, per cell type. Control substrates \(^{3}\text{H}\)-paraaminohippuric acid (0.5 µM), \(^{3}\text{H}\)-cyclic guanosine monophosphate (cGMP, 0.5 µM), \(^{3}\text{H}\)-estrone sulfate (0.2 µM) and \(^{14}\text{C}\)-metformin (20 µM) were used for OAT1, OAT2, OAT3 and OCT2/MATEs, respectively, to monitor the functionality of the cells. For inhibition studies HEK293 cells seeded at densities of 0.5 - 0.7 \(\times\) 10\(^5\) cells/well, in a volume of 0.1 mL/well. The required final assay concentrations for inhibitors were prepared using transport buffer spiked with probe substrates, 10 - 100 µM \(^{14}\text{C}\)-Creatinine for OCT2, OAT2 and MATEs, 0.2 µM penciclovir for OAT2 and 10 - 20 µM \(^{14}\text{C}\)-metformin for OCT2/MATEs. All solutions contained a final concentration of dimethylsulfoxide below 1% (v/v). Uptake was started by the addition of 0.1 mL of probe spiked transport buffer without or with inhibitors. The plates were then incubated for 3 minutes at 37°C with shaking at 150 rpm. The experiment was stopped by washing cells three washes with 0.15 ml/well ice-cold transport buffer. Radiolabeled samples from substrate and inhibition were retrieved by lysing the cells with 0.1 mL of 1% NP40 (nonyl phenoxypolyethoxylethanol) in double distilled water. Accumulated radioactivity was determined by mixing 0.05 mL of the cell lysate with 0.20 mL of scintillation fluid. Radioactivity in each sample was quantified by measurement on a Perkin Elmer MicroBeta TriLux Liquid Scintillation Counter (Perkin Elmer Life Sciences, Waltham, MA). Refer to Supplemental Methods for the bioanalysis of penciclovir.

Interaction potency (concentration yielding 50% inhibition or half-maximal inhibition, IC\(_{50}\) or EC\(_{50}\)) values for the tested inhibitors of OAT2, OCT2, MATEs were estimated using a four-parameter logistic equation (Mathialagan et al., 2017; Mathialagan et al., 2021):
The in vitro parameters for concentration-dependent uptake rates and transporter inhibition were estimated using Graphpad Prism V 9.5.1 (Graphpad Software, LLC, La Jolla, CA).

Preparation of hPTCs

hPTCs used in this study were isolated from normal human kidneys with a clamp time to isolation time of less than 18 h. Kidneys were sourced from the UK Transplantation Service by via a UK Research Tissue Bank with full ethical approval for use in research and commercial studies. Demographics of the donors include: 9 kidney donors, 45-74 year old, 8 male, 1 female and presented GFR >90 mL/min. Proximal tubule cells were isolated from kidney cortex as previously described (Brown et al., 2008). Briefly, kidneys were decapsulated and cortex slices taken, which were then minced to approximately 1 mm$^3$ pieces before suspending 5 g of minced tissue in 50 mL of isolation medium (RPMI-1640 media, 5% fetal calf serum, penicillin/streptomycin (200U/mL/200µM). Type 2 collagenase (activity of ≈300 units/mg, working concentration of 1 µg/mL) was added to the suspension to initiate the digestion of the tissue. The suspension was shaken for 1.5–2 h at 37°C and passed through a 40 µm nylon sieve to remove undigested material.

To separate out the hPTCs, the cell suspension was loaded on top of discontinuous Percoll gradients with densities of 1.04 g/mL and 1.07 g/mL and centrifuged at 1600g for 25 min. After centrifugation, hPTCs at the intersection of the gradients were aspirated and washed as previously described (Brown et al, 2008). The cells were resuspended in warm human renal...
epithelial growth medium (REGM plus SingleQuot supplements, Lonza, UK). The cell yield was estimated using a Cellometer Auto T4 Cell Counter (Nexcelom Bioscience LLC, USA) after passing the cell suspension through a large bore needle three times to separate aggregated cells.

Isolated cells were either used immediately after isolation as cells in suspension (Day 0 cells) or seeded onto 24-well Transwell® filter supports (surface area of 0.33 cm²). The medium was refreshed after 24 h of initial seeding, and at day 3 and 5 of culture. hPTCs were maintained in a humidified incubator at 37°C with 5% CO₂ and 95% air. Transepithelial electrical resistance (TEER) was measured using an electric voltohmmeter (EVOM2, World Precision Instruments, UK). Monolayers demonstrated TEERs of at least 60 Ω cm² before they were used in experiments.

**Transport Studies Using hPTCsls**

Measurement of creatinine, metformin and penciclovir transepithelial flux was performed as previously published (Brown et al., 2008). The studies to calculate the flux were carried out using three different donor kidneys.

To measure creatinine, metformin or penciclovir uptake in the presence and absence of specific inhibitors, aliquots of freshly isolated hPTCs were resuspended in 100 µL of Krebs buffer, which comprised NaCl (140 mM), KCl (5.4 mM), MgSO₄ (1.2 mM), KH₂PO₄ (0.3 mM), NaH₂PO₄ (0.3 mM), CaCl₂ (2 mM), glucose (5 mM), and 2-(N-morpholino)ethanesulfonic acid (10 mM), titrated to the pH 6.8 with Tris base at 1x10⁶ cells per aliquot. Suspended hPTCs were pre-incubated for 30 min adding either 100 µL Krebs buffer plus and minus a range of inhibitor concentrations at 2x the final desired concentration (as outlined in appropriate figure legend) at
37°C. Uptake was initiated by topping up each relevant suspension with 200 µL Krebs buffer containing either 14C-creatinine, 3H-metformin or 3H-penciclovir (1 µCi/mL) with or without its respective inhibitor at 37°C. At the appropriate time point, the uptake was terminated by addition of 1 mL of ice-cold Krebs solution followed by a rapid pelleting of cells in a microcentrifuge. The pellet was then gently resuspended in 150 µL ice cold Krebs buffer and washed by centrifugation once more. The final pellet was resuspended in ice-cold Krebs and three aliquots taken for liquid scintillation counting, three counts per sample.

To measure transepithelial flux of substrates, the media was removed from apical and basolateral chamber of confluent monolayers cultured on Transwell permeable filter supports (24-well plates, corning, part number 3413), by suction and the monolayers washed by sequential transfer of the inserts through three beakers of Krebs buffer at 37°C. Monolayers were then pre-incubated for 30 min in Krebs buffer or Krebs buffer containing a range of concentrations of the inhibitor being tested. At the end of this pre-incubation period, flux of the appropriate substrate was initiated when the Krebs buffer (200 µL pH 6.8 in apical and 800 µL pH 7.4 at basolateral chamber) was replaced with replaced with equivalent volumes of Krebs solutions containing either 14C-creatinine, 3H-metformin or 3H-penciclovir with or without its respective inhibitor. The flux solutions also contained radiolabelled 14C-mannitol or 3H-mannitol, which allowed for the measurement of paracellular flux. 50 µL from the contralateral chamber was sampled after 60 min of flux, and the samples placed into scintillation vials. Flux was terminated by sequentially transferring the inserts into three beakers of ice-cold Krebs buffer and left to air dry. The filters of the inserts, on which the monolayers were adhered, were then excised out and transferred to clean scintillation vials. The filter samples provided the amount of creatinine, metformin or
penciclovir accumulated over the 60 min flux period, which was indicative of the uptake of the substrates from across either the apical or basolateral membrane of the monolayers.

**Statistical Analysis**

The paired Student’s *t* test was used to compare effect of inhibitors, and *P* < 0.05 and **P** < 0.01 were considered statistically significant. All statistical analyses were performed using Graphpad Prism V 9.5.1 (Graphpad Software, LLC, La Jolla, CA).
RESULTS

Transport Characteristics of Creatinine, Metformin and Penciclovir in Transfected HEK293 Cells

Uptake was measured in transporter-transfected HEK293 cells using radiolabelled compounds. Creatinine showed specific transport by OAT2, OCT2 and MATEs, while metformin showed transport by OCT2 and MATEs only (Figure 1). Also, metformin and creatinine showed no transport activity via OAT1 and OAT3 – highly expressed OATs in human kidney (Supplementary Table 1). In contrast, penciclovir was selectively transported by OAT2, with no measurable specific transport by OAT1/3 and OCT2/MATEs (Figure 1C, Supplementary Table 1). Cell uptake was found to be linear within the measured timeframe across the transporters and probe substrates, and subsequent inhibition studies were conducted within the linear uptake phase (2 min). The initial concentration for all three substrates is well below the reported Km values for the specific transporter, and thus the obtained kinetics are assumed to be under sub-saturation conditions. Creatinine uptake clearance via OAT2 (0.56 µL/min/mg) under these conditions is about 4% of that observed for penciclovir (13.5 µL/min/mg). On the other hand, creatinine uptake clearance via OCT2 (1.35 µL/min/mg) is about 17% of OCT2-mediated clearance of metformin. Overall, this data confirms affinity of creatinine for OAT2 in addition to transport via OCT2 and MATEs.

Creatinine transporter-specific activity was inhibited by pyrimethamine in HEK293-OCT2, -MATE1 and -MATE2 cells with a measured IC\textsubscript{50} of 1.1 ± 0.05, 0.21 ± 0.02 and 0.35 ± 0.05 µM, respectively (Figure 2, Table 1). Decynium-22 also inhibited creatinine transport by OCT2, MATE1 and MATE2K presenting IC\textsubscript{50} values of 1.03 ± 0.05, 10.2 ± 0.56 and 16.4 ± 1.93 µM,
respectively. Decynium-22 showed >10x potent IC\textsubscript{50} against OCT2, compared to MATEs; while pyrimethamine was found to be relatively more potent inhibitor for MATEs over OCT2 (~3x). However, both pyrimethamine and decynium-22 showed <30% inhibition of OAT2-mediated creatinine transport up to a concentration of 100µM. In contrast, indomethacin reduced OAT2-mediated creatinine transport with an estimated IC\textsubscript{50} of 2.29 ± 0.15 µM. Indomethacin showed a minimal effect on OCT2, MATE1 and MATE2K transport in the concentration range tested (<50% inhibition at 300µM), implying a >100x selectivity towards OAT2 inhibition when using creatinine as probe substrate. Metformin is not transported by OAT2 (Figure 1), and therefore inhibition with indomethacin was not tested (Figure 2B). Finally, indomethacin showed similar IC\textsubscript{50} values when using creatinine, penciclovir and cGMP as probe substrates (Figure S1).

**Transport Characteristics of Creatinine, Metformin and Penciclovir in hPTCs**

In the freshly isolated and plated human PTCs, time-dependent uptake was observed for creatinine, metformin and penciclovir (Figure 3). The estimated mean (range, n=9) uptake clearance of creatinine, metformin and penciclovir is 0.42 (0.24-0.62), 0.73 (0.51-0.96) and 0.68 (0.43-1.05) µL/min/million cells, respectively. Uptake clearance was found to be similar for hPTCs obtained from 3 to 6 different organ donors.

Effect of pyrimethamine, decynium-22 and indomethacin on cellular accumulation of creatinine, metformin and penciclovir was evaluated in hPTCs also (Figure 4). Creatinine and metformin uptake was increased by pyrimethamine in a concentration-dependent manner, which is likely associated with preferential inhibition of MATEs over OCT2. The estimated hybrid inhibition constants of pyrimethamine were 3.1 ± 1.1 µM and 5.3 ± 2.2 µM when using creatinine and metformin as substrates, respectively. These values are within the range of the measured IC\textsubscript{50}s.
for MATE/OCT2 in the HEK293 cells (Figure 1). Decynium-22 showed concentration-dependent inhibition of both creatinine and metformin uptake by hPTCs with an estimated IC\(_{50}\) of approximately 1 µM. Pyrimethamine and decynium-22 did not modulate penciclovir uptake over the concentration range tested. In contrast, an OAT2 inhibitor, indomethacin, showed no notable effect on creatinine and metformin uptake, while a concentration-dependent inhibition was observed when using penciclovir as probe substrate (IC\(_{50}\) ~0.5 µM). The differential effects of these 3 inhibitors, demonstrate OCT2 and MATEs transport mechanisms for creatinine and metformin, and OAT2 transport for penciclovir in the hPTCs.

Transcellular Transport of Creatinine Across hPTCs

Bidirectional flux and accumulation of creatinine, in the absence and presence of indomethacin and pyrimethamine, was evaluated in hPTC monolayers cultured on Transwell inserts (Figure 5). Control monolayers demonstrated a secretory net flux driven by a greater flux in the basolateral to apical (J\(_{B-A}\)) direction. This was not significantly altered in the presence of the OAT2 inhibitor indomethacin. Incubation with pyrimethamine inhibited the J\(_{B-A}\) flux by ~50% (P<0.05), resulting in no net movement of creatinine. Creatinine accumulation in hPTCs was ~5x greater when incubated in the basolateral compartment over the apical compartment. Intracellular accumulation was not altered across either membrane when treated with indomethacin, but basolateral accumulation was significantly reduced (P<0.01) in the presence of pyrimethamine. Impact on basolateral uptake and net secretory flux of selective inhibition of OCT2 (versus OAT2) demonstrates its importance in proximal tubular creatinine secretion.
DISCUSSION

In this study, we revisited the transport mechanisms of creatinine renal secretion with the goal of assessing the relevance of OCT2- versus OAT2-mediated uptake. Previous studies showed creatinine as a substrate to OAT2, however the quantitative contribution in basolateral uptake and thus CrCL is unclear (Lepist et al., 2014; Shen et al., 2015). Our comprehensive transport studies using transporter-transfected cells and freshly isolated hPTCs from multiple donors depicted: i) creatinine transport by OAT2, along with OCT2/MATE1/2K, where the specific transport is inhibited by indomethacin and decynium-22/pyrimethamine, respectively, in transporter-transfected HEK293 cells; ii) creatinine uptake in hPTCs, which is inhibited by decynium-22 in a concentration-dependent manner but not by the OAT2 inhibitor, indomethacin; iii) time-dependent cell accumulation of metformin (OCT2/MATE substrate) and penciclovir (OAT2 substrate) by hPTCs, wherein metformin uptake is sensitive to OCT2/MATE inhibitors, pyrimethamine and decynium-22, and penciclovir uptake is only sensitive to an OAT2 inhibitor, indomethacin; iv) and finally, the specific effect of pyrimethamine, but not indomethacin, on creatinine and metformin hPTC-mediated transcellular transport. Overall, the data presented here using a physiologically relevant in vitro system provides functional evidence for the primary role of OCT2/MATE-mediated renal secretion of creatinine with limited contribution from OAT2.

Creatinine is a typical clinical marker for kidney function and thus understanding its disposition is important to comprehend mechanistic drivers of drug-induced kidney dysfunction in drug development. Creatinine secretory transport mechanisms are similar to those of metformin, which is a well characterized probe substrate drug recommended to assess in vivo OCT2/MATE mechanisms (Mathialagan et al., 2017; Zamek-Gliszczynski et al., 2018; Varma, 2023). In clinical studies, the effect of inhibitor drugs on the renal active secretion of metformin and
creatinine are generally consistent (Mathialagan et al., 2021). For instance, dolutegravir, an OCT2/MATE inhibitor with a minimal effect on OAT2 in vitro, presents a greater than 50% reduction in active secretion of creatinine and metformin (Song et al., 2016). In fact, the association between CrCL and metformin renal clearance change can be noted across a wide range of OCT2/MATE inhibitors, which show minimal in vitro inhibition of OAT2, corroborating OCT2/MATE as primary drivers of CrCL (Mathialagan et al., 2021). Clinically relevant OAT2 inhibitors are limited and there is no direct in vivo evidence to associate OAT2-mediated transport to CrCL. Of the identified OAT2 inhibitors, indomethacin has potential to inhibit OAT2 transport in vivo with the estimated maximal unbound plasma concentration ($I_{max,u}$)/$IC_{50}$ ratio (~0.30) at 50 mg dose ($I_{max,u}$, 0.67µM (Ryu et al., 2022); $IC_{50}$, 2.34µM (Table 1)) that is above the recommended in vivo inhibition risk criteria (European Medicines Agency, 2012; US Food & Drug Administration, 2020). However, no change was noted in CrCL with indomethacin 25 mg t.i.d. for 6 weeks (Baber et al., 1980) and 50 mg t.i.d. for 10 days (Jørgensen et al., 1991). It is noteworthy that indomethacin exhibits a similar in vivo inhibition risk against OAT3 ($IC_{50}$, 2.2µM (Parvez et al., 2017)) and has been shown to reduce the renal clearance of intravenous furosemide (a recommended OAT3 probe drug) by about 40% (Chennavasin et al., 1980). Therefore, the clinical observations with indomethacin do support the inhibition of basolateral renal transporters, and the observed lack of effect on CrCL can be interpreted as limited OAT2 contribution.

Several studies demonstrated creatinine transport by OAT2 using overexpressed cells. Ciarimboli (2012), Lepist et al. (2014) and Shen et al. (2015) reported that creatinine is transported by OAT2 in overexpressed cells (Madin-Darby canine kidney or HEK293 cells). Our results are consistent with these reports and confirm functional transport of creatinine by OAT2.
in overexpressed HEK293 cells (Figure 1). Additionally, Shen et al. (2015) showed markedly high intrinsic transport clearance via OAT2, estimated from the $V_{\text{max}}/K_m$ ratio (maximal uptake rate/concentration of substrate at half-maximal uptake rate) in HEK293 cells, compared to only about <3% relative transport by OCT2 and MATEs, and thus projecting OAT2 as a primary driver for secretory transport across the basolateral membrane. *In vitro-in vivo* extrapolation (IVIVE) strategies based on such $V_{\text{max}}/K_m$ ratios are not extensively validated for translating transport clearance and could be of limited value when the estimated $K_m$ values are very large and outside the concentration range tested (e.g., creatinine OCT2 $K_m$ >18mM versus OAT2 $K_m$ <1mM (Shen et al., 2015)). In contrast, the intrinsic clearance of creatinine at a concentration of 10µM (well below its $K_m$) is higher in our OCT2 cells compared to OAT2 cells. Transporter protein abundance, measured based on tandem liquid chromatography-mass spectrometry (LC-MS/MS) methodology previously reported (Prasad et al., 2016), were 8.6 ± 0.2 pmol/mg membrane protein (Kumar et al., 2020) and 89.7±5.1 pmol/mg membrane protein (unpublished) for our OAT2-HEK293 and OCT2-HEK293 cells, respectively. In comparison, kidney cortex samples analysed using the same methodology recovered an OAT2 abundance of 0.93 ± 0.32 pmol/mg membrane protein and OCT2 abundance of 7.42 ± 2.84 pmol/mg membrane protein (Prasad et al., 2016; Kumar et al., 2020). Considering the protein abundance difference and the resting membrane potential difference between the OCT2-expressing HEK293 cells and the renal epithelial cells (-35 millivolts and -70 millivolts, respectively) [as described in (Kumar et al., 2018)], the intrinsic clearance measured in overexpressing HEK293-OAT2 and HEK293-OCT2 cells (Figure 1) translates to fraction transported ($f_t$) by OCT2 of about 79%. Thus, our results support OCT2, but not OAT2, as a major driver for the uptake clearance of creatinine. That said, elaborate examples of relative expression factor-based scaling to estimate renal $f_t$ are limited and
should be carefully positioned. The significance of OCT2 is evident in hPTCs, with creatinine uptake modulation by OCT2/MATE inhibitors and the lack of inhibition by indomethacin (Figure 4). Therefore, it was critical to integrate the transfected/overexpression HEK293 cell data with the results obtained with freshly isolated hPTC to assess the contribution of individual basolateral solute carriers to renal CrCL. Use of the latter is important as it is known that the conditionally immortalized hPTCs (e.g., hPTC-telomerase reverse transcriptase/TERT or ciPTC), as well as other cell lines (e.g., HK-2 or Caki), may present transporter expression profiles and functional characteristics that are likely not reflective of native human kidney cortex tissue and lack the appropriate transport functionality to support solute carrier phenotyping of a substrate such as creatinine (Hilgendorf et al., 2007; Jenkinson et al., 2012; Aschauer et al., 2015; Bajaj et al., 2018).

An association between SLC22A7 polymorphism (rs70953677-T) and SCr and estimated GFR (eGFR) was found in a genome wide association study (GWAS) (https://www.ebi.ac.uk/gwas/home) (Sinnott-Armstrong et al., 2021). In the same GWAS report, a frame-shift variant in OCT2 gene SLC22A2 (rs8177505AT) showed strong lowering effects on eGFR (P-value $4 \times 10^{-54}$) and increasing effect on SCr (Sinnott-Armstrong et al., 2021). Additionally, four other SLC22A2 variants (rs79370442, rs3127573, rs596881, rs476235) are associated with SCr/eGFR with P-value larger than that observed with SLC22A7 polymorphism (rs70953677-T, P-value $2 \times 10^{-9}$). The observed SLC22A7 genotype effect is limited to one report and has not been confirmed elsewhere, while associations between SLC22A2 gene variants and SCr/eGFR were found in several independent studies (Pattaro et al., 2016; Barton et al., 2021; Richardson et al., 2022). While genotype-phenotype relationships are not clearly understood for these variants of SLC22A2 and SLC22A7, it can be alluded that OCT2-mediated transport
contributes to the SCr/eGFR while the evidence for relevance of OAT2 is lacking. It is accepted that SCr is highly dependent on a patient’s age, sex, ethnicity and body weight, and multiple models/equations accounting for these variables are implemented for translating SCr values to assess kidney function in clinical practice (Delanaye and Mariat, 2013; Delanaye et al., 2017; Porrini et al., 2019; Inker et al., 2021). Variability in SCr and eGFR is therefore complex and cannot be directly associated with secretory transport mechanisms in the absence of information regarding changes in renal clearance. Further studies primarily assessing CrCL (not SCr alone) will be needed to determine SLC22A7 genotypic effects, if any.

CONCLUSIONS

As described herein, it was possible to study the uptake of creatinine in vitro using freshly isolated hPTCs manifesting functional OCT2 and OAT2 transport activity. After the careful use of OCT2- and OAT2-selective inhibitors, our in vitro functional studies support OCT2-mediated transport as a primary pathway for basolateral uptake in hPTCs. Although creatinine is transported by OAT2 in overexpressed cell systems, its contribution to CrCL in vivo appears limited. Mechanistic models should therefore duly consider OCT2/MATE pathways in assessing kidney function and DDIs to minimize underestimation of SCr and CrCL modulation due to OCT2 inhibition, genotype, as well as age- and disease-related activity changes.
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AUTHORSHIP CONTRIBUTIONS


Conducted experiments: Mathialagan S, Chung G, Pye K.

Performed data analysis: Mathialagan S, Chung G, Pye K, Brown C, Varma M.

Wrote or contributed to the writing of the manuscript: Brown C, Rodrigues D, Mathialagan S, Varma M.
References


Footnotes:

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DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are contained within the paper.

CONFLICT OF INTEREST

SM, ADR, MVSV are full-time employees of Pfizer Inc. All authors declare no conflicts of interest that are directly relevant to this study.
Figure Legends:

Figure 1. Transport activity of creatinine, metformin and penciclovir in transporter-transfected cells. A. Creatinine showed transport by OAT2, OCT2 and MATE1/2K. B. Metformin was selectively transported by OCT2 and MATE1. C. Penciclovir was selectively transported by OAT2. Solid lines are fitted linear regression used to estimate uptake rates. *Uptake rate significantly different (F test with α-value of 0.05) compared to un-transfected HEK cells.

Figure 2. Concentration-dependent effect transporter inhibitors, pyrimethamine, decynium-22 and indomethacin, on creatinine (A) and metformin (B) uptake in the single transfected HEK293 cells. Each data point represents the mean ± S.D. of n = 3. Inhibition was measured at 2 min incubation time. Curves – data was fitted to Eq. 1 to estimate IC<sub>50</sub> values.

Figure 3. Time-course of cellular accumulation of 10 μM creatinine (A), 20 μM metformin (B) and 0.2 μM penciclovir (C) in hPTCs, across 30 min of continued exposure. Data is presented as mean ± S.D. of n=9 (3 donors, 3 replicates). Solid lines are linear fitting of data.

Figure 4. Concentration-dependent effect of pyrimethamine (I), decynium-22 (II) and indomethacin (III) on the cellular accumulation of 10 μM creatinine (A), 20 μM metformin (B) and penciclovir (C) in human PTCs, after 20-minutes of continued exposure. Each data point represents the mean ± S.D. of n = 9 (3 donors x 3 replicates). Curves – data was fitted to Eq. 1 to estimate interaction potency values.

Figure 5. Effect of indomethacin (100μM) and pyrimethamine (100μM) on transcellular flux (A) and cell accumulation (B) of creatinine in hPTCs. Apical-to-basolateral (J<sub>A-B</sub>) and basolateral-to-apical (J<sub>B-A</sub>) transport was measured in confluent monolayers with 10μM creatinine on the donor side, and cellular accumulation was measured at the end of incubation (30min). Secretory flux (J<sub>Secretion</sub>) is the difference between J<sub>B-A</sub> and J<sub>A-B</sub>. Data represent mean±SD from three independent experiments. * p<0.05, **p<0.01, significantly different from control. Paired one-tailed Student’s t-test.
Table 1. IC$_{50}$ values of decynium-22, indomethacin and pyrimethamine against different probe substrates, creatinine, metformin and penciclovir, as measured in transfected HEK293 cells and hPTCs

<table>
<thead>
<tr>
<th>Cell system</th>
<th>$^{14}$C-Creatinine (10 µM) as substrate</th>
<th>$^{14}$C-Metformin (20µM as substrate)</th>
<th>$^{3}$H-Penciclovir (0.2µM) as substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decynium-22</td>
<td>Indomethacin</td>
<td>Pyrimethamine</td>
</tr>
<tr>
<td>HEK-OAT2</td>
<td>215 ± 21</td>
<td>2.34 ± 0.15</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HEK-OCT2</td>
<td>1.03 ± 0.05</td>
<td>461 ± 39</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>HEK-MATE1</td>
<td>10.2 ± 0.56</td>
<td>&gt;250</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>HEK-MATE2K</td>
<td>16.4 ± 1.93</td>
<td>&gt;250</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>hPTCs</td>
<td>0.93 ± 0.4</td>
<td>&gt;1000</td>
<td>3.1 ± 1.1*</td>
</tr>
</tbody>
</table>

IC$_{50}$ (µM)

Values represent mean ± S.D. (n=3/6). $^*$ Creatinine 100 µM was used for MATE1 and MATE2K studies. * Hybrid constant based on concentration-dependent increase in cellular accumulation (Figure 4).
Figure 1

A. $^{14}$C-Creatinine (10μM)

B. $^{14}$C-Metformin (20μM)

C. Penciclovir (3μM)
Figure 2
Figure 3

(A) Creatinine uptake (pmol/5x10^4 cells) vs. Time (min)
(B) Metformin uptake (pmol/5x10^4 cells) vs. Time (min)
(C) Penciclovir uptake (pmol/5x10^4 cells) vs. Time (min)
Figure 4
Figure 5
Supplementary Material

Significance of organic anion transporter 2 and organic cation transporter 2 in creatinine clearance: Mechanistic evaluation using freshly-prepared human primary renal proximal tubule cells

Sumathy Mathialagan, Git Chung, Keith Pye, A. David Rodrigues, Manthena V. S. Varma, Colin Brown
Supplementary Figure 1. Inhibition of OAT2-mediated creatinine, penciclovir and cGMP uptake by indomethacin in transfected HEK293 cells. Datapoints represent mean ± S.D. (n=3).
Supplementary Table 1. Uptake ratios of creatinine, metformin and penciclovir in transfected-HEK293 cells.

<table>
<thead>
<tr>
<th>Transporters</th>
<th>(^{14}\text{C})-Creatinine (10 µM)</th>
<th>(^{14}\text{C})-Metformin (10 µM)</th>
<th>(^{3}\text{H})-Penciclovir (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAT1</td>
<td>0.55 ± 0.05</td>
<td>0.83 ± 0.13</td>
<td>0.89 ± 0.10</td>
</tr>
<tr>
<td>OAT2</td>
<td>7.36 ± 0.88</td>
<td>1.6 ± 0.08</td>
<td>23.03 ± 1.39</td>
</tr>
<tr>
<td>OAT3</td>
<td>0.81 ± 0.14</td>
<td>1.04 ± 0.13</td>
<td>1.65 ± 0.14</td>
</tr>
<tr>
<td>OCT2</td>
<td>28.14 ± 4.46</td>
<td>39.21 ± 0.71</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>MATE1</td>
<td>20.49 ± 0.94</td>
<td>22.15 ± 0.22</td>
<td>1.66 ± 0.08</td>
</tr>
<tr>
<td>MATE2K</td>
<td>7.72 ± 0.21</td>
<td>7.8 ± 0.55</td>
<td>1.06 ± 0.01</td>
</tr>
</tbody>
</table>

*Uptake ratio is ratio of cell accumulation in transfected- to wild-type cells, measured at 2 min.*
Supplementary Methods

Chemicals and Reagents

All the compounds used in the assay were obtained from Pfizer chemical inventory system or procured from Sigma-Aldrich (St.Louis, MO). HEK293 cells transfected with OCT2 were obtained from Dr. Kathleen Giacomini (UCSF, CA). HEK293 cells transfected with OAT2-variant 1 were obtained from Dr. Ryan Pelis (Halifex, Canada). HEK293 cells transfected with MATE1/2K were obtained from Dr. Katsuhisa Inoue (Nagoya City University). BioCoat™ 96-well poly-D-lysine 96-well plates were obtained from Corning Inc (Corning, NY). Fetal bovine serum was purchased from Sigma-Aldrich (St.Louis, MO). DMEM (Dulbecco's Modified Eagle Medium), Hygromycin B, Gentamicin and sodium pyruvate were obtained from Gibco life technologies (Waltham, MA). HBSS (Hank’s Balanced Salt Solution), HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) were obtained from Lonza Inc. (Allendale, NJ). 14C-Creatinine and 14C-Metformin were purchased from Moravek Biochemical Inc. (Brea,CA). NP-40 Surfact-Amps™ Detergent Solution was purchased from Thermo Scientific (Rockford, IL). 3H-cGMP, 3H-para aminohippuric acid and 3H-estrone sulfate were purchased from Perkin Elmer (Oat Brook, IL) and 3H-penciclovir were purchased from American Radiolabeled Chemicals (St. Louis, MO).

LC/MS/MS Method

LC-MS/MS analyses for penciclovir were performed on a SCIEX Triple Quad 6500 mass spectrometer equipped with an IonDrive Turbo V ion source. The HPLC systems consisted of an Agilent 1290 Infinity binary pump. An Apricot/Sound Analytics ADDA autosampler was used for sample introduction. All instruments were controlled and synchronized by SCIEX Analyst.
software (version 1.6.2 or higher) working in tandem with the ADDA software. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Either a Phenomenex Kinetex C18 (2.6 µm, 2.1 × 30 mm) column with a C18 guard column was used or a Phenomenex Synergi Polar-RP (2.6 µm, 2.1 × 30 mm). The following gradient was used to elute samples using the C18 column: flow rate was set at 0.8 mL/min, 5% solvent B for 0.2 min, increasing to 95% B for 0.5 min, held at 95% B for 0.3 min, reduction to 5% B over 0.02 min, and held at 5% solvent B for 0.48 min (total run time 1.5 min). For the Polar-RP column, the gradient was: flow rate was set at 0.6 mL/min, 0% solvent B increasing to 70% B for 1 min, held at 70% B for 0.2 min, reduction to 0% B over 0.05 min, and held at 0% solvent B for 0.75 min (total run time 2.0 min). Quantitative analysis was performed in multiple reaction monitoring (MRM) mode. The MRM transition monitored for penciclovir was m/z 254.3/152.2 and the internal standard was 687.0/320.0. Results were analyzed using MultiQuant version 2.1 or 3.0.