Use of a Double-Transfected System to Predict hOCT2/hMATE1-mediated Renal Drug-drug Interactions

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Running title (60 characters max including space and punctuation):

Improving the prediction of hMATE1-mediated renal DDIs

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Word Counts:

- Text pages: 34
- Tables: 2
- Figures: 6
- References: 38
- Abstract: 250 (max 250 words)
- Significance statement: 77 (max 80 words)
- Introduction: 741 (max 750 words)
- Discussion: 1,497 (max 1500 words)

Keywords: Drug-drug interactions, hOCT2, hMATE1, Transwell system

Abbreviations:

AUC, area under the concentration-time curve; BRI, brigatinib; CIM, cimetidine; DDI, drug-drug interaction; FAM, famotidine; HEK, Human embryonic kidney; HCQ, hydroxychloroquine; I_{max,u}, maximal unbound inhibitor plasma concentration; IC_{50}, half-maximal inhibitory concentration; KRH, Krebs-Ringer-HEPES; hMATE, human multidrug and toxin extrusion
protein; MDCK, Madin-Darby Canine Kidney; NME, new molecular entity; hOCT, human organic cation transporter; $P_{\text{app}}$, apparent permeability; PYR, pyrimethamine.
ABSTRACT

Accurate predictions of renal drug-drug interactions (DDIs) mediated by the human organic cation transporter 2 (hOCT2) and multidrug and toxin extrusion proteins (hMATEs) remain challenging. Current DDI evaluation using plasma maximal unbound inhibitor concentrations ($I_{\text{max,u}}$) and IC$_{50}$ values determined in single transporter-transfected cells frequently leads to false or overprediction especially for hMATE1. Emerging evidence suggests intracellular unbound inhibitor concentration may be more relevant for hMATE1 inhibition in vivo. However, determination of intrarenal inhibitor concentrations is impractical. Here we explored the use of hOCT2/hMATE1 double-transfected MDCK cells as a new in vitro tool for DDI risk assessment. Our results showed that potent in vitro hMATE1 inhibitors (hydroxychloroquine, brigatinib and famotidine) failed to inhibit metformin B-to-A flux in the double-transfected system. On the other side, the classical hOCT2/hMATE1 inhibitors, pyrimethamine and cimetidine, dose-dependently inhibited metformin apparent B-to-A permeability ($P_{\text{app}}$). The different behaviors of these hMATE1 inhibitors in the double-transfected system can be explained by their different ability to gain intracellular access either via passive diffusion or transporter-mediated uptake. A new parameter (IC$_{50,\text{flux}}$) was proposed reflecting the inhibitor’s potency on overall hOCT2/hMATE1-mediated tubular secretion. The IC$_{50,\text{flux}}$ values significantly differ from the IC$_{50}$ values determined in single transporter-transfected cells. Importantly, the IC$_{50,\text{flux}}$ accurately predicted in vivo DDIs (within 2-fold) when used in a static model. Our data demonstrated that the IC$_{50,\text{flux}}$ approach circumvents the need to measure intracellular inhibitor concentrations and more accurately predicted hOCT2/hMATE1-mediated renal DDIs. This system represents a new approach that could be used for improved DDI assessment during drug development.
SIGNIFICANCE STATEMENT

This study demonstrated that flux studies in double-transfected MDCK cells and the IC$_{50,\text{flux}}$ represents a better approach to assess in vivo DDI potential for the renal organic cation secretion system. Our study highlights the importance of inhibitor intracellular accessibility for accurate prediction of hMATE1-mediated renal DDIs. This approach has the potential to identify in vitro hMATE1 inhibitors that are unlikely to result in in vivo DDIs, thus reducing the burden of unnecessary and costly clinical DDI investigations.
INTRODUCTION

The renal organic cation secretion system, constituted by the organic cation transporter 2 (hOCT2) and multidrug and toxin extrusion proteins 1 and 2K (hMATE1/2K), is a major drug secretion system in the human kidney. Respectively expressed in the basolateral and the apical membrane of renal proximal tubular epithelial cells (PTECs), hOCT2 and hMATEs work sequentially to mediate renal secretion of many cationic drugs such as metformin, atenolol, and cimetidine (Morrissey et al., 2013; Yin and Wang, 2016). Inhibition of hOCT2/hMATEs can lead to drug-drug interactions (DDIs), which may impact not only systemic exposure and pharmacokinetics of substrate drugs but also their intrarenal accumulation potentially leading to nephrotoxicity (Morrissey et al., 2013; Yin and Wang, 2016). Evaluation of new molecular entities (NMEs) as substrates and inhibitors of renal hOCT2 and hMATE1/2K transporters is recommended by regulatory agencies including the US Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the Pharmaceuticals and Medical Devices Agency (PMDA).

Current guidelines for initial DDI assessment are based on the inhibitor’s maximal unbound plasma concentration ($I_{\text{max,u}}$) and its half-maximal inhibitory concentration ($IC_{50}$) towards a specific transporter determined through uptake studies using single-transfected nonpolarized cell lines (FDA. In vitro DDI guidance, 2020). If the $I_{\text{max,u}}/IC_{50}$ value is larger than the regulatory agency’s cutoff value (e.g. ≥ 0.1 for hOCT2 or hMATE1 by FDA), the sponsor is recommended to consider conducting an in vivo DDI study (FDA. In vitro DDI guidance, 2020). While this approach is widely adopted due to its simplicity and feasibility, there are gaps in translating in vitro data to successfully predict in vivo DDIs, especially for hMATEs. While many compounds exhibit potent hMATE1 inhibition in vitro with $I_{\text{max,u}}/IC_{50}$ surpassing the cutoff
value, many don’t translate to DDIs in vivo (Hibma et al., 2016; Mathialagan et al., 2021; Ogasawara et al., 2021; Krishnan et al., 2022). For instance, famotidine has been previously identified as a potent hMATE1 inhibitor in vitro, however, clinical evaluation showed no impact of famotidine on metformin exposure in vivo (Hibma et al., 2016). The high rate of false- or over-prediction for hMATE1-mediated DDIs is concerning as it can lead to costly and unnecessary clinical evaluations (Mathialagan et al., 2021; Krishnan et al., 2022).

One possible reason contributing to the false- or over-prediction of hMATE1-mediated DDIs is that plasma $I_{\text{max,u}}$ is used to predict the degree of hMATE1-inhibition in vivo. Physiologically, hMATE is located at the apical (urine-facing) membrane of PTECs not in direct contact with the plasma. Although unbound drug is also present in the filtrate, previous evidence suggests that hMATE inhibitors may need to enter PTECs in order to inhibit the transporter (Yin et al., 2016). Thus, unbound intracellular concentration of the inhibitor would be more relevant in predicting hMATE inhibition than $I_{\text{max,u}}$, which may significantly differ from unbound intracellular concentrations due to multiple factors including passive permeability, transporter-mediated uptake/efflux, and intracellular binding or sequestration of the inhibitor (Fig. 1).

However, accurate determination or prediction of intracellular concentrations in human PTECs can be challenging and laborious (Guo et al., 2018). Additionally, many inhibitors interact with both hOCT2 and hMATEs with varying inhibition potencies. While tubular secretion is sequentially mediated by hOCT2 and hMATEs, initial DDI assessment is based on inhibition of individual transporters, which does not consider the inhibitor’s overall impact on the net secretion process and possible interplay between apical and basolateral transporters.

Transwell system employing transporter-transfected Madin-Darby canine kidney (MDCK) cells are commonly used in drug transporter research. Unlike uptake studies which
measure cellular accumulation in nonpolarized cells, the transwell system allows the measurement of substrate flux across a monolayer of polarized MDCK cells. Single- or double-transfected MDCK cells are typically employed in substrate evaluation for efflux transporters (e.g. P-gp) or assessment of substrate transepithelial transport mediated by multiple transporters (e.g. the hOCT2/hMATE1 pathway) (Sato et al., 2008; König et al., 2011; Yin et al., 2015, 2016; López Quiñones et al., 2020). However, its utility in quantitative assessment of renal DDIs has been largely unexplored. Here, we used hOCT2/hMATE1 double-transfected cells as an in vitro model to assess the inhibitory potential of compounds on transepithelial flux of the organic cation probe substrate metformin. A new parameter reflecting the inhibition potency of a compound on metformin transepithelial basal-to-apical (B-to-A) flux (IC\text{50,flux}) is proposed and used to predict the in vivo DDI potential using a static model. Our results showed that this approach can more accurately predict hOCT2/hMATE1-mediated renal DDIs without the necessity to measure intracellular inhibitor concentrations.

**MATERIALS AND METHODS**

**Materials**

[^14]C]Metformin (112 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA).[^3]H]mannitol (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Hydroxychloroquine (HCQ) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Brigatinib (BRI) was purchased from MedChemExpress LLC (Monmouth Junction, NJ). Pyrimethamine (PYR) was purchased from TCI America, Inc. (Portland, OR). Cimetidine (CIM) and famotidine (FAM) were purchased from Sigma Aldrich.
All chemicals were analytical grade. Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA).

**Cell Lines and Cell Culture**

Flp-In human embryonic kidney (HEK) 293 cells stably transfected with hOCT2, hMATE1, or empty pcDNA5/FRT vector (control) were previously established in our laboratory (Duan et al., 2015; Yin et al., 2015). Both control and transporter-expressing HEK293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 150 µg/mL hygromycin B. The surface of the flasks and plates were coated with 0.1 mg/mL poly-D-lysine in Milli-Q water to improve HEK293 cell attachment. The Madin-Darby Canine Kidney (MDCK) cells double-transfected with hOCT2 and hMATE1 or empty vector were previously generated and validated in our laboratory (Yin et al., 2015) and were maintained in DMEM supplemented with 15% fetal bovine serum, 500µg/mL G418, and 200µg/mL hygromycin B. All cell lines were cultured in a 37°C humidified incubator with 5% CO₂.

**Inhibition Assays in HEK293 Cells**

Inhibition assays were performed as previously described (Duan and Wang, 2010; Yin et al., 2015, 2016) with slight modifications. Briefly, HEK293 cells were seeded in poly-D-lysine coated 96-well plates and grown to greater than 90% confluency (1–2 days). Cells were washed twice with prewarmed Krebs-Ringer-HEPES (KRH) buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM CaCl2, 1.2 mM MgSO4, and 25 mM HEPES, pH 7.4). Uptake of ¹⁴C-labeled metformin in vector-transfected and transporter expressing HEK293 cells were performed within the linear range of metformin uptake at 37°C in the presence or absence
of various inhibitor concentrations. For hOCT2-transfected cells uptake was performed using KRH at pH 7.4, while for hMATE1-transfected cells KRH at pH 8.0 was used to generate an outwardly directed proton gradient favoring metformin uptake. Uptake was quenched by washing the cells three times with ice-cold KRH buffer. After washing, cells were solubilized with 100 µL 1M NaOH at 37°C and neutralized with 100 µL 1M HCl after 1 hour. Radioactivity of 150 µL of the lysates was measured by a Tri-Carb Liquid Scintillation Counter (Perkin Elmer, Waltham, MA). The protein content in the lysates was measured through the BCA method using 20 µL of the lysate, and the uptake in cells was normalized to their total protein content. Transporter-specific uptake was calculated by subtracting the uptake in the vector-transfected cells, and uptake in the presence of inhibitor was normalized to uptake in the absence of inhibitor.

**Transwell Studies in hOCT2/hMATE1 Double-Transfected MDCK Cells**

Transepithelial flux of metformin across MDCK cell monolayers was determined as previously described (Yin *et al.*, 2015, 2016). Briefly, empty vector and hOCT2/hMATE1-expressing MDCK cells were seeded at a density of 2 x 10^5 cells/cm^2 on 12-well Corning™ Transwell™ inserts (PET membrane, 0.4 µM pore size). Transport experiments were performed 5 days after seeding. Integrity of the MDCK monolayer and proper formation of tight junctions was verified by measurement of transepithelial flux of mannitol as described previously (Yin *et al.*, 2015), and only data from inserts in which mannitol apparent permeability (P_{app}) value was below 1 x 10^{-6} cm/s were accepted. After removal of cell culture media from both sides of the inserts, cells were carefully washed three times with warm KRH buffer pH 7.4. For apical-to-basal (A-to-B) transport, 1.5 mL of KRH buffer (pH 7.4) was added to the B chamber, and transport was initiated by adding 0.5 mL of KRH (pH 6.0) containing [1^4C]metformin (1µCi/mL, 8.9µM) and...
[3H]mannitol (1µCi/mL, 50nM) to the A chamber. Similarly, for basal-to-apical (B-to-A) transport, 0.5 mL of KRH buffer (pH 6.0) was added to the A chamber, and transport was initiated by adding 1.5 mL of KRH (pH 7.4) containing radiolabeled metformin and mannitol to the B chamber. An apical pH of 6.0 was used based on previously published methods by our and other groups to mimic the average urine pH (Tsuda et al., 2009; Rowland and Tozer, 2010; Müller et al., 2011; Yin et al., 2015). To measure transcellular transport, at each time point 50 µL of sample from the receiving chamber were collected for metformin and mannitol quantification and replaced with an equal volume of the appropriate KRH buffer. Inhibitors studied (HCQ, BRI, PYR, CIM and FAM) were applied to the basal, apical or both chambers at the start of the transwell study as specified in the figure legends. Throughout the transepithelial flux time-course, cells were maintained in a water bath at 37°C under gentle shaking. At the end of the experiment (120 minutes), inserts were washed three times with ice-cold KRH buffer and cells were lysed using 1M NaOH and then neutralized with 1M HCl after 1 hour. Radioactivity of the cell lysate and buffer aliquots collected during the transcellular transport time course was measured by a Tri-Carb Liquid Scintillation Counter (Perkin Elmer, Waltham, MA). Intracellular accumulation of metformin was normalized to total protein content in cell lysate, which was measured by the BCA method.

Functionality of the hOCT2 and hMATE1 transporters in the double-transfected MDCK cells was confirmed by a much higher metformin transepithelial flux and $P_{\text{app}}$ (> 19-fold) from the B-to-A direction when compared to the A-to-B direction (supplementary Fig. S1). The minimal permeability ($< 1.0 \times 10^{-6}$ cm/s) of the paracellular marker ($[3H]$-mannitol) over the 120-minute transwell assay, as well as the linear nature of the $^{14}$C-metformin transepithelial transport were used as indicators of the viability and stability of the cells throughout the transwell experiment.
Inhibition studies in HEK293 cells were performed in triplicate and repeated at least three times independently. Transwell studies using MDCK cells were performed in two individual apparatuses and were repeated at least three times. Data is reported as mean ± SD. Data was plotted and fitted through nonlinear regression using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA). IC$_{50}$ values were obtained by fitting the data to the following four-parameter dose-dependent inhibition equation:

\[
V = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{[1 + (I/\text{IC}_{50})^H]}
\]

where $V$ is the rate of uptake – or apparent permeability ($P_{\text{app}}$) value – in the presence of inhibitor, Bottom is the residual non inhibitable baseline value constrained to values higher or equal to zero, Top is the rate of uptake – or $P_{\text{app}}$ value – in the absence of inhibitor, $I$ is the inhibitor concentration, and $H$ is the Hill coefficient. For the transwell experiments, the $P_{\text{app}}$ of metformin was calculated using the following equation:

\[
P_{\text{app}} = \frac{(dQ/dt)}{(A \times C_0)}
\]

where $dQ/dt$ is the amount of metformin transported over time, $A$ is the insert membrane surface area, and $C_0$ is the initial metformin concentration in the donor chamber. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s post hoc test to correct for multiple comparisons. A $P$ value less than 0.05 was considered statistically significant.

**Prediction of hOCT2 and hMATE1-mediated DDIs with Metformin**
The effect of inhibition of hOCT2 and hMATE1 on plasma exposure of metformin was assessed using the mechanistic static model previously proposed by Feng et al. (Feng et al., 2013; Feng and Varma, 2016):

\[
AUCR = \frac{1}{1 - f_{sec} \cdot \frac{[I_{u,\text{max}}]/IC_{50}}{1+[I_{u,\text{max}}]/IC_{50}}}
\]

Where AUCR represents the area under the concentration-time curve ratio in the presence over absence of an inhibitor, \([I_{\text{max,u}}]\) represents the plasma maximum inhibitor unbound concentration, \(IC_{50}\) represents the concentration of inhibitor necessary to inhibit 50% of either individual transporter activity or transepithelial flux, and \(f_{sec}\) represents the fraction of metformin’s renal clearance mediated by secretion in the absence of inhibitors. In our calculations, glomerular filtration rate of healthy young adults was assumed to be 120 mL/min, and metformin \(f_{sec}\) was calculated as 0.8 considering that metformin is exclusively renally eliminated, has a renal clearance of 600 mL/min (Bristol-Myers Squibb Company. Glucophage (metformin hydrochloride) [package insert], 2017) and is negligibly bound to plasma proteins.

**Estimation of Compounds Physicochemical Properties**

Strongest basic pKa values for tested inhibitors were collected from [https://go.drugbank.com/](https://go.drugbank.com/). Calculated LogP values (computed by XLogP3 3.0) were collected from [https://pubchem.ncbi.nlm.nih.gov/](https://pubchem.ncbi.nlm.nih.gov/). LogD at pH 6.0 and 7.4 were calculated using ChemAxon’s Calculator Plugins. Percentage of ionization at pH 7.4 was calculated using the Henderson-Hasselbalch equation.

**RESULTS**

*Hydroxychloroquine (HCQ) and Brigatinib (BRI) selectively inhibit hMATE1.*
In order to evaluate the impact of inhibitor intracellular accessibility on hMATE1 inhibition in the double-transfected system, it is necessary to have compounds that are selective for hMATE1 inhibition. To expand the profile of hMATE1 selective inhibitors, we chose HCQ and BRI based on literature evidence suggesting these compounds may preferentially inhibit hMATEs over hOCT2 (Takeda Pharmaceutical Company Ltd. ALUNBRIG (brigatinib) [package insert], 2020; Yee et al., 2021). Next, we determined IC$_{50}$ values of HCQ and BRI towards hOCT2 and hMATE1 in HEK293 cells expressing a single transporter. Metformin was used as a probe substrate at concentration well below its apparent affinity (K$_{m}$) value (Yin et al., 2016). For hOCT2-expressing cell line, experiments were performed at pH 7.4 and for hMATE1-expressing cell line, experiments were performed at pH 8.0 (Tanihara et al., 2007). Our results showed that both HCQ and BRI preferentially inhibit hMATE1 (Fig. 2). HCQ inhibited hMATE1 with a calculated IC$_{50}$ of 2.4 ± 1.3 µM, and no significant inhibition of hOCT2 was observed up to 100 µM, in agreement to what was previously reported by Yee et al., 2021. BRI was found to be approximately 10-fold more potent towards hMATE1 than hOCT2, with IC$_{50}$ values of 0.63 ± 0.24 µM and 6.5 ± 2.3 µM respectively.

**MATE1 inhibition by HCQ and BRI does not translate to inhibition of metformin flux in hOCT2/hMATE1 double-transfected MDCK cells.**

Next, we tested the inhibitory potential of HCQ and BRI towards the B-to-A transepithelial flux of metformin in a hOCT2/hMATE1 double-transfected MDCK polarized cells in a Transwell system. A HCQ concentration of 40 µM and BRI concentration of 6.25 µM were selected for this study. Based on the IC$_{50}$ values determined in single-transfected nonpolarized HEK293 cells, at these concentrations HCQ was expected to completely inhibit hMATE1, with no impact on hOCT2 transport (Fig. 2A), and BRI would lead to near complete inhibition of hMATE1 but
only partial inhibition of hOCT2 (Fig. 2B). In addition, due to stronger hMATE1 inhibition, both
264 drugs were expected to lead to an increase in metformin intracellular accumulation. To assess
265 inhibitor accessibility, inhibitors were applied to the apical, basolateral or to both transwell
266 chambers. Interestingly, contrary to the prediction, HCQ had no or minimal impact on
267 metformin B-to-A transepithelial flux regardless of which chamber it was applied to (Fig 3A).
268 Similarly, BRI had no or minimal impact on metformin B-to-A flux over the two-hour time
269 course (Fig. 3B). When $P_{\text{app}}$ of metformin was calculated, no inhibitory effect was observed by
270 either HCQ or BRI, except for a small inhibitory effect of HCQ and BRI when applied to the
271 basal chamber (approximately 15% and 25% reduction respectively) (Fig. 4A/B). The small
272 inhibitory effect observed by basally added BRI is likely due to partial inhibition of basolateral
273 hOCT2. Consistent with the lack of significant hMATE1 inhibition, there were no changes in
274 metformin intracellular accumulation in the presence of either inhibitor (Fig. 4C/D). These
275 results demonstrate that although HCQ and BRI are potent hMATE1 inhibitors in the single-
276 transfected cell system, these compounds are unable to inhibit hMATE1 in the hOCT2/hMATE1-
277 transfected MDCK monolayer.

Classic MATE/OCT Inhibitors Dose-Dependently Reduced Metformin Transepithelial Flux in
278 Double-transfected cells.

The lack of inhibition by HCQ and BRI observed in the double-transfected system indicates that
280 these compounds are less likely to produce significant DDIs involving hOCT2/hMATE1 in vivo.
281 Currently clinical metformin DDI studies using these compounds as perpetrators are not
282 available in literature. However, clinical DDI studies have been conducted for several classical
283 inhibitors of the renal organic cation secretion system. Therefore, we have explored the
284 usefulness of the transwell system employing hOCT2/hMATE1 double-transfected MDCK cells
in the assessment of DDI potential of pyrimethamine (PYR) and cimetidine (CIM), compounds recognized by the FDA as classic OCT2 and MATE1 inhibitors. We also chose to evaluate famotidine (FAM), which was reported by Hibma et al. as a selective and potent in vitro hMATE1 inhibitor that did not lead to significant DDI with metformin despite reaching plasma \( I_{\text{max,u}} \) 4-fold higher than its reported IC\(_{50}\) value (Supplementary Table S1) (Hibma et al., 2016).

The IC\(_{50}\) values used for pyrimethamine (PYR) and cimetidine (CIM) in Table 1 were previously generated in our laboratory in the same hOCT2 and hMATE1 stable-transfected HEK293 cell lines (Yin et al., 2016). To confirm the inhibitory potency of FAM and to account for inter-laboratory variability in the IC\(_{50}\) determination, we also determined FAM IC\(_{50}\) towards hOCT2 and hMATE1 and collected literature data from the Certara® Drug Interactions Database (DIDB) regarding hMATE1 inhibition. The IC\(_{50}\) values determined in our laboratory confirmed preferential inhibition of hMATE1 by FAM, with hOCT2 IC\(_{50}\) > 100 \( \mu \text{M} \) and hMATE1 IC\(_{50}\) = 3.0 ± 1.0 \( \mu \text{M} \) (Table 1 and supplementary Fig. S2), which is in the range of the hMATE1 IC\(_{50}\) values collected from DIDB (metformin as substrate, ranging 0.25 to 3.11 \( \mu \text{M} \)) (supplementary Fig. S2). We next determined the dose-dependent effect of PYR, CIM and FAM in metformin transepithelial flux and intracellular accumulation in the double-transfected MDCK system. Inhibitors were applied to both apical and basal chambers simulating the in vivo microenvironment of the nephron where inhibitors are present in both the plasma and the filtrate.

As shown in Figure 5, PYR and CIM dose-dependently inhibited metformin transepithelial flux and increased metformin intracellular accumulation up to 3-fold in comparison to the absence of inhibitor, suggesting predominant inhibition of apical hMATE1. FAM, however, had minimal impact in metformin transepithelial flux, and led to only a marginal increase in metformin intracellular accumulation at the highest concentration tested (40 \( \mu \text{M} \)), which is 13-fold above its
IC₅₀ value towards hMATE1 (3.0 µM). These results are consistent with outcomes from clinical DDI studies, in which co-administration of PYR or CIM with metformin led to a significant increase in metformin AUC, whereas FAM had no impact on metformin exposure (Somogyi et al., 1987; Kusuhara et al., 2011; Hibma et al., 2016).

**Flux IC₅₀ (IC₅₀,flux) Values Obtained from Transwell Studies More Accurately Predicted in vivo metformin DDI.**

The results from our transwell inhibition study suggest that the hOCT2/hMATE1 double-transfected in vitro system has significant predictive value. To further assess if this approach can be used to quantitatively predict the degree of hOCT2/hMATE1 inhibition in vivo, we propose the calculation of a flux IC₅₀ (IC₅₀,flux) based on the dose-dependent impact of inhibitor on metformin Pₐₚ values. The IC₅₀,flux takes into consideration the overall impact of the inhibitor on metformin secretion clearance mediated sequentially by hOCT2 and hMATE1. Metformin Pₐₚ values were calculated using Equation 2 at varying inhibitor concentrations, and IC₅₀,flux values were then estimated from the dose response curves (Fig. 6 and Table 1). As shown in Table 1, the IC₅₀,flux values obtained from Pₐₚ are different than those IC₅₀ reported for either transporter using single-transfected HEK293 cells. According to the latest 2020 FDA guidance, an in vivo DDI study is recommended if Iₘₐₓ,u/IC₅₀ ≥ 0.1 for either hOCT2 or hMATE1. To determine whether the use of different in vitro systems (uptake in single-transfected cell line vs. transwell flux in double-transfected cells) leads to different recommendations for in vivo DDI assessment, the Iₘₐₓ,u/IC₅₀ values of CIM, PYR and FAM were calculated using IC₅₀ values determined from hOCT2-transfected, hMATE1-transfected or hOCT2/hMATE1 double-transfected cell lines (Table 1). While the use of hOCT2 IC₅₀ values led to calculated ratios below 0.1, the use of hMATE1 IC₅₀ values resulted in ratios well above the 0.1 cutoff value (1.36, 5.1 and 0.33 for
PYR, CIM and FAM respectively), leading to recommendations of in vivo DDI studies for all three compounds. In contrast, when using the IC\(_{50,\text{flux}}\) values, the calculated ratios resulted in positive DDI prediction for PYR and CIM (0.29 and 0.36 \(I_{\text{max},u}/IC_{50,\text{flux}}\) respectively), but not for FAM (\(I_{\text{max},u}/IC_{50,\text{flux}} < 0.025\)). The recommendation based on transwell inhibition studies matches the results observed in clinical metformin DDI studies (Table 1). Next, we predicted the percent change in metformin AUC using a static model (Equation 3). As shown in Table 1, while the prediction using hOCT2 IC\(_{50}\) value for FAM is consistent with in vivo metformin DDI observation, the use of these values for PYR and CIM led to 35-fold and 8-fold underprediction respectively. For hMATE1, the use of hMATE1 IC\(_{50}\) led to overprediction of PYR and CIM-mediated DDIs by 2.4 and 4-fold respectively, and a false prediction for FAM. In contrast, predictions using the IC\(_{50,\text{flux}}\) values were all within 2-fold of the observed AUC change from in vivo DDI studies (Table 1). This data suggests that compared to prediction based on individual transporter IC\(_{50}\) values, the use of IC\(_{50,\text{flux}}\) values obtained from hOCT2/hMATE1 double-transfected cells more accurately predicted in vivo metformin DDI in both a qualitative and a quantitative manner.

**Considerations on Physicochemical Properties, Membrane Permeability and Substrate Status May Help Explain Inhibition Discrepancies.**

An interesting observation in our study is that the degree to which our IC\(_{50,\text{flux}}\) values differed from IC\(_{50}\) values obtained from hMATE1-transfected cells varied significantly among the inhibitor studied (5-fold for PYR, 14-fold for CIM and >13-fold for FAM) (Table 1). Such differences are likely rooted in their different ability to enter the cells, either by passive diffusion or/and carrier-mediated transport, to access the hMATE1 inhibition site (Fig. 1). We thus examined the physicochemical properties of the inhibitors explored in this study as an indicator.
of their ability to cross membrane by passive diffusion (Table 2). All inhibitors are weak bases ionized to different degrees under physiological pH. FAM, HCQ and BRI are highly ionized (>90%), while PYR and CIM are ionized to a lower extent. LogD values were calculated at physiologically relevant pH of 7.4 (blood) and 6.0 (urine). LogP and LogD values suggest that CIM, FAM and HCQ are highly hydrophilic, whereas PYR and BRI are more lipophilic. The low molecular weight of PYR in combination with its lipophilicity indicates that PYR may be successful in passively permeating through the membrane and reaching hMATE1 site of inhibition. Despite its high lipophilicity, BRI is a large molecule (molecular weight > 500 g/mol), which could limit its passive permeability (Lipinski et al., 2001) and potentially explain its lack of inhibition in the double-transfected system. In contrast, CIM, FAM and HCQ are small and highly hydrophilic molecules, which have difficulty crossing the membrane without the assistance of an uptake transporter. This could explain the lack of inhibitory effect of FAM and HCQ in the double-transfected system. CIM is the exception, but it can enter the cells through carrier-mediated uptake as CIM has been previously reported to be a substrate of hOCT2 and to accumulate in hOCT2/hMATE1-transfected MDCK cells (Yin et al., 2016). Together this data suggests that the ability of the inhibitor to enter cells, either by passive diffusion or carrier-mediated uptake, is an important determinant in its ability to produce clinically significant DDIs through hMATE1 inhibition in vivo.

DISCUSSION

Accurate prediction of hOCT2/hMATEs-mediated renal DDIs based on in vitro data remains a challenge (Mathialagan et al., 2021; Krishnan et al., 2022). Current DDI risk assessment relies on the use of plasma \( I_{\text{max,u}} \) and IC\(_{50} \) determined for a single transporter, which
frequently leads to false or overprediction for hMATEs. In this study, we explored the use of 
hOCT2/hMATE1 double-transfected MDCK polarized cells in a transwell system as a new in 
vitro tool for DDI risk assessment. A new inhibition parameter (IC$_{50,\text{flux}}$) reflecting inhibitor’s 
overall impact on renal secretory clearance was obtained and used to predict in vivo metformin 
DDIs.

hMATE functions as a proton/organic cation antiporter, with proton binding occurring on 
the opposite side from substrate binding. In inhibition studies using nonpolarized hMATE1 
single-transfected HEK293 cells, the extracellular or intracellular pH is modified to create an 
outwardly directed proton gradient, driving hMATE1 to function as an uptake transporter. In this 
state, the organic cation binding site is exposed to the extracellular space, allowing IC$_{50}$ 
determination through conventional uptake studies where substrates and inhibitors are both 
added to the extracellular side. PTECs, however, are polarized cells in which hMATE1 is 
expressed in the apical membrane and mediates organic cation efflux utilizing the inwardly 
directed physiologic proton gradient. In this state, organic cation binds from the intracellular 
side whereas proton binds from the lumen side. Our previous work suggested that in the 
physiological state, hMATE1 inhibitor binding occurs from the intracellular side (Yin et al., 
2016). Our inhibition study with HCQ and BRI further corroborate to this observation as these 
potent hMATE1 inhibitors (Fig. 2) showed no inhibitory effect on metformin B-to-A flux when 
applied apically at concentrations much higher than their IC$_{50}$ determined in single-transfected 
cells (Fig. 3 and 4). Collectively, these data strongly suggest that when hMATE1 functions as an 
efflux transporter, inhibitors must be present intracellularly, i.e. at the same side with the 
substrate, to exert an inhibitory effect.
hMATEs inhibitors can gain intracellular access via passive diffusion or transporter-mediated uptake. Their intracellular concentrations may be additionally influenced by intracellular binding/sequestration and transporter-mediated efflux (Fig. 1). The lack of inhibitory effect of HCQ and BRI on metformin B-to-A flux (Fig. 3 and 4) indicates that they may not reach sufficient intracellular concentration to inhibit hMATE1. HCQ is highly ionized at physiological pH and has a low LogD, limiting its passive membrane permeability. BRI is also highly ionized, and although it has an intermediate LogD, its larger molecular size may also limit its passive permeability (Lipinski et al., 2001) (Table 2). Neither HCQ or BRI have been reported as a substrate of the basolateral uptake transporter hOCT2. Instead, both were reported as substrates of the efflux transporter P-glycoprotein (P-gp) present in human kidney (Li et al., 2018; Weiss et al., 2020) and MDCK cells (Goh et al., 2002). Additionally, HCQ is known to accumulate within lysosomes (Derendorf, 2020). Together, these factors may contribute to low unbound intracellular concentrations resulting in the lack of hMATE1 inhibition in the double-transfected system. These data also suggests that HCQ and BRI may not affect metformin renal secretion in vivo. Unfortunately, no in vivo metformin DDI information is available for HCQ or BRI to corroborate with our hypothesis.

PYR and CIM are classic inhibitors of the renal organic cation secretion system known to impact metformin exposure in vivo (Somogyi et al., 1987; Kusuhara et al., 2011). Both PYR and CIM display much higher inhibitory potency towards hMATE1 than for hOCT2 (Table 1). FAM is also a potent hMATE1 inhibitor (IC\textsubscript{50} = 3.0 µM) in vitro but has no in vivo effect on metformin exposure (Hibma et al., 2016). When evaluated in our polarized double-transfected transwell system, PYR and CIM dose-dependently inhibited metformin P\textsubscript{app} (Fig. 5A/B). The observed increase in metformin intracellular accumulation (Fig. 5D/E) further suggests that the
main site of inhibition is hMATE1. In contrast, FAM had minimal impact on metformin transepithelial flux and intracellular accumulation at concentrations up to 40 µM (Fig. 5C/F), demonstrating a disconnect between inhibition in nonpolarized single-transfected cells in a standard well plate versus polarized double-transfected cells in a transwell system. These observations corroborate with clinical DDI study outcomes, in which co-administration of PYR or CIM with metformin led to a significant increase in metformin AUC whereas FAM did not (Somogyi et al., 1987; Kusuhara et al., 2011; Hibma et al., 2016).

The different impact of these three inhibitors on metformin flux could be explained by their inherent characteristics impacting intracellular accessibility (Fig.1). PYR is a small lipophilic molecule only partially ionized at pH 7.4 (Table 2), and thus more likely to enter cells by passive diffusion. In contrast, FAM is predominantly ionized at physiologic pH, highly hydrophilic (LogD$_{7.4}$ = -2.6), is a substrate of P-gp (Dahan and Amidon, 2009), and is not (or is a poor) hOCT2 substrate (Motohashi et al., 2004; Tahara et al., 2005). These factors likely contribute to poor FAM intracellular accessibility and lack of hMATE1 inhibition in the transwell system (Fig. 5 and 6). Although CIM is also a small and highly hydrophilic compound, it is a substrate of hOCT2 (Tahara et al., 2005; Tanihara et al., 2007). Previously, we demonstrated that hOCT2-mediated uptake is crucial for granting CIM intracellular access to inhibit hMATE1 (Yin et al., 2016), given that CIM accumulated intracellularly when applied basally and also failed to inhibit transepithelial flux of substrates when added apically to the double-transfected cells in the transwell system. Hence, despite CIM’s low passive permeability, the presence of hOCT2 in our double-transfected system facilitates CIM intracellular accessibility, resulting in the observed dose-dependent inhibition of metformin $P_{app}$ (Fig. 5 and 6).
Our results suggest that the use of unbound intracellular inhibitor concentrations instead of plasma $I_{\text{max,u}}$ could better predict hMATE-mediated DDIs. However, determination of such concentrations within PTECs can be challenging and impractical during early drug-development. We thus proposed a new approach that accounts for inhibitor’s intracellular accessibility and allows the prediction of DDI potential using plasma inhibitor concentrations. In this approach, a new inhibition parameter ($IC_{50,\text{flux}}$) reflecting inhibitor’s potency on overall renal secretory clearance was determined in hOCT2/hMATE1 double-transfected MDCK polarized cells in a Transwell system (Fig. 6). The $IC_{50,\text{flux}}$ directly links the inhibitory effect on substrate flux with extracellular inhibitor concentrations in the transport buffer, which reflects plasma $I_{\text{max,u}}$ (Fig. 1).

As shown in Table 1, the $IC_{50,\text{flux}}$ values calculated from metformin $P_{\text{app}}$ for PYR, CIM and FAM were quite different from $IC_{50}$ obtained by uptake studies in single transporter-transfected cells, with difference being 5-fold for PYR, 14-fold for CIM and >13-fold for FAM for hMATE1. In quantitative prediction of in vivo DDIs using a static prediction model (Equation 3), the use of $IC_{50,\text{flux}}$ performed significantly better than individual transporter $IC_{50}$ values for PYR and CIM (Table 1). Importantly, it accurately predicted the lack of in vivo DDI for FAM, which would otherwise be incorrectly predicted when using hMATE1 $IC_{50}$. Therefore, the $IC_{50,\text{flux}}$ approach could mitigate the risk of false and over-predictions commonly observed for predicting hMATE1-mediated DDIs, reducing the burden of unnecessary and costly clinical DDI investigations.

The use of flux studies in hOCT2/hMATE1 double-transfected MDCK cells and the $IC_{50,\text{flux}}$ approach have several advantages for predicting renal transporter-mediated DDIs. First, the system is robust for identifying hMATE1 inhibitors that are likely to lead to false DDI prediction as it accounts for inhibitor intracellular accessibility without the need to measure
intracellular inhibitor concentrations. Second, it measures the overall inhibitory effect on hOCT2/hMATE1-mediated secretory clearance without assuming a single transporter as the site of interaction. Third, intracellular substrate accumulation can be measured, which could serve as a useful indicator of intrarenal drug accumulation and drug-induced nephrotoxicity. Lastly, compared to other emerging in vitro platforms such as cultured organoids and microphysiological systems, which require expensive human renal cells and specialized culturing techniques, the double-transfected MDCK system is less complex, cost-effective, and scalable for high throughput screening, which could be incorporated into the current scheme of in vitro DDI assessments during preclinical drug development. A similar concept can be implemented to predict DDIs for other transport pathways such as hOATP1B/hMRP2 in human hepatocytes.

Our approach nevertheless has limitations. While our results indicate that some inhibitors (i.e. FAM, HCQ and BRI) do not reach sufficient unbound intracellular concentrations to inhibit MATE1 in our double-transfected system, we did not directly measure these inhibitor concentrations given that reliable measurement of unbound intracellular concentrations is challenging. We only considered hMATE1 as the apical efflux transporter. Although recent proteomic studies have reported hMATE2K expression levels are much lower than hMATE1 in human PTECs (Prasad et al., 2016; Kikuchi et al., 2021), we cannot exclude the possibility that hMATE2K may also contribute to renal DDIs. Nevertheless, the concept and approach developed in this study should be applicable to hMATE2K using double- or triple-transfected cell systems expressing hMATE2K. Additionally, our double-transfected model uses a canine-derived cell line (MDCK) that was derived from distal tubule and also expresses endogenous canine transporters (e.g. P-gp) (Goh et al., 2002). Further, it does not account for the potential role of other human PTEC transporters in the basolateral uptake of hMATE1 inhibitors.
Additionally, differences in hOCT2 and hMATE1 expression levels in MDCK-transfected cells in comparison to human PTECs could impact quantitative predictions. Finally, although IC\textsubscript{50,flux} performed well in our predictions with PYR, CIM and FAM, this strategy should be further validated with more inhibitors for which clinical DDI information is available.

In summary, our findings highlight the importance of considering inhibitor’s accessibility to intracellular space – via passive diffusion or transporter-mediated uptake – for accurate prediction of hMATE1-mediated DDI in vivo. The use of a double-transfected system and IC\textsubscript{50,flux} have the potential to bridge the gap in current prediction of hMATE1-mediated DDIs and reduce the burden of unnecessary and costly clinical DDI investigations.
DATA AVAILABILITY

Data generated and analyzed in this study are available upon request from the corresponding author.

AUTORSHIP CONTRIBUTIONS

Participated in research design: Vieira and Wang

Conducted experiments: Vieira

Performed data analysis: Vieira and Wang

Wrote or contributed to writing of the manuscript: Vieira and Wang
REFERENCES


This study was supported by the National Institutes of Health (NIH) National Institute of General Medical Sciences [Grant R01 GM066233]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The authors declare no potential conflicts of interest.
Figure 1. Representation of the renal secretion of organic cations (OC\(^+\)) sequentially mediated by hOCT2 and hMATE1. hOCT2 is expressed in the basolateral membrane of renal proximal tubule cells and mediates OC\(^+\) uptake driven by the inside-negative membrane potential. hMATE1 is expressed in the apical membrane and functions as a proton/OC\(^+\) exchanger mediating the efflux of OC\(^+\) from intracellular space to the proximal tubule lumen. \(I_{\text{max,u}}\) denotes the maximal unbound plasma concentration of the inhibitor which also presents in the filtrate due to glomerular filtration. Although \(I_{\text{max,u}}\) is often used for assessment of DDI potential, intracellular inhibitor concentration (\([I]_{\text{intra}}\)) may be more relevant for assessment of hMATE1-mediated DDI, which can be influenced by many factors.

Figure 2. Inhibition of hOCT2 and hMATE1 by hydroxychloroquine (A) or brigatinib (B) using metformin as probe substrate. Cells were incubated in KRH buffer containing \(^{14}\text{C}\)-labeled metformin (8.9 \(\mu\text{M}\)) for 2 minutes in the presence or absence of inhibitor at indicated concentrations. Transporter-specific uptake was calculated by subtracting the uptake in vector-transfected HEK293 cells and data is presented as percentage of the uptake in the absence of inhibitor. Each data point represents mean \(\pm\) SD from at least three independent experiments.

Figure 3. Effect of 40 \(\mu\text{M}\) hydroxychloroquine (A) or 6.25 \(\mu\text{M}\) brigatinib (B) on B-to-A transcellular flux of metformin in hOCT2/hMATE1-expressing MDCK cells. The pH in the basal and apical chambers were maintained at 7.4 and 6.0, respectively. Cells were incubated in KRH buffer containing \(^{14}\text{C}\)-metformin (8.9 \(\mu\text{M}\)) in the basal chamber, and B-to-A flux of metformin was measured in the absence or presence of inhibitors added to apical, basolateral or both chambers. An aliquot of buffer from the apical chamber was sampled periodically and
replenished with an equal volume of KRH buffer with or without inhibitor. Data is presented as mean ± SD of three independent experiments.

Figure 4. Effect of hydroxychloroquine and brigatinib in metformin apparent permeability (P_app) and intracellular accumulation in MDCK-hOCT2/hMATE1 cells. The B-to-A P_app value of metformin in the absence or presence of 40µM hydroxychloroquine (A) or 6.25 µM brigatinib (B) was calculated using equation 2 described in Materials and Methods. Intracellular accumulation of metformin was measured at the end of the transwell study in the absence and presence of hydroxychloroquine (C) or brigatinib (D). Inhibitors were added to apical, basal or both chambers at the beginning of the transwell experiment. Permeability and accumulation in the presence of inhibitors was compared to those in absence of inhibitor (* P < 0.05). Statistical significance was determined using one-way ANOVA followed by Dunnett’s post hoc test to correct for multiple comparisons. Data is presented as mean ± SD of three independent experiments.

Figure 5. Effect of pyrimethamine, cimetidine and famotidine in metformin B-to-A transcellular flux (A, B and C) and intracellular accumulation (D, E and F) in hOCT2/hMATE1-expressing MDCK cells. The pH in the basal and apical chambers were maintained at 7.4 and 6.0, respectively. Cells were incubated in KRH buffer containing 14C-metformin (8.9 µM) in the basal chamber in the absence or presence of inhibitors. Inhibitors were added to both apical and basal chambers at indicated concentrations. An aliquot of buffer from the apical chamber was sampled periodically up to 120 minutes and B-to-A flux of metformin was measured in the absence and presence of varying concentrations of pyrimethamine (A), cimetidine (B) and famotidine (C). At the end of the transport experiment, cells were lysed, and intracellular...
accumulation of metformin was measured in the absence and presence of pyrimethamine (D), cimetidine (E) and famotidine (F). *** $P<0.001$, **$P < 0.01$, *$P < 0.05$ indicates significantly higher accumulation in comparison to control (absence of inhibitor). Statistical significance was determined using one-way ANOVA followed by Dunnett’s post hoc test to correct for multiple comparisons. Data is presented as mean ± SD of at least three independent experiments.

**Figure 6.** Dose-dependent inhibition of metformin B-to-A $P_{app}$ in hOCT2/hMATE1 double-transfected MDCK cells by pyrimethamine, cimetidine and famotidine. $P_{app}$ values in the absence or presence of varying inhibitor concentrations were calculated using equation 2 described in *Materials and Methods*. Inhibitors were added to both basal and apical chambers at the start of the transwell experiment at indicated concentrations. Each data point represents mean ± SD from three to four independent experiments.
## Table 1. Prediction of hOCT2 and hMATE1 mediated DDIs utilizing IC$_{50}$ values determined in single- vs. double-transfected cells (IC$_{50,\text{flux}}$). Data is presented as mean ± SD from three or four independent experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Transporter assessed</th>
<th>IC$_{50}$ (µM)</th>
<th>$I_{\text{max,u}}$/IC$_{50}$</th>
<th>Predicted change in Metformin AUC (%)</th>
<th>Observed change in Metformin AUC (%)</th>
<th>Difference from observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYR</td>
<td>hOCT2</td>
<td>22.9 A</td>
<td>0.01</td>
<td>1</td>
<td>35 B</td>
<td>35-fold ↓</td>
</tr>
<tr>
<td></td>
<td>hMATE1</td>
<td>0.22 A</td>
<td>1.36</td>
<td>85</td>
<td></td>
<td>2.4-fold ↑</td>
</tr>
<tr>
<td></td>
<td>hOCT2/hMATE1 (Flux)</td>
<td>1.04 ± 0.15</td>
<td>0.29</td>
<td>22</td>
<td></td>
<td>1.6-fold ↓</td>
</tr>
<tr>
<td>CIM</td>
<td>hOCT2</td>
<td>93.5 A</td>
<td>0.08</td>
<td>6</td>
<td>50 C</td>
<td>8.3-fold ↓</td>
</tr>
<tr>
<td></td>
<td>hMATE1</td>
<td>1.5 A</td>
<td>5.1</td>
<td>202</td>
<td></td>
<td>4-fold ↑</td>
</tr>
<tr>
<td></td>
<td>hOCT2/hMATE1 (Flux)</td>
<td>21.4 ± 10.0</td>
<td>0.36</td>
<td>27</td>
<td></td>
<td>1.9-fold ↓</td>
</tr>
<tr>
<td>FAM</td>
<td>hOCT2</td>
<td>&gt; 100</td>
<td>&lt; 0.01</td>
<td>&lt; 1</td>
<td>0 D</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>hMATE1</td>
<td>3.0 ± 1.0</td>
<td>0.33</td>
<td>25</td>
<td></td>
<td>False prediction</td>
</tr>
<tr>
<td></td>
<td>hOCT2/hMATE1 (Flux)</td>
<td>&gt; 40</td>
<td>&lt; 0.025</td>
<td>&lt; 2</td>
<td></td>
<td>No difference</td>
</tr>
</tbody>
</table>

$I_{\text{max,u}}$ used in calculations for PYR, CIM and FAM are 0.3, 7.6 and 1 µM respectively. Further details of these values can be found in Supplementary Table S1. Predicted change in metformin AUC was calculated based on equation 3 described in Materials and Methods. Overprediction or underprediction are respectively indicated by ↑ or ↓.

- A Value from Yin et al., 2016.
- B Value from Kusuhara et al., 2011.
- C Value from Somogyi et al., 1987.
- D Value from Hibma et al., 2016.
Table 2. Physicochemical properties and membrane permeability values of compounds investigated in the present study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (g/mol)</th>
<th>pKa</th>
<th>% ionized at pH 7.4</th>
<th>LogP (XLogP3 3.0)</th>
<th>LogD (ChemAxon) pH 7.4</th>
<th>LogD (ChemAxon) pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYR</td>
<td>248.7</td>
<td>7.34</td>
<td>46.6</td>
<td>2.7</td>
<td>2.25</td>
<td>1.19</td>
</tr>
<tr>
<td>CIM</td>
<td>252.3</td>
<td>6.91</td>
<td>24.4</td>
<td>0.4</td>
<td>-0.25</td>
<td>-0.84</td>
</tr>
<tr>
<td>FAM</td>
<td>337.5</td>
<td>8.38</td>
<td>90.5</td>
<td>-0.6</td>
<td>-2.6</td>
<td>-3.35</td>
</tr>
<tr>
<td>HCQ</td>
<td>335.9</td>
<td>9.7</td>
<td>99.5</td>
<td>3.6</td>
<td>0.62</td>
<td>-3.7</td>
</tr>
<tr>
<td>BRI</td>
<td>584.1</td>
<td>8.54</td>
<td>93.2</td>
<td>4.6</td>
<td>2.49</td>
<td>1.1</td>
</tr>
</tbody>
</table>

MW – molecular weight. Only the strongest basic pKa is reported. Percent ionized at pH 7.4 was calculated using the Henderson-Hasselbalch equation.
Figure 1

Blood pH 7.4

-70 mV

Proximal Tubule lumen pH 6.0 - 6.8

Influenced by:
- Passive permeability
- Uptake/efflux by transporters
- Binding to intracellular proteins
- Sequestration into organelles

$\text{I}_{\text{max,u}}$

$\text{hOCT2}$

$\text{hMATE1}$

$\text{OC}^+$

$\text{OC}^+$

$[\text{I}]_{\text{intra}}$

$\text{H}^+$

$\text{I}_{\text{max,u}}$
Figure 2

A

\[
\text{\textsuperscript{14}C-Metformin uptake (\% of Control)}
\]

\[
[\text{Hydroxychloroquine (\mu M)}]
\]

- hMATE1
- hOCT2

B

\[
\text{\textsuperscript{14}C-Metformin uptake (\% of Control)}
\]

\[
[\text{Brigatinib (\mu M)}]
\]

- hMATE
- hOCT2
Figure 3

A Hydroxychloroquine

B Brigatinib

$^{14}$C-Metformin B to A Transport (pmol/cm$^2$)

Time (min)
Figure 4

Hydroxychloroquine

A

B

Brigatinib

C

D

Metformin (P<sub>app</sub> x 10<sup>-5</sup> cm/sec)

No Inhibitor, Apical, Basal, Both

No Inhibitor, Apical, Basal, Both

Intracellular Metformin (pmol/mg protein at 120 min)

No Inhibitor, Apical, Basal, Both

No Inhibitor, Apical, Basal, Both

* indicates significant difference.
Figure 5

A. Pyrimethamine

B. Cimetidine

C. Famotidine

D. Pyrimethamine (µM)

E. Cimetidine (µM)

F. Famotidine (µM)
Figure 6

![Graph showing the concentration of Metformin Papp versus concentration in μM for Pyrimethamine, Cimetidine, and Famotidine.](image-url)