Impact of Substrate-Dependent Inhibition on Renal Organic Cation Transporters hOCT2 and hMATE1/2-K-Mediated Drug Transport and Intracellular Accumulation[S]

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ABSTRACT

Renal transporter–mediated drug-drug interactions (DDIs) are of significant clinical concern, as they can adversely impact drug disposition, efficacy, and toxicity. Emerging evidence suggests that human renal organic cation transporter 2 (hOCT2) and multidrug and toxin extrusion proteins 1 and 2-K (hMATE1/2-K) exhibit substrate-dependent inhibition, but their impact on renal drug secretion and intracellular accumulation is unknown. Using metformin and atenolol as the probe substrates, we found that the classic inhibitors (e.g., cimetidine) of renal organic cation secretion were approximately 10-fold more potent for hOCT2 when atenolol was used, suggesting that atenolol is a more sensitive in vitro substrate for hOCT2 than metformin. In contrast, inhibition of hMATE1/2-K was influenced much less by the choice of substrate. Cimetidine is a much more potent inhibitor for hMATE1/2-K when metformin is the substrate but acts as an equally potent inhibitor of hOCT2 and hMATE1/2-K when atenolol is the substrate. Using hOCT2/hMATE1 double-transfected Madin-Darby canine kidney cells, we evaluated the impact of substrate-dependent inhibition on hOCT2/hMATE1-mediated transepithelial flux and intracellular drug accumulation. At clinically relevant concentrations, cimetidine dose dependently inhibited basal-to-apical flux of atenolol and metformin but impacted their intracellular accumulation differently, indicating that substrate-dependent inhibition may shift the major substrate-inhibitor interaction site between apical and basolateral transporters. Cimetidine is effective only when applied to the basal compartment. Our findings revealed the complex and dynamic nature of substrate-dependent inhibition of renal organic cation drug transporters and highlighted the importance of considering substrate-dependent inhibition in predicting transporter-mediated renal drug interaction, accumulation, and toxicity.

Introduction

Renal excretion is a major elimination pathway for many drugs and drug metabolites. Besides glomerular filtration, circulating drugs are actively secreted by carrier-mediated pathways in the renal proximal tubules. In humans, secretion of organic cation (OC) drugs is primarily accomplished by basolateral uptake via the electrogenic human organic cation transporter 2 (hOCT2) followed by apical efflux via the proton/OC exchangers human multidrug and toxin extrusion proteins 1 and 2-K (hMATE1 and 2-K) (Li et al., 2006; Giacomini et al., 2010; Morrissey et al., 2013; Motohashi and Inui, 2013). Anionic drug molecules, on the other hand, are generally first transported into tubular cells by the basolateral organic anion transporters 1 and 3 (hOAT1 and 3) and then effluxed into the lumen by apical transporters such as the

multidrug resistance-associated proteins 2 and 4 (Li et al., 2006; Giacomini et al., 2010; Morrissey et al., 2013). These kidney transporters are important pharmacokinetic and pharmacodynamic determinants for a wide array of clinically used drugs (Giacomini et al., 2010; Morrissey et al., 2013). Furthermore, an imbalance between transporter-mediated uptake and efflux may result in drug accumulation in proximal tubule cells, leading to drug-induced nephrotoxicity and kidney injury (Li et al., 2006; Morrissey et al., 2013).

Numerous clinically significant drug-drug interactions (DDIs) in the kidney are attributed to the inhibition of renal organic cation or anion secretion systems (Masereeuw and Russel, 2001; Li et al., 2006; Morrissey et al., 2013). Historically, cimetidine has been used as the classic inhibitor of the OC system, whereas probenecid is the prototypical inhibitor of the anion system (Masereeuw and Russel, 2001; Li et al., 2006; Morrissey et al., 2013). Renal transporter–mediated DDIs are of significant clinical concern, as they can adversely impact drug disposition, efficacy, and toxicity. Recognizing the importance of transporters in drug disposition and interactions, the US Food and Drug Administration (FDA) and the International Transporter Consortium (ITC) have published a

ABBREVIATIONS: C_{max}, maximal plasma concentration; DDI, drug-drug interaction; FDA, Food and Drug Administration; HEK, human embryonic kidney; hMATE, human multidrug and toxin extrusion protein; hOAT, human organic anion transporter; hOCT, human organic cation transporter; KRH, Krebs-Ringer-Henseleit; MDCK, Madin-Darby canine kidney; MPP$^+$, 1-methyl-4-phenylpyridinium; NME, new molecular entity; OC, organic cation; TEER, transepithelial electrical resistance.

series of recommendations to guide industry in assessing the drug interaction potentials of new molecular entities (NMEs) toward clinically important transporters, including hOCT2, hOAT1/3, and hMATE1/2-K (Giacomini et al., 2010; Zhang et al., 2011; FDA, 2012; Brouwer et al., 2013; Hillgren et al., 2013). In general, if an NME is an in vitro inhibitor for these transporters and its unbound maximal plasma concentration (C_{max}) is greater than one-tenth of its half-maximal inhibitory concentration (IC_{50}), further in vivo DDI assessment is recommended (Giacomini et al., 2010; FDA, 2012). A key parameter in the prediction of DDI risk is the IC_{50} (or the inhibition constant K_i) of the NME, which is typically determined in transporter-expressing cell lines using a recommended probe substrate (Brouwer et al., 2013). Several in vitro substrates, including metformin and 1-methyl-4-phenylpyridinium (MPP^+), have been recommended as the probe substrates in preclinical DDI assessment with hOCT2 and hMATEs (FDA, 2012; Hillgren et al., 2013). This approach assumes that the K_i or IC_{50} value of an NME determined with a probe substrate is a constant and can be extrapolated to predict the in vivo interaction of the NME with clinically used drugs. However, several drug-metabolizing enzymes, including CYP3A4 and 2D6, are known to exhibit substrate-dependent inhibition, where the K_i or IC_{50} value of an inhibitor can vary considerably depending on the probe substrate used (Kenworthy et al., 1999; VandenBrink et al., 2012). To mitigate the risk of false-negative prediction, the use of two or more probe substrates or a sensitive drug substrate for these enzymes has been suggested (Yuan et al., 2002; FDA, 2012).

Emerging evidence suggests substrate-dependent inhibition can also occur with drug transporters (Belzer et al., 2013; Martinez-Guerrero and Wright, 2013; Izumi et al., 2015). Using metformin and several experimental compounds as probe substrates, Wright and coworkers showed that inhibition of hOCT2 and hMATE1 is substrate-dependent (Belzer et al., 2013; Martinez-Guerrero and Wright, 2013). However, except for metformin, all other substrates used in these studies were experimental compounds not used in humans, making it difficult to infer the clinical impact of substrate-dependent inhibition on DDI prediction. Moreover, the influence of substrate-dependent inhibition on overall tubular secretion has never been studied. Its impact on intracellular drug accumulation, which is an important predictor for drug-induced nephrotoxicity, is also unknown. In this study, we investigated the influence of probe substrate choice on predicting transporter-mediated DDIs using metformin and a newly identified drug substrate of hOCT2 and hMATEs, atenolol (Yin et al., 2015). The overall impact of substrate-dependent inhibition on tubular secretion and intracellular accumulation was also investigated using a double-transfected cell culture model of renal OC secretion.

Materials and Methods

Materials. [³H]Atenolol (3.3 Ci/mmol) and [¹⁴C]metformin (98 mCi/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). [³H]Cimetidine (80 Ci/mmol) and [³H]mannitol (20 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade.

Cell Lines and Cell Culture. Control (pcDNA5-transfected) and Flp-In human embryonic kidney (HEK)-293 cell lines stably expressing hOCT2, hMATE1, and hMATE2-K were previously established and maintained in Dulbecco's modified Eagle's medium (Duan et al., 2015; Yin et al., 2015). Transporter-expressing cells generally showed transport activities much higher (∼10- to 30-fold) than control HEK-293 cells for a probe substrate (e.g., metformin or MPP^+) (Yin et al., 2015). Both transporter-expressing and control HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 150 μg/ml hygromycin B. Vectors (pcDNA3.1+ and pcDNA3.1/Hygro(+)) and hOCT2/hMATE1 double-transfected Madin-Darby canine kidney (MDCK) cell lines were previously established in our laboratory (Yin et al., 2015). MDCK cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 500 μg/ml G418, and 200 μg/ml hygromycin B. All cell lines were cultured in a humidified incubator at 37°C with 5% CO_2.

Uptake and Inhibition Assays in HEK-293 Cells. In vitro transport and inhibition studies were performed as previously described (Duan and Wang, 2010; Yin et al., 2015). Briefly, transporter-expressing and control HEK-293 cells were seeded in poly-D-lysine–coated plates and allowed to grow for 2–3 days to reach 80–90% confluence. Growth medium was then aspirated, and each well was rinsed two times with prewarmed Krebs-Ringer-Henseleit (KRH) buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM HEPES, pH 7.4). Uptake of [¹⁴C]metformin or [³H]atenolol in control and transporter-expressing HEK-293 cells were performed at 37°C in KRH for 2 minutes in the presence or absence of an inhibitor. Metformin and atenolol are highly hydrophilic compounds ($LogD_{7.4}$ of –5.5 and –2.0, respectively) with low nonspecific binding to cells and cell culture plates. The 2-minute incubation time was chosen because our previous studies showed that it is within the initial rate period of atenolol and metformin uptake for all three transporters (Yin et al., 2015). hMATE uptake studies were performed in KRH buffer with pH adjusted to 8.0 to maintain an outwardly directed proton gradient to drive cellular uptake of the OC substrate, because hMATEs function as proton/organic cation exchangers (Otsuka et al., 2005). For hOCT2, uptake was performed at pH 7.4. Uptake was terminated by washing cells three times with ice-cold KRH buffer. Cells were then solubilized with 0.5 ml of 1 M NaOH at 37°C and neutralized with an equal volume of 1 M HCl. The radioactivity of cell lysates was quantified by a liquid scintillation counter (Tri-Carb B3110TR; PerkinElmer, Waltham, MA) and the protein content was determined by a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL). Cellular uptake was normalized by the total protein content in each well. In all studies, uptake in vector-transfected cells was performed at each data point and used to control for nonspecific substrate uptake and binding. Transporter-specific uptake was calculated by subtracting background uptake in vector-transfected cells from the uptake measured in transporter-expressing cells. Each data measurement was performed in triplicate in three different wells. Each experiment was repeated at least two times.

Transwell Studies in MDCK-hOCT2/hMATE1 Cells. Transepithelial flux of atenolol or metformin across MDCK cell monolayers was determined as previously described (Yin et al., 2015). Briefly, control and hOCT2/hMATE1-expressing MDCK cells were seeded on Falcon cell culture inserts at a density of 2×10^5 cells/cm². Transport experiments were performed 5 days after seeding in Transwell apparatuses. The integrity of the MDCK monolayer was verified by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS system (EMD Millipore, Bedford, MA) before and after each experiment. Only data from monolayers with TEER values greater than 150 $\Omega \cdot cm^2$ were accepted. In addition, proper formation of tight junctions was also verified by measurement of transepithelial flux of mannitol as described previously (Yin et al., 2015). After

removing cell culture medium from both sides of the inserts, cells were preincubated at 37°C for 10 minutes in KRH buffer with the pH in the apical and basal chambers maintained at 6.0 and 7.4, respectively. Transwell transport studies were then initiated after aspirating the preincubation buffer from both apical and basal chambers. For apical-to-basal (A-to-B) transport, 2 ml of KRH buffer (pH 7.4) was first added to the basal chamber, and the transport was initiated by adding 0.8 ml of KRH buffer (pH 6.0) containing a radiolabeled substrate to the apical chamber. Likewise, for basal-to-apical (B-to-A) transport, 0.8 ml of KRH buffer (pH 6.0) was first added to the apical chamber and 2 ml of KRH buffer (pH 7.4) containing a radiolabeled substrate was added to the basal chamber. To measure time-dependent transcellular transport, an aliquot of the incubation medium (100 μl from the basal chamber, 50 μl from the apical chamber) in the receiving chamber was periodically collected and replaced with an equal volume of fresh buffer. For inhibition studies, cimetidine was applied to the basal, apical, or both chambers at the start of the Transwell study. At the end of the Transwell experiment, inserts were rinsed three times with ice-cold KRH buffer and cells were lysed with 1 M NaOH and then neutralized with 1 M HCl. Intracellular accumulation of the radiolabeled substrate was quantified by liquid scintillation counting and normalized to total protein content in cell lysate. Metformin and atenolol are not significantly metabolized in MDCK cells (Zhou et al., 2007; Yin et al., 2015). Experiments were performed in two to three individual Transwell apparatuses and repeated twice. Data were presented as mean ±S.D. of all apparatuses with acceptable TEER values.

Data Analysis. Uptake studies in HEK-293 cells were performed in triplicate in different wells, whereas Transwell studies in MDCK cells were performed in two to three individual apparatuses. All experiments were repeated at least two times. Data were presented as mean ±S.D. of all pooled measurements ($n \geq 6$). The IC$_{50}$ values were obtained by nonlinear regression fitting of the uptake data to Log (inhibitor concentration) using a four-parameter function model in GraphPad Prism 6.0 using the equation: V = Bottom + (Top − Bottom) / [1 + (I/IC$_{50}$)^{nH}], where V is the rate of uptake in the presence of inhibitor, Bottom is the residual noninhibitable baseline value, Top is the rate of uptake in the absence of inhibitor, I is the inhibitor concentration, and nH is the Hill coefficient. For transport experiments, the apparent permeability (P$_{app}$) of compounds across cell monolayers was calculated as P$_{app}$ = (dQ/dt) / (A*C$_0$), where Q is the amount of compound transported over time t, A is the insert membrane surface area, and C$_0$ is the initial compound concentration in the donor chamber. Statistical significance was determined using unpaired Student's t test. A P value less than 0.05 was considered statistically significant.

Results

Inhibition of hOCT2 by Classic Inhibitors Is Highly Dependent on Substrate. To determine if cimetidine, the classic inhibitor of renal OC secretion, exhibits substrate-dependent inhibition of hOCT2 and hMATE1/2-K, inhibition studies were conducted using two drug substrates, metformin and atenolol. Metformin is a widely used antidiabetic drug and a well-established probe substrate for hOCT2 and hMATE1/2-K, and atenolol is a major antihypertensive that was recently identified as an excellent substrate for these transporters (Yin et al., 2015). Two additional known drug inhibitors of these transporters, pyrimethamine (Kusuhara et al., 2011) and carvedilol (Zolk et al., 2009; Wittwer et al., 2013), were also tested. Inhibition assays of the two substrates were conducted side-by-side in control and transporter-expressing HEK-293 cells and the IC$_{50}$ values were determined at a substrate concentration much lower than their K$_m$ values (see Supplemental Table 1). For hOCT2, substantial substrate-dependent

inhibition was observed for all three tested inhibitors (Fig. 1, Table 1). Cimetidine and pyrimethamine were approximately 10-fold more potent toward hOCT2 when atenolol is used as the substrate. Carvedilol also showed 6-fold higher potency for hOCT2 when atenolol is used. In contrast, substrate-dependent inhibition was much less pronounced for hMATE1 and 2-K (Fig. 1, Table 1). Not all inhibitors showed substrate-dependent inhibition of hMATE transporters. Those that showed dependency had much smaller differences in IC$_{50}$ (<5-fold) compared with hOCT2. Interestingly, atenolol is a more sensitive substrate to detect hOCT2 inhibition, whereas metformin appears to be a more sensitive substrate for hMATE1 and 2-K inhibition.

To examine the inhibition mechanisms of cimetidine toward hOCT2 and hMATE1/2-K, concentration-dependent uptake of atenolol or metformin was performed in the presence of different concentrations of cimetidine (0, IC$_{50}$, and 5 × IC$_{50}$). The complete kinetic data along with Lineweaver-Burk plots are also shown (Supplemental Figs. 1 and 2). A close-up view of the double-reciprocal plots at the lower x and y range (Fig. 2) indicates that cimetidine acted as a mixed-type inhibitor of hOCT2 for atenolol but became a competitive inhibitor when metformin was used. Cimetidine acted as a mixed-type inhibitor for both hMATE1 and 2-K regardless of the substrate used.

Substrate-Dependent Inhibition Results in Different Prediction of DDI Potential for hOCT2. According to the latest FDA draft guidance for hOCT2-mediated DDI studies, an in vivo DDI study is recommended for a compound if its unbound C$_{max}$/IC$_{50}$ is greater than 0.1 (FDA, 2012). Cimetidine is 20% protein bound in the plasma, and the reported unbound C$_{max}$ after a typical 400-mg oral dose is around 8 μM (Somogyi et al., 1980, 1989). Our study showed that the IC$_{50}$ values of cimetidine toward hOCT2 differ by 10-fold when different drug substrates are used. To examine if substrate-dependent inhibition leads to different recommendations for in vivo DDI assessment, the unbound C$_{max}$/IC$_{50}$ values of cimetidine were calculated for atenolol and metformin. In addition, the reported cimetidine IC$_{50}$ toward MPP$^+$ (Ito et al., 2012), a recommended in vitro probe for hOCT2, was also used for prediction. Very different recommendations for cimetidine in vivo DDI study were obtained for hOCT2 with a "Yes" for atenolol, a "No" for MPP$^+$, and a borderline value for metformin (Table 2). These data demonstrated that substrate-dependent inhibition can significantly impact the outcome of DDI prediction for hOCT2. Compared with hOCT2, cimetidine is generally a more potent inhibitor for hMATE1/2-K and showed less pronounced substrate-dependent inhibition (Table 1). The unbound C$_{max}$/IC$_{50}$ values of cimetidine are all greater than 0.1 for hMATE1/2-K, resulting in a consensus in recommendations for all three substrates.

Cimetidine Applied in the Basal Chamber Inhibited B-to-A Flux of Atenolol and Metformin in hOCT2/hMATE1-Expressing MDCK Monolayer. In the human kidney proximal tubule, OC secretion is sequentially mediated by basolateral hOCT2 and apical hMATE1/2-K. To determine the overall effect of cimetidine on hOCT2/hMATE-mediated OC secretion, we examined the inhibitory effect of cimetidine on basal-to-apical (B-to-A) flux of radiolabeled atenolol (1 μM) and metformin (5.5 μM) using an hOCT2/hMATE1 double-transfected MDCK cell line previously established in our
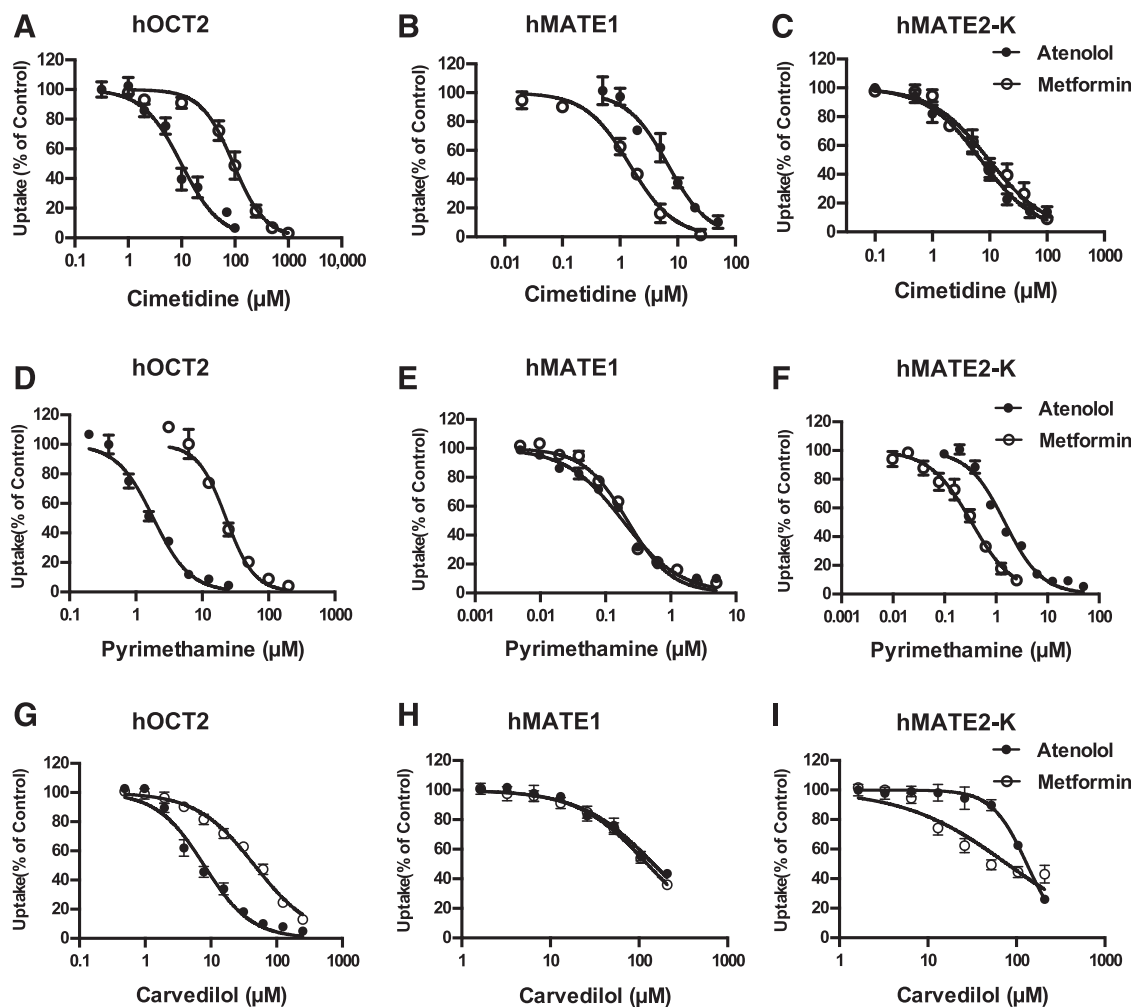
Fig. 1. Inhibition of hOCT2, hMATE1, and hMATE2-K by cimetidine, pyrimethamine, and carvedilol using atenolol or metformin as the probe substrate. Cells were incubated in KRH buffer containing either atenolol (1 μM) or metformin (5.5 μM) for 2 minutes in the presence of an inhibitor at graded concentrations. Transporter-specific uptake was calculated by subtracting the uptake in vector-transfected HEK-293 cells and presented as percentage of the uptake determined in the absence of an inhibitor. All experiments were performed in triplicate and repeated two times. Data were presented as mean ±S.D. of all pooled measurements (n = 9).

laboratory (Yin et al., 2015). Two clinically relevant concentrations of cimetidine (2 and 10 μM) were chosen on the basis of their IC_{50} values toward hOCT2 and hMATE1 (Table 1). The substrate concentrations used in our study were also within the reported clinical concentration range of atenolol

and metformin (Goodman et al., 2011). To evaluate whether different membrane access of the inhibitor plays a role on its inhibitory effect, cimetidine was applied to the basal, apical, or both chambers in the Transwell studies. As expected, B-to-A flux of atenolol and metformin in the absence of cimetidine

TABLE 1

Cimetidine IC_{50} values for atenolol and metformin

Experiments were performed in triplicate and repeated two times. Data are presented as mean ± S.D. of all pooled measurements (n = 9).

Inhibitor	Transporter	IC_{50} (μM)		P Value	Fold Difference
		Atenolol	Metformin		
Cimetidine	hOCT2	9.9 ± 1.1	93.5 ± 1.1	$P < 0.001$	9.4[a]
	hMATE1	6.7 ± 1.2	1.5 ± 1.0	$P < 0.01$	4.5[b]
	hMATE2-K	7.1 ± 1.1	9.7 ± 1.3	$P = 0.057$	1.4[a]
Pyrimethamine	hOCT2	1.8 ± 0.2	22.9 ± 1.9	$P < 0.001$	12.7[a]
	hMATE1	0.18 ± 0.006	0.22 ± 0.01	$P < 0.01$	1.2[a]
	hMATE2-K	1.5 ± 0.07	0.3 ± 0.06	$P < 0.001$	5.0[b]
Carvedilol	hOCT2	7.9 ± 0.6	46.4 ± 0.4	$P < 0.001$	5.9[a]
	hMATE1	146.4 ± 0.9	124.2 ± 4.3	$P < 0.001$	1.2[b]
	hMATE2-K	131.2 ± 4.2	78.6 ± 12.5	$P < 0.001$	1.7[b]

[a]IC_{50} for metformin/IC_{50} for atenolol.
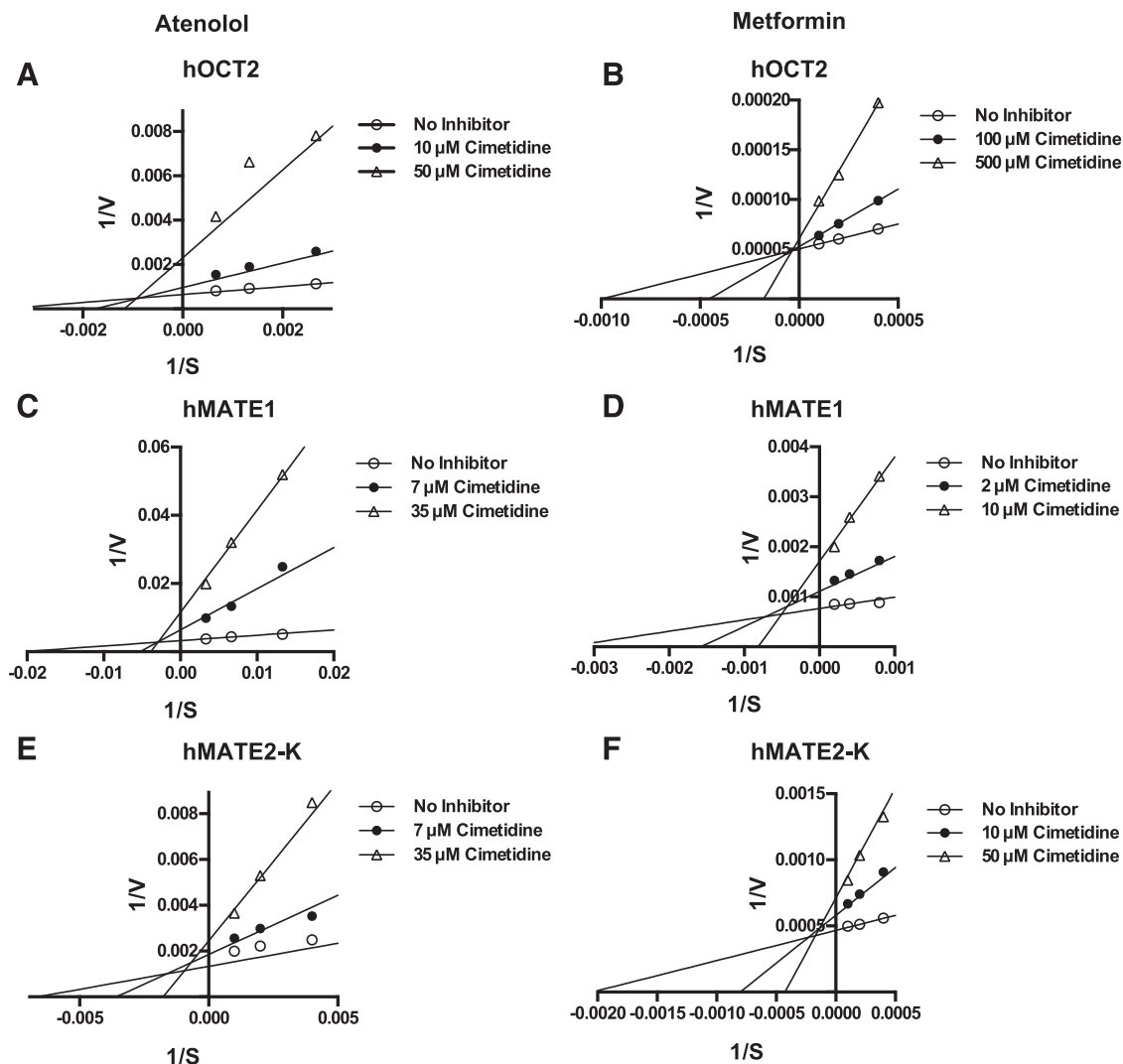[b]IC_{50} for atenolol/IC_{50} for metformin.

Fig. 2. Effect of cimetidine inhibition on atenolol or metformin transport kinetics of hOCT2, hMATE1, and hMATE2-K. Concentration-dependent uptake of atenolol and metformin was measured in both transporter-expressing and vector-transfected (control) HEK-293 cells after a 2-minute incubation in the presence of cimetidine at specified concentration (0, IC_{50}, and $5 \times IC_{50}$). Transporter-specific uptake was obtained by subtracting the uptake in control cells from the uptake in transporter-expressing cells. All experiments were performed in triplicate and repeated twice. The mean values of the kinetic data were used to transform data into Lineweaver-Burk plots. Although all kinetic data points were used for the generation of Lineweaver-Burk plots, only the close-up view at lower x and y ranges was presented to clearly show the intersection point. The kinetic data and corresponding Lineweaver-Burk plots with all data points are provided in Supplemental Figs. 1 and 2.

was much greater in hOCT2/hMATE1-transfected cells than in vector-transfected cells (Fig. 3). When applied to the basal or both chambers, cimetidine dose dependently inhibited B-to-A flux of atenolol and metformin (Fig. 3, A, C, D, F). Surprisingly, when applied to the apical chamber, cimetidine at the concentrations used had little effect on B-to-A flux of either atenolol or metformin (Fig. 3, B and E).

Cimetidine Differentially Impacted Intracellular Drug Accumulation in a Substrate-Dependent Manner. Transporter-mediated drug accumulation within renal tubular cells is a frequent cause underlying drug-induced nephrotoxicity (DeGorter et al., 2012; Morrissey et al., 2013). Depending on whether the interaction occurs at the site of basolateral uptake or apical efflux, an inhibitor can have very different effects on intracellular drug accumulation. Our inhibition data (Table 1 and Fig. 1) showed that cimetidine is an equally potent inhibitor of hOCT2 and hMATE1 when atenolol is the substrate but becomes an hMATE1-selective

inhibitor if metformin is used. These data suggest that although cimetidine reduces tubular secretion for hOCT2/hMATE substrates, its effect on intracellular drug accumulation may be substrate-dependent. Indeed, when the apparent permeability was calculated, cimetidine applied to the basal or both chambers showed a clear inhibitory effect on B-to-A permeability of atenolol and metformin (Fig. 4, A and B). When the intracellular drug accumulation was measured in the absence of cimetidine at the end of the Transwell study, intracellular drug accumulation was significantly higher in hOCT2/hMATE1 cells than in vector-transfected cells (Fig. 4, C and D). Interestingly, cimetidine, applied to either basal or both chambers, had no effect on intracellular atenolol accumulation (Fig. 4C), which is consistent with its similar inhibition potencies for hOCT2 and hMATE1 when atenolol is used as the substrate (Table 1). In contrast, cimetidine dose dependently increased the intracellular accumulation of metformin when applied to basal or both chambers (Fig. 4D), suggesting a predominant inhibition

TABLE 2
Prediction of hOCT2 DDIs following FDA decision tree

	Atenolol	Metformin	MPP$^+$
hOCT2			
IC$_{50}$	9.9	93.5	146^b
Cimetidine unbound C$_{max}$/IC$_{50}$^{a}	0.8	0.09	0.05
Recommendation for in vivo DDI study	Yes	Yes/No	No
hMATE1			
IC$_{50}$	6.7	1.5	2.7^b
Cimetidine unbound C$_{max}$/IC$_{50}$^{a}	1.2	5.3	3.0
Recommendation for in vivo DDI study	Yes	Yes	Yes
hMATE2-K			
IC$_{50}$	7.1	9.7	4.0^b
Cimetidine unbound C$_{max}$/IC$_{50}$ ^{a}	1.1	0.8	2.0
Recommendation for in vivo DDI study	Yes	Yes	Yes

^{a}Cimetidine unbound C$_{max}$ = 8 μM was used for calculation. Value is from Brunton LL, Chabner BA, Knollman BC eds (2011) *Goodman & Gilman's The Pharmacological Basis of Therapeutics,* 12th ed, McGraw-Hill Medical, New York.
^{b}Value is from Ito et al. (2012).

of the apical hMATE1 when metformin is used as the substrate (Table 1). These data, obtained from a cell culture model of renal OC secretion, suggest that inhibitors of tubular secretion can impact intracellular drug accumulation in a substrate-dependent manner and the major substrate-inhibitor interacting site for a given inhibitor can shift between the apical and basolateral OC transporters depending on the substrate used.

Basally Applied Cimetidine Accumulated in MDCK-hOCT2/MATE1 Cells. An intriguing observation in our studies is that the effect of cimetidine on hOCT2/hMATE1-mediated substrate flux and intracellular accumulation is only manifested when the inhibitor is applied to the basal chamber. To explore what could be the possible cause of the differential effect of cimetidine on substrate transport when placed on a different side of MDCK monolayer, we measured and compared monolayer permeability and intracellular accumulation of ^{3}H-labeled cimetidine under several experimental conditions. Cimetidine showed a minor directional transport in vector-transfected control cells. Consistent with being a reported substrate for hOCT2 and hMATE1 (Tahara et al., 2005; Tanihara et al., 2007), cimetidine exhibited a B-to-A/A-to-B ratio of 2.3 in hOCT2/hMATE1-transfected MDCK cells (Fig. 5A), suggesting the role of hOCT2/hMATE1 in its renal secretion. Importantly, intracellular accumulation of cimetidine (10 μM) is much greater when it was applied to the basal side than the apical side in MDCK-hOCT2/hMATE1 cells (Fig. 5B). Similar results were also observed in the double-transfected cells when cold atenolol (1 μM) or metformin (5.5 μM) was added in the basal chamber (Fig. 5, C and D) to simulate the experimental conditions used in the inhibition studies in Figs. 3 and 4. In vector-transfected control cells, cimetidine intracellular accumulation was also slightly higher when applied basally, which probably results from its uptake
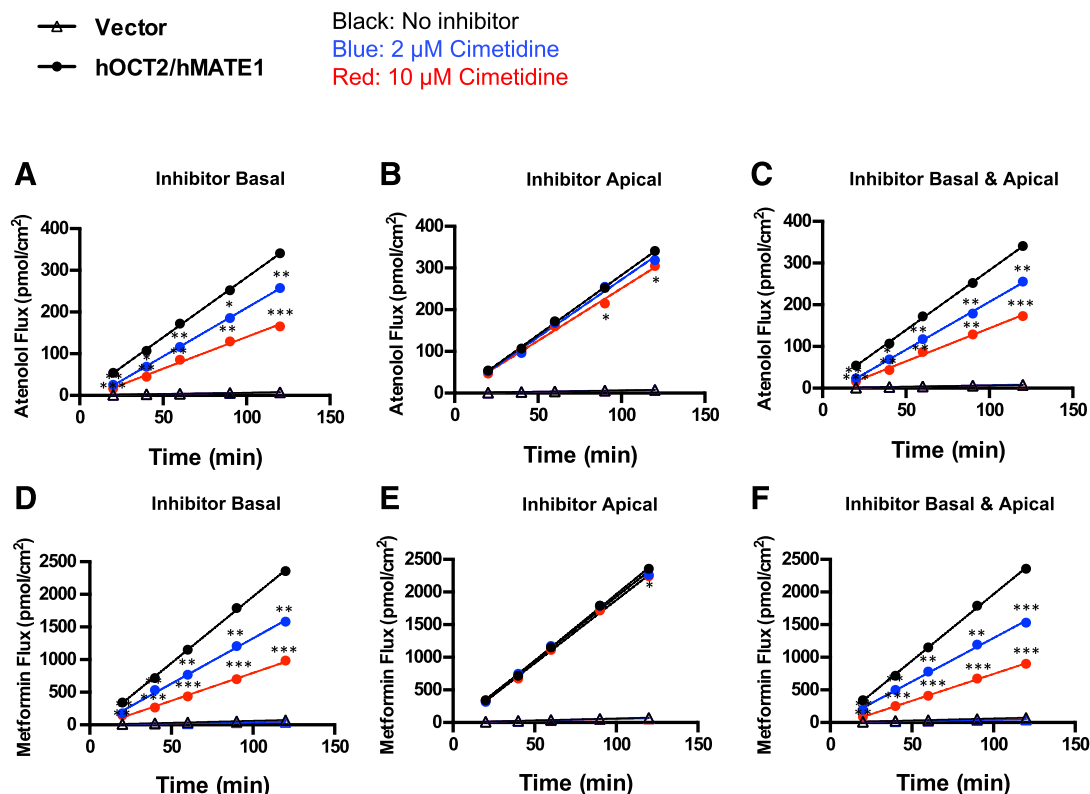

Fig. 3. Effects of cimetidine on B-to-A flux of atenolol and metformin in hOCT2/hMATE1-expressing MDCK cells. Cells were incubated in KRH buffer containing atenolol (1 μM) or metformin (5.5 μM) in the basal chamber. The pH in the apical and basal chambers was maintained at 6.0 and 7.4, respectively. B-to-A flux of atenolol and metformin was measured in the absence or presence of 2 μM or 10 μM of cimetidine. Cimetidine was added to the basal (A, D), apical (B, E), or both chambers (C, F). At various time points, 50 μl of sample was taken from the apical chamber and replenished with an equal volume of fresh KRH buffer. Experiments were performed in two to three individual Transwell apparatuses and repeated twice. Data were presented as mean ±S.D. ($n = 6$) of all apparatuses with acceptable TEER values. The B-to-A flux of atenolol and metformin in MDCK-hOCT2/hMATE1 cells in the presence of cimetidine was compared with that in the absence of cimetidine. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ indicates significant difference from the no inhibitor control.
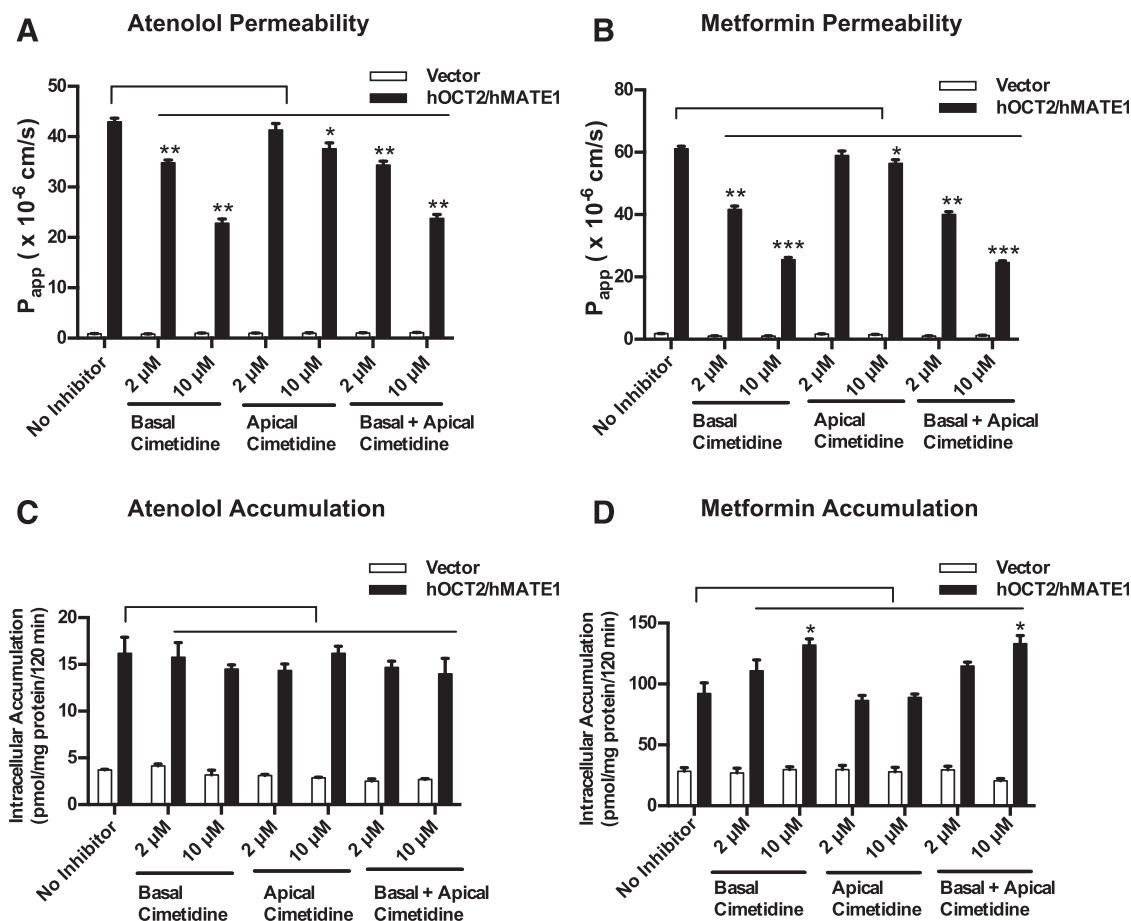
Fig. 4. Effect of cimetidine inhibition on permeability and intracellular accumulation of atenolol and metformin in MDCK-hOCT2/hMATE1 cells. The B-to-A permeability of atenolol (A) and metformin (B) was calculated using the equation described in *Materials and Methods*. Intracellular accumulation of atenolol (C) and metformin (D) was measured at the end of the Transwell study in the absence or presence of cimetidine (2 or 10 μM). Cimetidine was added to basal, apical, or both chambers at the start of the Transwell experiment. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ indicates significant difference from no inhibitor control. Experiments were performed in two to three individual Transwell apparatuses and repeated twice. Data were presented as mean $\pm$S.D. ($n = 6$) of all apparatuses with acceptable TEER values.

by a low level of endogenously expressed canine Oct2 in MDCK cells (Shu et al., 2001). These data suggest that under the experimental condition, when the acidic pH (6.0) in the apical chamber drives hMATE1-mediated substrate efflux from the cells, the configuration of the hMATE1 transporter may allow the substrate/inhibitor binding site(s) to be accessible only from the intracellular side. The lack of an inhibitory effect of apically applied cimetidine on hMATE1 appears to be attributable to a low intracellular accumulation of the inhibitor (Fig. 5, B–D).

Discussion

In this study, we investigated the impact of substrate-dependent inhibition on transporter-mediated drug interaction and accumulation by focusing on the hOCT2/hMATE-mediated renal secretion pathway. Several novel findings were obtained. First, our data showed that the choice of a probe substrate has a larger impact on hOCT2 than hMATEs in their interactions with inhibitors. Secondly, atenolol is a more sensitive substrate than metformin for evaluating hOCT2-inhibitor interactions in vitro. Further, substrate-dependent inhibition can complicate in vitro-to-in vivo DDI prediction for hOCT2 and lead to different recommendations

for in vivo DDI studies. Thirdly, we showed that although cimetidine inevitably reduced hOCT2/hMATE1-mediated transepithelial flux of a drug substrate, its impact on intracellular drug accumulation, thus toxicity, can be unpredictable owing to substrate-dependent shifting of the major interaction site between apical and basolateral transporters. Lastly, our data provided the first evidence that cimetidine concentrations in the basolateral side (i.e., blood) may be more relevant than those in the apical side (i.e., filtrate) for estimating its inhibitory effects on renal hOCT2 and hMATE1/2-K transporters.

In our study, metformin and atenolol were used as the substrates to probe potential substrate-dependent inhibition of renal OC transporters. Our data showed that cimetidine, as well as pyrimethamine and carvedilol, are much more potent inhibitors of hOCT2 when atenolol is used as the substrate (Fig. 1, Table 1). In contrast, substrate-dependent inhibition was less pronounced for hMATE1 and 2-K (Fig. 1, Table 1). These data suggest that the choice of a probe substrate can greatly influence the inhibition potency of a potential inhibitor (e.g., an NME), especially for hOCT2, leading to very different recommendations for in vivo DDI studies as exemplified in Table 2. Accordingly, it may be necessary to evaluate two or more probe substrates in in vitro studies for drug transporters
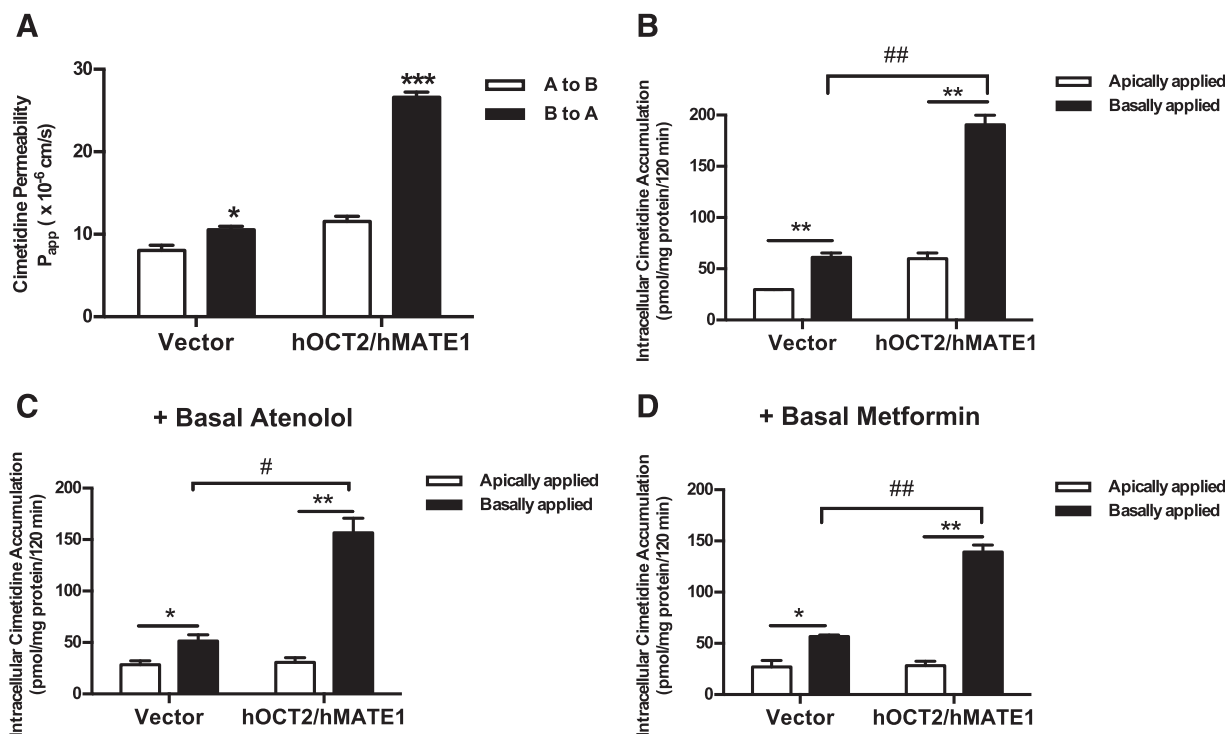
A

B

C + Basal Atenolol

D + Basal Metformin

Fig. 5. Cimetidine permeability and intracellular accumulation in MDCK-hOCT2/hMATE1 cells. Vector- and hOCT2/hMATE1-transfected MDCK cells growing on inserts were incubated in KRH buffer with [^{3}H]cimetidine (10 μM) added to either apical (pH 6.0) or basal chamber (pH 7.4). An aliquot of buffer in the receiving chamber was sampled periodically up to 120 minutes. (A) Cimetidine permeability in the B-to-A and A-to-B directions was measured and compared (***P < 0.001, *P < 0.05). (B–D) Intracellular accumulation of [^{3}H]cimetidine (10 μM) after application to basal or apical chamber alone or with cold atenolol (1 μM) or metformin (5.5 μM) added in the basal chamber. **P < 0.01, *P < 0.05 indicates significantly higher accumulation when cimetidine was applied to the basal chamber compared with the apical chamber. ##P < 0.01, #P < 0.05 indicate significantly higher accumulation in hOCT2/hMATE1 cells compared with vector control. Experiments were performed in two to three individual Transwell apparatuses and repeated twice. Data were presented as mean ±S.D. (n = 6) of all apparatuses with acceptable TEER values.

that display substantial substrate-dependent inhibition. To mitigate the risk of false-negative predictions, a sensitive substrate, which consistently produces lower IC$_{50}$ or K$_i$ values for a range of known inhibitors, should be included. Lastly, direct in vitro DDI analysis should be performed using a commonly comedicated drug as the substrate if frequent coadministration is anticipated (e.g., two antihypertensive drugs used simultaneously).

In the current International Transporter Consortium and FDA recommendations, MPP$^+$ and metformin are suggested as the probe substrates for assessing the inhibition potential of an NME toward hOCT2 (Giacomini et al., 2010; FDA, 2012; Hillgren et al., 2013). Belzer et al. (2013) previously reported that structurally diverse hOCT2 inhibitors were generally more effective (up to 9- to10-fold) for inhibition of metformin than of MPP$^+$, suggesting that metformin is a more sensitive hOCT2 substrate than MPP$^+$. Our data revealed that atenolol is an even more sensitive (up to 10-fold) hOCT2 substrate than metformin. Like metformin, atenolol is minimally metabolized in vivo and exclusively eliminated unchanged by the kidney. With a LogD$_{7.4}$ of –2.0, atenolol is highly hydrophilic with minimal passive membrane diffusion and nonspecific binding. We recently showed that atenolol is an excellent hOCT2 substrate and also has a higher apparent affinity for hOCT2 than metformin (Supplemental Table 1) (Yin et al., 2015). These characteristics bestow on atenolol clinical relevance as a probe substrate more sensitive than metformin for assessing hOCT2 inhibitors in vitro. However, available DDI data did not suggest a positive in vivo interaction for atenolol

and cimetidine (Houtzagers et al., 1982; Kirch et al., 1982; Mutschler et al., 1984). The total renal clearance of atenolol (~168 ml/min) is only 1.4-fold of its glomerular filtration clearance (~120 ml/min), suggesting that active tubular secretion only contributes to a minor (~30%) part of atenolol total clearance. This makes it difficult to observe clinically significant pharmacokinetic interactions of atenolol in vivo. In contrast, metformin has a total renal clearance of ~454 ml/min with a much larger (75%) contribution from tubular secretion. Thus, to assess hOCT2-mediated DDIs in vivo, metformin may be more revealing than atenolol for detection of systemic exposure or renal clearance changes caused by inhibition of tubular secretion.

Drug-induced nephrotoxicity is a major concern during drug discovery and development (Morrissey et al., 2013). Renal transporter–mediated DDIs may not only affect the systemic exposure of a victim drug but could also have profound toxicological effect on renal proximal tubule cells. Inhibition of a basolateral uptake transporter is generally nephron-protective as it reduces transporter-mediated drug accumulation within renal tubular cells. Indeed, coadministration of probenecid to inhibit hOAT1-mediated uptake of cidofovir is used clinically to prevent intrarenal accumulation and nephrotoxicity of cidofovir (Cundy et al., 1995; Cihlar et al., 1999). Inhibition of hOCT2-mediated renal cisplatin uptake is also being explored as a strategy to ameliorate cisplatin nephrotoxicity (Filipski et al., 2009; Sprowl et al., 2013; Pabla et al., 2015). On the other hand, inhibition of apical efflux transporters diminishes drug exit from renal cells, which may lead

to increased drug accumulation and nephrotoxicity (Yonezawa and Inui, 2011). Therefore, knowing the major site of interaction (i.e., apical versus basolateral) is critical for predicting a nephron-toxic or a nephron-protective effect of a potential inhibitor. In the case of cimetidine, inhibition of hOCT2-mediated uptake was historically thought to underlie its numerous clinical DDIs (Li et al., 2006; Giacomini et al., 2010). However, on the basis of the in vitro K_i or IC_{50} values of cimetidine determined with metformin and other experimental substrates, Ito et al. (2012) suggested that inhibition of apical hMATEs, but not basolateral hOCT2, is the major mechanism underlying clinically observed renal DDIs with cimetidine. Here, our data revealed that cimetidine is an hMATE-selective inhibitor when metformin is the substrate but acts as an equally potent inhibitor for hOCT2 and hMATE1 when atenolol is the substrate (Table 1). These data suggest that depending on the substrate (i.e., the victim drug), the site of cimetidine interaction is changeable between the apical and basolateral OC transporters. Consequently, the effect of an inhibitor on intracellular accumulation is also substrate-dependent, leading to either a nephron-toxic or a nephron-protective effect. This concept, supported by our Transwell studies on atenolol and metformin (Figs. 3 and 4), is illustrated in Fig. 6. Our studies demonstrated that the effect of a transport inhibitor on intracellular drug accumulation is dynamic and substrate-dependent; and information obtained with one inhibitor-substrate pair may not be extrapolated to other drug combinations containing the same inhibitor.

To predict transporter-mediated renal DDIs, the unbound plasma concentration of the inhibitor is often used (Giacomini et al., 2010; FDA, 2012). An intriguing observation in our study is that although cimetidine inhibited hMATE1-mediated atenolol or metformin uptake in HEK-293 cells, it had no inhibitory effect on hMATE1-mediated substrate efflux in Transwell studies in MDCK cells when applied apically

(Figs. 3 and 4). These seemingly paradoxical observations may be explained by the unique transport mechanism of the MATE transporters, which function as proton/OC antiporters and simultaneously transport proton in the opposite direction of OC substrate (Otsuka et al., 2005). For these transporters, proton binding always occurs on the opposite side of OC substrate binding. In the uptake studies in HEK-293 cells, an extracellular pH of 8.0 was used to create an outwardly directed proton gradient to drive cellular uptake of the OC substrate. Proton binds intracellularly whereas substrate/inhibitor binds from the extracellular side, which allows the measurement of the inhibitory effect of cimetidine in these studies. In contrast, in the Transwell studies in MDCK cells, the pH in the basal and apical chambers was maintained, respectively, at 7.4 and 6.0 to mimic the physiologic environment in the nephron. The acidic pH in the apical compartment creates an inwardly directed proton gradient to drive hMATE1-mediated substrate efflux from MDCK cells. Under this condition, proton binds from the outside whereas substrate/inhibitor binds from the inside of the MDCK cells (see Fig. 6). The configuration of the hMATE1 transporter may allow the substrate/inhibitor binding site(s) to be accessible only from the intracellular side. As apically applied cimetidine cannot diffuse into MDCK cells owing to its low passive diffusion (LogP = 0.4), the inhibitor may not gain access to the intracellularly orientated binding site under this condition. Thus, apically applied cimetidine lacks an effect on hMATE1 whereas basally applied cimetidine, after being transported into cells by hOCT2, can exert an inhibitory effect on hMATE1. This hypothesis is in agreement with data presented in Fig. 5 demonstrating a much higher intracellular accumulation of cimetidine when applied to the basal compartment. Together, our studies suggest that plasma and intracellular concentrations of cimetidine, compared with its filtrate or urine concentrations, may be more relevant for
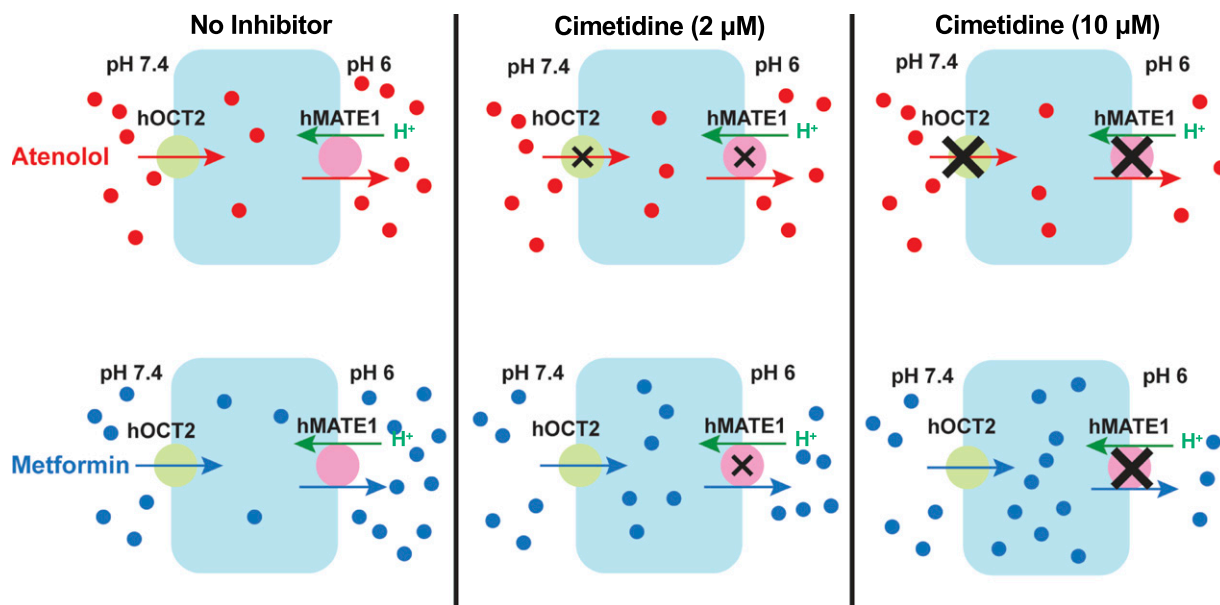

Fig. 6. Hypothesized effect and site of interaction for cimetidine on renal tubular secretion and intracellular accumulation of atenolol and metformin. For atenolol, cimetidine inhibits hOCT2 and hMATE1 with equal potencies. At clinically relevant concentrations, cimetidine dose dependently reduces atenolol tubular secretion but has no effect on intracellular accumulation. For metformin, cimetidine mainly inhibits hMATE1. Cimetidine not only reduces metformin tubular secretion but also increases its intracellular accumulation in a dose-dependent manner.

assessing hOCT2/hMATE1-mediated cimetidine-drug interactions in the kidney.

In summary, we demonstrated that the choice of a probe substrate can greatly impact the prediction of hOCT2-mediated renal drug interaction and accumulation. Our findings revealed the complex and dynamic nature of substrate-inhibitor interactions in multispecific drug transporters and highlighted the importance of considering substrate-dependent inhibition in assessing transporter-mediated renal drug interaction, accumulation, and toxicity.

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