Substrate-Dependent Ligand Inhibition of the Human Organic Cation Transporter OCT2

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ABSTRACT

Organic cation transporter 2 (OCT2) mediates the initial step in renal secretion of organic cations: uptake from the blood, across the basolateral membrane, and into the renal proximal tubule cells. Because of its potential as a target for unwanted drug-drug interactions (DDIs), considerable attention has been directed toward understanding the basis of OCT2 selectivity. These studies typically assess selectivity based on ligand inhibition profiles for OCT2-mediated transport of a probe substrate. However, little attention has been given to the potential influence of the substrate on the profile of ligand inhibition. Here we compared the IC50 values obtained for a set of structurally distinct inhibitors against OCT2-mediated transport of three structurally distinct substrates: 1-methyl-4-phenylpyridinium (MPP); metformin; and a novel fluorescent substrate, N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium iodide (NBD-MTMA). The median IC50 value for inhibition of MPP transport was 9-fold higher than that for inhibition of metformin transport. Similarly, the median IC50 value for inhibition of MPP transport was 5-fold higher than that for NBD-MTMA transport. However, this was not a systematic difference in inhibitory efficacy; the ratio of IC50 values, MPP versus NBD-MTMA, ranged from 88-fold (ipratropium) to 0.3-fold (metformin). These data show that 1) the choice of OCT2 substrate significantly influences both quantitative and qualitative inhibitory interactions with cationic drugs; and 2) ligand interactions with OCT2 are not restricted to competition for a common ligand binding site, consistent with a binding surface characterized by multiple, possibly overlapping interaction sites. Development of predictive models of DDIs with OCT2 must take into account the substrate dependence of ligand interaction with this protein.

Introduction

At physiologic pH, approximately 40% of all prescribed drugs are cationic (Neuhoff et al., 2003; Ahlin et al., 2008), and these organic cations (OCs) are potential substrates for one or more of the transport processes that mediate renal secretion of endogenous and exogenous OCs. The latter group includes many commonly prescribed drugs; consequently, OC transport influences their pharmacokinetic and pharmacodynamic profiles. The existence within the kidney of a common pathway(s) for the secretion of OCs (e.g., cimetidine, trimethoprim, metformin) sets the stage for unwanted drug-drug interactions (DDIs) (Endres et al., 2006). In addition, genetic variations of drug transporters have been linked to interindividual variations in drug efficacy, toxicity, and pharmacokinetics (Sissung et al., 2012). The clinical cost of DDIs is substantial and responsible for approximately 1% of hospital admissions (almost 5% in elderly populations) (Becker et al., 2007; Zolk et al., 2009; Kido et al., 2008), typically identify charge and some measure of hydrophobicity as key determinants of ligand binding (see also Ullrich, 1997). However, the several three-dimensional

ABREVIATIONS: CHO, Chinese hamster ovary; DDI, drug-drug interaction; DMSO, dimethylsulfoxide; HEK293, human embryonic kidney cell line; MATE, multidrug and toxin extrusion transporter; MPP, 1-methyl-4-phenylpyridinium; NBD-MTMA, N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium iodide; NBD-TMA, [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium; OC, organic cation; OCT, organic cation transporter; P-gp, P-glycoprotein; WB, Waymouth buffer.
pharmacophores that have attempted to highlight the molecular determinants of ligand-transport interaction (Bednarczyk et al., 2003; Suhre et al., 2005; Moaddel et al., 2007; Zolk et al., 2009) display similar but structurally distinct placements of hydrophobes, cationic features, and hydrogen bond donor and acceptor sites. Whereas the bases of these discrepancies could include methodological or analytical differences between these studies, we suggest another, generally unacknowledged, complicating factor, namely, the influence of substrate on OCT interaction with an inhibitor. In this regard, it is interesting that the three modeling efforts with OCT2 (and the three with OCT1) were based on profiles of inhibition of structurally distinct substrates produced by inhibitory ligands: 1-methyl-4-phenylpyridinium (MPP) (Moaddel et al., 2007; Zolk et al., 2009), TEA (Bednarczyk et al., 2003; Suhre et al., 2005), or 4-((dimethylamino)styril)-N-methylpyridinium iodide (Ahlin et al., 2008; Kido et al., 2011). Thus, the differences in the resulting models, noted earlier, may have reflected, at least to some degree, substrate-dependent ligand inhibition of these processes.

An influence of substrate on the profile of ligand interaction has been noted for other multidrug transporters, including P-glycoprotein (P-gp) (Ekins et al., 2002; Garrigues et al., 2002) and the organic anion transporting polypeptides (Westholm et al., 2009; Roth et al., 2011). In their extensive review of the literature, Nies et al. (2011) compiled the inhibitory profiles for a 100+ inhibitory ligands against transport activity of human OCT2 (as well as OCT1, OCT3, multidrug and toxin extrusion transporter 1 (MATE1) and MATE2-K). Noting the variations in IC50 values that were often associated with differences in probe substrate, they suggested: “The existence of various substrate and inhibitor binding sites and the complex interactions between different sites explains why largely different IC50 values were obtained for individual transporters when different substrates were used for transport measurements.” Although a number of observations in the literature appear to support this view (as discussed by Nies et al., 2011), so, too, are there multiple examples of similar degrees of variance in kinetic constants when different studies report IC50 values for inhibition of transport of the same substrate by the same test inhibitor (cf. Nies et al., 2011). Consequently, it is difficult to draw conclusions on the issue of substrate-dependent ligand interactions based on data generated by different groups. However, a recent study by Zolk et al. (2009) provided a set of observations that warrants discussion. They compared IC50 values for seven structurally distinct cationic drugs against OCT2-mediated transport of MPP and metformin. Lending particular credence to the idea that substrate identity may influence the interactions of inhibitory ligands with OCT2, their results showed that each test compound was a more effective (~10-fold) inhibitor of metformin than of MPP.

In the present study, we test the hypothesis that the effectiveness of cationic drugs as inhibitors of OCT2 can be significantly influenced by the substrate used in the transport assay. We confirmed the observation of Zolk et al. (2009) that cationic ligands are substantially more effective inhibitors of metformin than of MPP. This observation was then extended by comparing the inhibition of OCT2-mediated transport of the novel fluorescent cation N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium iodide (NBD-MTMA) that was produced by a structurally distinct battery of inhibitory ligands to the inhibition these same ligands exerted against OCT2-mediated transport of MPP and metformin. The results show that 1) the choice of OCT2 substrate significantly influences both the quantitative and the qualitative inhibitory interactions with cationic drugs; and 2) ligand interactions with OCT2 must not be restricted to competition for a common ligand binding site, consistent with a binding surface characterized by multiple, possibly overlapping interaction sites.

Materials and Methods

Reagents. Chinese hamster ovary (CHO) cells containing a single integrated flipase (Flip) recombination target site (CHO Flip-In), Flip recombinase expression plasmid, platinum high-fidelity DNA polymerase, hygromycin, Zeocin, and mammalian expression vector pcDNA3/PRTV5-His TOPO were acquired from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). Ham’s F-12 nutrient mixture, amitriptyline, chloroquine, quinine, propramoline, cinetidine, clonidine, diphenhydramine, disopyramide, doxepin, flecainide, imipramine, ipratropium, metformin, propafenone, paroxetine, quinidine, TEA, triethylmethylammonium, trimethoprim, and verapamil were all purchased from Sigma-Aldrich (St. Louis, MO). NBD-MTMA, 1-methyl-4-phenylepyridinium iodide (MPP), and N1-ethylacridinium were synthesized by the Synthesis Core of the Southwest Environmental Health Sciences Center/Department of Chemistry of the University of Arizona (Tucson, AZ) according to (Aavula et al., 2006). [3H]MPP (80 Ci/mmol) was synthesized by the Synthetic Chemistry Core of the Southwest Environmental Health Sciences Center, as was [3H-NBD-MTMA (80 Ci/mmol; see below). [14C]Metformin (107 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA).

Synthesis of [3H]NBD-MTMA. Titrated methyl iodide (31.0 mCi, 55.0 µCi, 0.38 µmol; American Radiochemicals, St. Louis, MO) in benzene (200 µl) was added to amine 1 (see Fig. 1 in Aavula et al., 2006) (1.0 mg, 3.7 µmol); the mixture was contained in a clean, conical, heavy-walled glass vial (3 ml), and the vial was sealed with a polytetrafluoroethylene-lined screw cap. The vial was kept in a secondary container (glass bottle) within a tertiary container (glass bottle) along with a piece of filter paper. The mixture was stirred for 3 weeks at room temperature. The filter paper was then checked for radioactivity, which was found to be negligible. The reaction vial was then cooled to approximately 5°C, and a mixture of water (0.5 ml) and ethyl acetate (0.5 ml). The reaction vial was subjected to additional washing with ethyl acetate (2 × 0.5 ml). The aqueous solution was then assayed for radioactivity and was found to contain 27.3 mCi of the desired salt (138.8 µg, 0.34 µmol, 88%). Analysis by paper chromatography showed that the compound was >90% pure.

Cell Culture and Stable Expression of OCT2. CHO cells and human embryonic kidney (HEK293) cells containing the Flip recombination target site were obtained from Invitrogen. CHO cells were grown in Ham’s F-12 Nutrient Mixture with 10% fetal bovine serum and supplemented with 100 µg/ml Zeocin. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum with 100 µg/ml blasticidin. Cells that stably expressed OCT2 were prepared using methods described previously (Pelis et al., 2007). The OCT2-expressing cells were grown and maintained under hygromycin pressure (100 µg/ml) in plastic cell culture flasks at 37°C in a humidified atmosphere with 5% CO2. Cells were passed every 3 to 4 days and seeded into 48-well plates at 137,500 cells/well and typically used 48 hours later.

Transport Studies: Radiolabeled Substances. CHO and HEK293 cells grown to confluence in 48-well plates were rinsed twice with Waymouth buffer (WB) (135 mM NaCl, 13 mM HEPES, 2.5 mM CaCl2·2H2O, 1.2 mM MgCl2·6H2O, 0.8 mM MgSO4·7H2O, 5 mM KCl, and...
Results

Kinetics of MPP Transport. We routinely use CHO cells as an expression system to study OCT2 transport (e.g., Suhrs et al., 2005; Pelis et al., 2007, 2012; Harper and Wright, 2013). However, it is more common to use HEK293 cells as an expression for studying OCT-mediated transport (Nies et al., 2011). In the present study, we compare data obtained using OCT2-expressing CHO cells to data obtained using HEK293 cells as the expression system, so it is reasonable to ask whether the nature of the expression system introduces its own bias into the quantitative (and qualitative) characteristics of a heterologously expressed transport protein. To address this issue, we compared the kinetics of MPP transport, in experiments performed “side-by-side,” in CHO cells and HEK293 cells that stably expressed OCT2. Both cell lines used the Flp-recombinase system of Invitrogen to introduce stable expression of OCT2, which places a single copy of the transcript into the cell genome. The uptake of [3H]MPP into CHO cells and HEK293 cells that stably expressed OCT2 was linear for at least 90 seconds (unpublished data), so 1-minute uptakes were used for all subsequent studies of transport kinetics. To establish the kinetics of OCT2-mediated MPP transport, the rate of [3H]MPP (10–30 nM) into cells that stably expressed the transporter was measured in the presence of increasing concentrations of unlabeled MPP (Fig. 1) (there was little or no blockable transport of labeled MPP in nontransfected cells; e.g., Zhang et al., 2012). The relationship was adequately described by the Michaelis-Menten equation (eq. 1) for competitive interaction of labeled and unlabeled substrate (Malo and Berteloot, 1991):

\[ J^* = \frac{J_{\text{max}}[S]^*}{K_t + [S]^* + D_{\text{nl}}[S]^*} \tag{1} \]

where \( J^* \) is the rate of transport of the radiolabeled substrate (in this case, [3H]MPP) from a concentration of the labeled substrate equal to \([S]^*\); \( J_{\text{max}} \) is the maximal rate of mediated substrate transport; \( K_t \) is the Michaelis constant of the transported substrate; \([S]\) is the concentration of unlabeled substrate; and \( D_{\text{nl}} \) is a first-order rate constant that describes the nonsaturable component of labeled substrate accumulation (reflecting the combined influence of diffusion, non-specific binding, and incomplete rinsing of labeled substrate from the cell culture well). As determined in three separate, paired experiments, assessing the kinetics of OCT2-mediated MPP transport when stably expressed in HEK293 cells or CHO cells, there was no difference in the values of either \( K_t \) (9.2 ± 0.4 versus 9.4 ± 0.3 μM). In these paired experiments, there was no difference in \( J_{\text{max}} \) either (29.2 ± 5.9 versus 24.0 ± 0.6 pmol cm\(^{-2}\) min\(^{-1}\) (Fig. 1), although over the course of our entire study, the OCT2-expressing HEK293 line of cells tended to display slightly higher rates of transport compared with the CHO line of cells [tracer clearance of 2.8 ± 0.6 (S.D.; n = 29) versus 2.0 ± 0.8 (S.D.; n = 31) μl cm\(^{-2}\) min\(^{-1}\), respectively]. Expressed per milligram of membrane protein, these \( J_{\text{max}} \) values were (approximately) 650 pmol mg\(^{-1}\) min\(^{-1}\). These data suggest that kinetic values for OCT2 determined when expressed in CHO cells can be expected to be reasonably comparable to those obtained when OCT2 is expressed in HEK293 cells. Importantly, these values for the kinetics of OCT2-mediated MPP transport, obtained using our experimental and analytical methods, are also quite similar to those reported by Zolk et al. (2009); \( K_t \) of 19.5 μM and \( J_{\text{max}} \) of 579 pmol mg\(^{-1}\) min\(^{-1}\), lending further credence to the upcoming comparisons of data reported here with those reported by the Zolk group.

Ligand Inhibition of OCT2-Mediated MPP Transport. To expand our comparison of the influence of expression system on the kinetics of ligand interaction with OCT2, we compared the influence of an inhibitory ligand on MPP transport when OCT2 was expressed in CHO versus HEK293 cells. Figure 1B shows the kinetic profile for inhibition of OCT2-mediated MPP transport as expressed in CHO and HEK293 cells, which was produced by the structurally distinct inhibitory ligand (and OCT2 substrate) metformin (see Fig. 4), a comparison that also displays our analytical approach for such measurements. For this (and all the other) compound(s) tested, uptake of [3H]MPP was inhibited by increasing concentrations of test agent according to the relationship shown in eq. 2:
where IC\textsubscript{50} is the concentration of the inhibitor [I] that reduced mediated (i.e., blockable) \[^{3}H\]MPP transport by 50%; \(J\text{\textsubscript{mapp}}\) is the product of the maximum rate of \(S\) (i.e., \([^{3}H\]MPP) uptake (\(J\text{\textsubscript{max}}\)) and the ratio of the IC\textsubscript{50} of the test agent and \(K_t\) for transport of \(S\) (Groves et al., 1994). In three separate experiments using OCT2-expressing CHO cells, the IC\textsubscript{50} for metformin inhibition of MPP transport was 545 ± 26 \(\mu M\). In five separate experiments using OCT2-expressing HEK293 cells, the IC\textsubscript{50} for metformin’s inhibition of MPP transport was 580 ± 129 \(\mu M\); neither of these values was different from the IC\textsubscript{50} value of 398 \(\mu M\) reported by Zolk et al. (2009). Figure 1C compares the IC\textsubscript{50} values for the inhibition of MPP transport for three additional compounds (imipramine, clonidine, and cimetidine), as well as apparent \(K_t\) values obtained for three transported substrates (MPP, TEA, and metformin), determined using our transport protocol with OCT2-expressing CHO and HEK293 cells; the values effectively fell on the line of identity. We conclude from these observations that the transport kinetics for OCT2 determined using these different expression systems are, at least on average, reasonably comparable.

Additional issues arise, however, when comparing the kinetic values determined in separate studies because of the possibility that differences in the technical approach to the measurement of transport may influence the outcome. Therefore, given our intention to draw conclusions based on an “intergroup” comparison of kinetic values, we selected 10 test compounds included in the Zolk study and compared the IC\textsubscript{50} values for inhibition of OCT2-mediated MPP transport determined using our methods with those reported for the same compounds by Zolk et al. (2009). These data are presented in Fig. 2 as the log of our values versus the log of those reported by Zolk et al. (2009) (Fig. 2; Table 1). Only one of the compounds, chloroquine (filled circle in Fig. 2), displayed markedly different (8-fold) IC\textsubscript{50} values in the two studies. To examine the potential basis for this discrepancy, we compared directly the IC\textsubscript{50} value for chloroquine’s inhibition of 10 nM MPP (129 \(\mu M\), \(n = 4\)) with that obtained in two experiments that used a substrate concentration of 10 \(\mu M\) MPP (the condition used in the Zolk et al., 2009 study); that value was 271 \(\mu M\) (± 4.5 \(\mu M\)). It is noteworthy that, presuming chloroquine is a competitive inhibitor of OCT2-mediated MPP transport (an untested assumption), the increase in substrate concentration from 10 nM to 10 \(\mu M\) (the latter value approximately equal to the \(K_t\) for OCT2-mediated MPP transport in our experiments; Fig. 1A) should approximately double the IC\textsubscript{50} value (i.e., from 129 \(\mu M\) to approximately 260 \(\mu M\)), similar to our value of 271 \(\mu M\) but still 4-fold lower than the 1096 \(\mu M\) IC\textsubscript{50} value reported by Zolk.
which represented equal IC50 values for the two data sets. From those of the line of identity (dashed line in the plot), the relationship for all 10 compounds did not differ significantly. However, for upcoming comparisons, the slope and intercept of the overall relationship, calculated as 0.96 ± 0.22 (95% confidence interval), differs by approximately 3-fold. Thus, within the limit of resolution of present analytical methods, the kinetic values for OCT2-mediated MPP transport we measured are (generally) comparable to those reported by Zolk et al. (2009), which supports the upcoming comparisons of our results with theirs.

Comparison of IC50 values for the inhibition of OCT2-mediated transport observed here and in the study by Zolk et al. (2009) reflects an, admittedly undefined, technical issue. Nevertheless, of the 10 compounds tested, six had IC50 values that differed by less than 2-fold, and two differed by approximately 3-fold. The point we wish to emphasize, and that is of particular importance to the upcoming comparisons, is the slope and intercept of the overall relationship for all 10 compounds did not differ significantly from those of the line of identity (dashed line in the plot), which represented equal IC50 values for the two data sets. Thus, within the limit of resolution of present analytical methods, the kinetic values for OCT2-mediated MPP transport we measured are (generally) comparable to those reported by Zolk et al. (2009), which supports the upcoming comparisons of our results with theirs.

**TABLE 1**

Comparison of IC50 values for the inhibition of OCT2-mediated transport in the present study (column A) or in the study of Zolk et al. (2009) (column B)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>A*</th>
<th>IC50</th>
<th>μM</th>
<th>B*</th>
<th>IC50</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>3.9 ± 0.9 (6, 3)</td>
<td>6</td>
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<tr>
<td>Clonidine</td>
<td>6.0 ± 1.1 (4, 2)</td>
<td>16</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Carvedilolin</td>
<td>55.2 ± 4.2 (3)</td>
<td>63</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Propranolol</td>
<td>71.6 ± 18.1 (2)</td>
<td>229</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>91.9 ± 35.6 (2, 2)</td>
<td>85</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Chloroquine</td>
<td>129 ± 22.0 (4)</td>
<td>1096</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Quinidine</td>
<td>91.6 ± 27.6 (5, 2)</td>
<td>87</td>
<td></td>
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</tr>
<tr>
<td>Cimetidine</td>
<td>171 ± 51.0 (7, 4)</td>
<td>120</td>
<td></td>
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<tr>
<td>Metformin</td>
<td>566 ± 107 (3, 5)</td>
<td>398</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Trimethoprim</td>
<td>775 ± 264 (4)</td>
<td>1318</td>
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</table>

*IC50 values represent the mean (± S.E.) of the number of separate experiments indicated in parentheses. In some cases the values include experiments with OCT2 expressed in both Chinese hamster ovary and HEK293 cells (HEK293 values indicated in italics).

The values were obtained from graphical interpolation of data presented in Fig. 6c from Zolk et al. (2009).

**Kinetic Profiles of Ligand Inhibition of OCT2-Mediated Transport of MPP and Metformin.** Figure 3A is a redrawing of data from the study by Zolk et al. (2009) that compared IC50 values for inhibition of OCT2-mediated metformin transport with those for the inhibition of MPP transport. The observation emphasized by these researchers was the clear correlation between the IC50 values for inhibition of these structurally distinct substrates produced by a common set of inhibitory ligands. Not commented on, however, was the observation that the inhibitory ligands were, on average 10.1 (±2.2) times more effective inhibitors of metformin transport than of the MPP transport. This substrate-dependent inhibitory profile has intriguing implications concerning the mechanism of ligand interaction with OCT2. Simply put, if MPP, metformin, and inhibitor compete for a common binding site, then the inhibitor should have a single IC50 value (i.e., Ki) for inhibition of the two substrates. It should be noted that whereas the metformin substrate concentration used in the determination of inhibitory ligand IC50 values by Zolk et al. (2009) was < < than the apparent KI for OCT2-mediated metformin transport, that was not the case for MPP; as noted earlier, the MPP substrate concentration used in those experiments was 10 μM, which is sufficiently close to the apparent KI for MPP transport these authors measured (20 μM) that it is likely that at least some of the IC50 values they reported represented modest (~50%) overestimates of the “true” Ki values for any of the ligands studied that interacted competitively with MPP. However, to the extent that was the case (and we have shown that for at least some of these ligands, the interactions are noncompetitive or mixed-type rather than competitive) (Harper and Wright, 2013), the MPP IC50 values still exceed by more than 7-fold those for metformin. We build on this idea in an upcoming section, but here we show that this observation by Zolk et al. is one we could reproduce. Figure 3B presents our own comparison of IC50 values for the inhibition of OCT2-mediated metformin and MPP transport, including four of the same inhibitory ligands used in the study by Zolk et al. (2009) (shaded gray symbols). We also saw a strong correlation between the relative inhibition of metformin and MPP transport; but of particular relevance, and consistent with the observations of Zolk and colleagues (Zolk et al., 2009), the test inhibitory ligands were 9.2 (±0.5) times more effective inhibitors of metformin transport than of MPP transport (median value, 9.4) (P < 0.001). This observation suggests that the disparity in IC50 values for the inhibition of OCT2-mediated MPP and metformin transport evident in data reported by Zolk et al. is a real phenomenon that argues for a substrate dependence for the interaction of at least these ligands with OCT2.

**Ligand Inhibition of OCT2-Mediated Transport of the Fluorescent Cation NBD-MTMA.** To determine whether the substrate dependence of inhibitory ligand interaction with OCT2 might be a general phenomenon (rather than restricted to MPP and metformin), we determined the profile of inhibition of OCT2-mediated transport of a structurally distinct substrate. We also took this opportunity to introduce to this type of work a novel OCT substrate with optical (i.e., fluorescent) properties that may prove useful for high-throughput applications. Some years ago, we described the synthesis of a novel fluorescent compound, [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium (NBD-TMA) and its use as a substrate of basolateral OC transport into isolated, single,
nonperfused renal proximal tubules (Bednarczyk et al., 2000). We subsequently reported (Aavula et al., 2006) the synthesis of a modest structural variation of NBD-TMA that both eliminated the modest pH sensitivity of NBD-TMA and shifted the excitation and emission peaks of the resulting compound, NBD-MTMA (Fig. 4), to overlap those of the widely used fluorescent organic anion, fluorescein (490 and 540 nm, respectively). NBD-MTMA is 1) highly soluble; 2) insensitive to pH and halide concentration (Aavula et al., 2006); 3) quite fluorescent in aqueous solution (compared with fluorescein, fluorescence of NBD-MTMA in aqueous solution is approximately 10% that of an equimolar solution of fluorescein; in DMSO, NBD-MTMA fluorescence is approximately 30% that of fluorescein in aqueous solution; unpublished data); and 4) does not bind to or intercalate into cell membranes. Figure 5 shows that increasing concentrations of NBD-MTMA inhibited the OCT2-mediated transport of MPP and metformin. However, the IC$_{50}$ values differed significantly: in three separate experiments comparing the inhibition by NBD-MTMA of OCT2-mediated transport, the IC$_{50}$ values for MPP and metformin were 64 ± 10 µM and 8.3 ± 1.7 µM, respectively.

We next determined whether the fluorescence of NBD-MTMA is sufficient to assess whether it is a substrate, as well as an inhibitor, of OCT2. Figure 6A shows the time course of 10 µM NBD-MTMA uptake into OCT2-expressing and wild-type CHO cells. Accumulation of fluorescence by CHO-wild-type cells was minimal, and uptake into CHO-OCT2 cells was linear for at least 5 minutes. Figure 6B compares the time course of OCT2-specific uptake of 1, 10, and 100 µM NBD-MTMA; at all concentrations, uptake was linear for at least 5 minutes. The kinetic values obtained by Zolk et al. (2009) that served as a standard for the upcoming comparisons with data obtained using the fluorescent properties of NBD-MTMA differed significantly: in three separate experiments comparing the inhibition by NBD-MTMA of OCT2-mediated transport, the IC$_{50}$ values for MPP and metformin were 64 ± 10 µM and 8.3 ± 1.7 µM, respectively.

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Fig. 3. (A) Relationship between IC$_{50}$ values for the inhibition of OCT2-mediated transport of metformin (x-axis) and MPP (y-axis), as shown in Fig. 6c in Zolk et al. (2009). The dashed line indicates the line of identity [equal IC$_{50}$ values; light curved lines indicate 95% confidence interval (CI)]. The heavy solid line reflects linear regression (r = 0.92 or 0.97 of the log of the IC$_{50}$ values; light curved lines indicate 95% CI). In both figures, the points represent mean values (± S.E.) determined in separate experiments (2–6 in the present study; reported as 3 in Zolk et al., 2009). Gray symbols indicate ligands tested by both Zolk et al. (2009) and the present study; circular symbols indicate ligands tested in the present study only; square symbols by Zolk et al. (2009) only (refer to Table 3). Different ligands indicated by different shapes: octagon, imipramine; triangle, clonidine; inverted triangle, verapamil; diamond, quinidine; open square, carvedilol; half-filled square up, mexiletine; half-filled square down, flecanide; open circle, cimetidine; half-filled circle left, chloroquine (refer to Table 3).

Fig. 4. Two-dimensional structures of the three OCT2 substrates used in this study: MPP, metformin, and NBD-MTMA. The Tanimoto similarities were calculated using ChemMine Web Tools (http://chemmine.ucr.edu/).

Fig. 5. The effect of increasing NBD-MTMA concentration on uptake of [3H]MPP (●) or [14C]metformin (○) into CHO cells that stably expressed OCT2. Each point is the mean (± S.E.) of 1-minute uptakes (expressed as the percentage of uptake measured in the absence of NBD-MTMA) of 16 nM [3H]MPP or 12 µM [14C]metformin (determined in triplicate) measured in three separate experiments.
were obtained using conventional radiotracer methods. Although similar in general design, the protocols associated with optical determination of transport rates differ in several respects from protocols that use radiolabeled substrates. To that end, we synthesized \[^3H\]NBD-MTMA and determined the kinetics of OCT2-mediated transport in the same cells. Figure 6C shows the time course of 6 nM \[^3H\]NBD-MTMA into OCT2-expressing and wild-type CHO cells. As seen with the accumulation of fluorescence, wild-type CHO cells displayed no mediated uptake of the radiolabeled substrate, and OCT2-mediated uptake of \[^3H\]NBD-MTMA was effectively linear for 5 minutes. We then measured in the same experiments 1) the fluorescence of accumulated, radiotracer-labeled NBD-MTMA and 2) accumulated radioactivity. As shown in Fig. 6D, the experimentally determined kinetics of OCT2-mediated NBD-MTMA transport were not influenced by the means of assessing the rate of uptake (i.e., the kinetic parameters based on the uptake of fluorescence (\(K_t\) of 9.0 \(\pm\) 3.0 \(\mu\)M and \(J_{\text{max}}\) of 6.8 \(\pm\) 0.7 pmol cm\(^{-2}\) min\(^{-1}\)) were not different from those determined from uptake of radiolabeled substrate (\(K_t\) of 9.6 \(\pm\) 3.8 \(\mu\)M and \(J_{\text{max}}\) of 5.4 \(\pm\) 0.6 pmol cm\(^{-2}\) min\(^{-1}\)).

The IC\(_{50}\) values for the inhibition of OCT2-mediated NBD-MTMA transport produced by 22 inhibitory ligands are listed in Table 2. These values spanned more than three orders of magnitude, with ipratropium displaying the highest apparent affinity for OCT2 (IC\(_{50}\) of 170 nM) and metformin the lowest (1.2 mM) (Fig. 7). Figure 8A compares the IC\(_{50}\) values for the inhibition of transport of these two substrates with those reported by Zolk et al. (2009) for the inhibition of MPP transport. Whereas there was a significant correlation between the inhibition of transport of these two substrates (\(P < 0.0001\)), the test inhibitory ligands were generally substantially more effective inhibitors of OCT2-mediated NBD-MTMA transport than of MPP transport (median value, 4.8). A more restricted (10-compound) comparison of IC\(_{50}\) values for the inhibition of OCT2-mediated NBD-MTMA (Table 2) transport with those we generated against MPP transport (Table 1) showed a similar correlation: the test compounds were generally more effective (2.7-fold; \(P < 0.05\))
IC50 values may overestimate (by approximately 2-fold) the highest IC50 values were effectively equivalent in their affinities for OCT2. Indeed, the inhibitory ligands with the most effective inhibitors of NBD-MTMA transport than of MPP transport. Interestingly, the disparity between IC50 values was more evident for inhibitory ligands with relatively high apparent affinities for OCT2. Thus, the difference in the inhibitory efficacy of the two substrates or even skewed toward being true for OCT1 (and other multidrug transporters), may have broad-reaching pharmacological and clinical implications for the development of models of ligand interaction with OCTs.

The results presented here support two broad conclusions. First, the profile of inhibition of OCT2 activity is both quantitatively and qualitatively influenced by the choice of substrate used to assess transport. Specifically, OCT2-mediated metformin transport was approximately 10 times more sensitive to inhibition by a structurally diverse array of cationic drugs than was OCT2-mediated transport of MPP; transport of the structurally distinct OCT2 substrate, NBD-MTMA, displayed an intermediate sensitivity to these inhibitory ligands (i.e., it is less sensitive to inhibition than transport of metformin but more sensitive than transport of MPP). The resulting substrate dependence of drug inhibition of OCT2 transport activity, which we suggest is likely to be true for OCT1 (and other multidrug transporters), may have broad-reaching pharmacological and clinical implications with respect to practical issues of assessing the susceptibility for DDIs of new chemical entities. Second, the failure of inhibitory ligands to display the same inhibitory interaction with different substrates indicates that structurally distinct ligands may not bind at a common binding site, an observation with marked implications for the development of ligand interaction with OCTs.

With respect to the latter point, the simplest model, arguably, of OC interaction with OCT2 involves competition for a common binding site or with sites that, although spatially distinct, overlap sufficiently to render their occupancy to be mutually exclusive. In the absence of contrary evidence, it is

### Table 2

Comparison of IC50 values for the inhibition of OCT2-mediated NBD transport (column A) or of OCT2-mediated transport of MPP (column B).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>A) NBD IC50</th>
<th>B) MPP IC50</th>
<th>IC50\textsubscript{MPP}/IC50\textsubscript{NBD}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylacridinium</td>
<td>0.08 ± 0.01</td>
<td>15</td>
<td>88</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>0.17 ± 0.05</td>
<td>13</td>
<td>8.5</td>
</tr>
<tr>
<td>Imipramine</td>
<td>1.4 ± 0.3</td>
<td>14</td>
<td>5.7</td>
</tr>
<tr>
<td>Doxepin</td>
<td>1.5 ± 0.5</td>
<td>25</td>
<td>7.0</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>2.5 ± 0.5</td>
<td>14</td>
<td>5.4</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>2.6 ± 0.4</td>
<td>14</td>
<td>5.4</td>
</tr>
<tr>
<td>MPP</td>
<td>3.0 ± 0.4</td>
<td>25</td>
<td>7.0</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.6 ± 0.5</td>
<td>14</td>
<td>5.4</td>
</tr>
<tr>
<td>Clonidine</td>
<td>4.5 ± 0.9</td>
<td>14</td>
<td>5.4</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>5.5 ± 0.6</td>
<td>36</td>
<td>6.8</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>13.1 ± 0.7</td>
<td>63</td>
<td>4.8</td>
</tr>
<tr>
<td>Verapamil</td>
<td>21.0 ± 1.7</td>
<td>85</td>
<td>4.1</td>
</tr>
<tr>
<td>Quinidine</td>
<td>29.6 ± 5.6</td>
<td>87</td>
<td>2.9</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>39.0 ± 2.6</td>
<td>324</td>
<td>8.3</td>
</tr>
<tr>
<td>Propranolol</td>
<td>67.2 ± 7.0</td>
<td>229</td>
<td>3.4</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>70.4 ± 8.5</td>
<td>120</td>
<td>1.7</td>
</tr>
<tr>
<td>TEA</td>
<td>110 ± 14.0</td>
<td>1318</td>
<td>12</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>113 ± 14.0</td>
<td>1318</td>
<td>12</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>117 ± 9.0</td>
<td>1096</td>
<td>9.3</td>
</tr>
<tr>
<td>TEMA</td>
<td>125 ± 14.0</td>
<td>191</td>
<td>0.82</td>
</tr>
<tr>
<td>Metformin</td>
<td>1180 ± 138</td>
<td>397</td>
<td>0.34</td>
</tr>
</tbody>
</table>

### Discussion

The results presented here support two broad conclusions. First, the profile of inhibition of OCT2 activity is both quantitatively and qualitatively influenced by the choice of substrate used to assess transport. Specifically, OCT2-mediated metformin transport was approximately 10 times more sensitive to inhibition by a structurally diverse array of cationic drugs than was OCT2-mediated transport of MPP; transport of the structurally distinct OCT2 substrate, NBD-MTMA, displayed an intermediate sensitivity to these inhibitory ligands (i.e., it is less sensitive to inhibition than transport of metformin but more sensitive than transport of MPP). The resulting substrate dependence of drug inhibition of OCT2 transport activity, which we suggest is likely to be true for OCT1 (and other multidrug transporters), may have broad-reaching pharmacological and clinical implications with respect to practical issues of assessing the susceptibility for DDIs of new chemical entities. Second, the failure of inhibitory ligands to display the same inhibitory interaction with different substrates indicates that structurally distinct ligands may not bind at a common binding site, an observation with marked implications for the development of models of ligand interaction with OCTs.
IC50 values for inhibition of MPP than for either metformin or showing that inhibitory ligands routinely displayed higher (Segel, 1975). Consequently, the evidence presented here have same for a common binding site is that an inhibitory ligand will tive. One of the principal predictions of mutual competition conclusion that ligand interaction with OCT2, including the Wright, 2013). The data presented here similarly support the mixed-type as well as competitive interactions (Harper and OCT2-mediated MPP transport involves noncompetitive and that such assumptions are unwarranted; ligand inhibition ofsuch inhibitors of MATE1-mediated metformin transport, suggests the generally stronger effectiveness of the tested ligands as inhibitors of metformin transport than of MPP and NBD-MTMA transport, both of which possess multiple ring structures. The absence of an aromatic feature in metformin, and the generally stronger effectiveness of the tested ligands as inhibitors of MATE1-mediated metformin transport, suggests the presence of an alternative binding site that is not shared by MPP or NBD-MTMA.

In light of the evidence that profiles of ligand inhibition of OCT-mediated transport are influenced, both qualitatively and quantitatively, by the choice of substrate (e.g., Fig. 4), the “universality” of pharmacophores based on inhibition of a single substrate must be called into question. It may also explain the differences in the principal features identified in studies using different probe substrates. For example, we previously described a pharmacophore, based on OCT2-mediated TEA transport that was characterized by 1) specificity” (Ullrich, 1997) of renal OC transporters makes it difficult to reconcile the structural diversity of molecules like MPP and NBD-MTMA (Fig. 4) and the observation that the apparent affinity of OCT2 for both is quantitatively similar (K_i of 9 μM; Figs. 1 and 6) with the idea of their binding to a common site. Furthermore, there is growing acceptance that ligand binding with other multidrug binding proteins involves interaction with spatially distinct sites within a larger binding surface. The kinetics of ligand inhibition of P-gp-mediated transport shows a clear substrate dependency (e.g., Ekins et al., 2002; Garrigues et al., 2002), as does the interaction of selected inhibitors with OATP-mediated transport Westholm et al., 2009; Roth et al., 2011). Moreover, X-ray crystal structures of P-gp (Aller et al., 2009) and the multidrug-binding transcription regulator QacR (Schumacher and Brennan, 2003) reveal the presence of multisite binding pockets. With respect to the OCTs, a number of studies have suggested that inhibitors of OCT transport display allosteric interactions at multiple sites (e.g., Volk et al., 2003, 2009; Popp et al., 2005; Lee et al., 2009; Minuesa et al., 2009; Egenberger et al., 2012). Furthermore, the results of site-directed studies of OCT function consistently suggest that substrates must interact with spatially distinct sites within the large hydrophilic translocation pathway suspected to exist with OCT1 (Popp et al., 2005) and OCT2 (Zhang et al., 2005; Pelis et al., 2006). Mutation of a single residue in rOct1 (D475E) exerts a profound effect on the affinity of the transporter for TEA and choline without having an effect on the kinetics of MPP transport (Gorboulev et al., 1999). Similarly, changing residue 447 in OCT2 (which imparts the marked difference in selectivity of OCT1 versus OCT2 for cimetidine) from glutamate to leucine effectively eliminates the transport of TEA and cimetidine but has no effect on MPP transport (Zhang et al., 2005), and mutation of C474 (adjacent to the aforementioned D475) markedly influences the apparent affinity of OCT2 for MPP but not TEA (Pelis et al., 2012). Taken with the observations reported here, the results of these studies support the hypothesis that OCTs have multiple, spatially distinct binding sites within a large polyspecific substrate binding pocket.

Insight into structural features that may influence spatially distinct ligand interaction with MATE1 may be found by comparing the structures of the three probe substrates. Of the three, metformin stands out in the absence of an aromatic feature (Fig. 4). With this in mind, it is interesting that the tested inhibitory ligands were generally more effective inhibitors of metformin transport than of MPP and NBD-MTMA transport, both of which possess multiple ring structures. The absence of an aromatic feature in metformin, and the generally stronger effectiveness of the tested ligands as inhibitors of MATE1-mediated metformin transport, suggests the presence of an alternative binding site that is not shared by MPP or NBD-MTMA.

Fig. 8. (A) Relationship between IC50 values for inhibition of OCT2-mediated transport of NBD-MTMA (x-axis; Table 1 in the present report) and MPP (y-axis; as reported in Table 1 of Zolk et al., 2009). The dashed line indicates the line of identity (equal IC50 values; light curved lines indicate 95% confidence interval). The heavy solid line reflects linear regression (r = 0.84) of the log of the IC50 values. The points represent mean values (± S.E.) determined in separate experiments (two to six in the present study, Table 1; reported as three in Zolk et al., 2009). (B) Ratio of IC50 value for inhibition of OCT2-mediated MPP transport to the value for inhibition of NBD-MTMA transport. The dashed line indicates the median value (5.1) for this ratio.

frequently assumed, either explicitly (e.g., Tahara et al., 2005; Amphoux et al., 2006) or implicitly (e.g., Muller et al., 2005), that the inhibition of OCT-mediated transport produced by small cations is competitive. However, we recently reported that such assumptions are unwarranted; ligand inhibition of OCT2-mediated MPP transport involves noncompetitive and mixed-type as well as competitive interactions (Harper and Wright, 2013). The data presented here similarly support the conclusion that ligand interaction with OCT2, including the interaction of transported substrates, need not be competitive. One of the principal predictions of mutual competition for a common binding site is that an inhibitory ligand will have same K_i (IC50) value for inhibition of different substrates (Segel, 1975). Consequently, the evidence presented here showing that inhibitory ligands routinely displayed higher IC50 values for inhibition of MPP than for either metformin or NBD-MTMA argues that these compounds (inhibitors and substrates) cannot interact with a common (or mutually exclusive set of) binding site(s). In retrospect, this is not a surprising observation. The hallmark “multisubstrate
training inhibitors, to explore adequately the chemical space distinct probe substrates, as well as a structurally rich set of will require identification of a suitable cohort of structurally suggest that adequate description of ligand binding to OCT2 with other inhibitory ligands, at spatially distinct sites. We consistent with their interaction, both with each other and

However, interaction of MPP and TEA with OCT2 is mixed

Diego, CA, and Inte:Ligand, Vienna, Austria, respectively).

(Catalyst v 4.9 versus LigandScout v 1.03; Accelrys, San

analytical methods used for pharmacophore generation

noted earlier, these differences may well have been influenced by the composition of the respective training sets or the analytical methods used for pharmacophore generation (Catalyst v 4.9 versus LigandScout v 1.03; Accelrys, San Diego, CA, and Inte:Ligand, Vienna, Austria, respectively).

However, interaction of MPP and TEA with OCT2 is mixed type rather than competitive (Harper and Wright, 2013), consistent with their interaction, both with each other and with other inhibitory ligands, at spatially distinct sites. We suggest that adequate description of ligand binding to OCT2 will require identification of a suitable cohort of structurally distinct probe substrates, as well as a structurally rich set of training inhibitors, to explore adequately the chemical space available for substrate or inhibitor interaction.

In addition to the influence that substrate-dependent inhibition of OCT2 has on understanding the molecular basis of drug interaction with this transporter, it also has a marked impact on the use of defined protocols (“decision trees”) designed to identify potential DDIs with OCTs, as well as other multidrug transporters, the results of which influence key decisions on the clinical and commercial future of these compounds (Giacomini et al., 2010; Lepist and Ray, 2012). For example, the current recommended guidance for initiating a clinical study of a drug’s potential for DDI with OCT1 or OCT2 is a value >0.1 for the ratio of unbound \( C_{\text{max}} \) to the drug’s \( IC_{50} \) for the inhibition of the transport of MPP, the prototypic OCT substrate (Giacomini et al., 2010; see U.S. Food and Drug Administration at http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/Drug-InteractionsLabeling/ucm110632.htm). Now consider: imipramine, a tricyclic antidepressant, has an unbound \( C_{\text{max}} \) of 70 nM (Thummel and Shen, 2001), and an \( IC_{50} \) of 7 \( \mu \)M against OCT2-mediated MPP transport (Zolk et al., 2009), resulting in a \( C_{\text{max}}/IC_{50} \) ratio of 0.01, well below the guidance limit. However, imipramine’s \( IC_{50} \) against OCT2-mediated metformin transport (determined in the same study; Zolk et al., 2009) is 0.4 \( \mu \)M, resulting in a ratio of 0.18, which would argue for a clinical DDI study (Giacomini et al., 2010; see U.S. Food and Drug Administration at http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/Drug-InteractionsLabeling/ucm110632.htm). We suggest that the decision-making process be more fully informed of a substrate-dependence for inhibitor interaction with OCTs that appears to be the rule rather than the exception.

In summary, structurally diverse cationic drugs were generally approximately 10 times more effective inhibitors of OCT2-mediated metformin transport than of MPP transport. A substrate-dependent inhibition profile was also seen for transport of the novel fluorescent cation NBD-MTMA; test drugs were generally less effective inhibitors of NBD-MTMA than metformin but more effective than MPP. These results suggest that substrate-dependent inhibitory ligand interaction is a common characteristic for OCT2. The results also argue for the presence within the translocation pathway of this multidrug transporter of a structurally complex binding surface than can accommodate ligand interactions with

![Fig. 9. Relationship between IC\(_{50}\) values for inhibition of OCT2-mediated transport of NBD-MTMA (x-axis; Table 2 in the present report) and metformin (y-axis; open symbols indicate values measured by us; solid symbols show the values reported by Zolk et al., 2009) (refer to Table 3). The dashed line indicates the line of identity (equal IC\(_{50}\) values; light curved lines indicate 95% confidence interval). The heavy solid line reflects linear regression (r = 0.89) of the log of the IC\(_{50}\) values.](image-url)

TABLE 3

Comparison of \( IC_{50} \) values for the inhibition of OCT2-mediated NBD-MTMA transport (column A) or of OCT2-mediated transport of metformin (Met) determined in the present study (column B) or in the study by Zolk et al. (2009) (column C).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>A( ^{\text{a}} ) NBD-MTMA IC(_{50})</th>
<th>B( ^{\text{a}} ) Met IC(_{50})</th>
<th>C( ^{\text{a}} ) Met IC(_{50})</th>
<th>IC(<em>{50}) Met/IC(</em>{50}) NBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu )M</td>
<td>( \mu )M</td>
<td>( \mu )M</td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>1.4 ± 0.3 (5)</td>
<td>0.77 ± 0.29 (3)</td>
<td>0.4</td>
<td>0.55 (0.3)</td>
</tr>
<tr>
<td>Clonidine</td>
<td>4.5 ± 0.9 (5)</td>
<td>0.68 ± 0.22 (4)</td>
<td>1.5</td>
<td>0.15 (0.3)</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>13.1 ± 0.7 (3)</td>
<td>15</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>21.0 ± 1.7 (3)</td>
<td>20.0 ± 7.6 (2)</td>
<td>5</td>
<td>0.95 (0.2)</td>
</tr>
<tr>
<td>Quinidine</td>
<td>29.6 ± 5.6 (4)</td>
<td>6.4 ± 0.9 (3)</td>
<td>8</td>
<td>0.22 (0.3)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>70.4 ± 8 (5)</td>
<td>25.4 ± 11.3 (4)</td>
<td>—</td>
<td>0.36</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>117 ± 9.0 (3)</td>
<td>9.6 ± 1.2 (2)</td>
<td>—</td>
<td>0.08</td>
</tr>
<tr>
<td>Flecaïnide</td>
<td>234 ± 3.0 (2)</td>
<td>—</td>
<td>61</td>
<td>(0.3)</td>
</tr>
</tbody>
</table>

\( ^{\text{a}} \) Values were obtained from Fig. 6C in Zolk et al. (2009).

\( ^{\text{b}} \) Values in italics represent the ratio of \( IC_{50} \) for inhibition of metformin transport measured by Zolk et al. (2009) versus the \( IC_{50} \) for inhibition of NBD-MTMA transport measured in the present report, whereas nonitalicized values stem from the present report.
spatially distinct sites. Development of predictive models of drug interaction with OCT2 should acknowledge the need to consider interaction profiles with structurally distinct substrates. Decision tree protocols for assessing the potential for DDIs between new and existing drugs must acknowledge that a weak interaction between a test compound and one probe substrate may mask a more potent interaction with others.

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Authorship Contributions

Participated in research design: Belzer, Jagadish, Mash, Wright.
Conducted experiments: Belzer, Morales, Jagadish.
Contributed new reagents or analytic tools: Belzer, Jagadish.
Performed data analysis: Belzer, Morales, Wright.
Wrote or contributed to writing of the manuscript: Belzer, Mash, Wright.

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