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Correlation between apparent substrate affinity and OCT2 transport turnover

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Running title: Correlation between substrate affinity and transport rate for OCT2

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Non-Standard Abbreviations: Intrinsic Clearance, Cl_{int}; MATE, Multidrug And Toxin Extrusion transporter; MPP, 1-methyl-4-phenylpyridinium; NBD-MTMA, N,N,N-Trimethyl-2-[methyl(7-nitrobenzo[c][l,2,5]oxadiazol-4-yl)amino]ethanaminium iodide; OCT, Organic Cation Transporter.

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Abstract

Organic cation transporter 2 (OCT2) mediates the first step in the renal secretion of many cationic drugs: basolateral uptake from blood into proximal tubule cells. The impact of this process on the pharmacokinetics of drug clearance as estimated using a physiologically-based pharmacokinetic (PBPK) approach relies on an accurate understanding of the kinetics of transport because the ratio of the maximal rate of transport to the Michaelis constant (i.e., J_{max}/K_t) provides an estimate of the intrinsic clearance (Cl_{int}) used in *in vitro-in vivo* extrapolation of experimentally determined transport data. Although the multispecificity of renal organic cation (OC) secretion, including that of the OCT2 transporter, is widely acknowledged, the possible relationship between relative affinity of the transporter for its diverse substrates and the maximal rates of their transport has received little attention. Here we determined the J_{max} and apparent Michaelis constant (K_{tapp}) values for six structurally distinct OCT2 substrates and found a strong correlation between J_{max} and K_{tapp}; 'high affinity' substrates (K_{tapp} values <50 µM; including 1-methyl-4-phenylpyridinium, or MPP, and cimetidine) displayed systematically lower J_{max} values (<50 pmol cm^{-2} min^{-1}) than did 'low affinity' substrates (K_{tapp} >200 µM; including choline and metformin). Similarly, preloading OCT2-expressing cells with low affinity substrates resulted in systematically larger *trans*-stimulated rates of MPP uptake than did preloading with high affinity substrates. The data are quantitatively consistent with the hypothesis that dissociation of bound substrate from the transporter is rate limiting in establishing maximal rates of OCT2-mediated transport. This systematic relationship may provide a means to estimate Cl_{int} for drugs for which transport data is lacking.

Introduction

At physiological pH about 40% of prescribed drugs carry a net positive charge (Neuhoff et al., 2003; Ahlin et al., 2008), and the kidney is the principal site of clearance of these 'organic cations' (OCs) (Hagenbuch, 2010). Renal OC clearance is supported by the two-step process of active secretion that occurs in the proximal tubule: uptake of OC from the blood into proximal tubule cells across the peritubular (basolateral) membrane, followed by efflux into the tubular filtrate across the luminal (apical) membrane. The sequential activity of these two processes effectively defines the pharmacokinetics of many cationic drugs. In humans, basolateral OC transport is dominated by the Organic Cation Transporter, OCT2, whereas apical OC transport involves the combined influence of the Multidrug And Toxin Extruders, MATE1 and MATE2/2-K (Pelis and Wright, 2011; Motohashi and Inui, 2013). Although MATE-mediated OC/H^+ exchange represents the 'active' step in net OC secretion, the process is initiated by the electrogenic, albeit passive, accumulation of OCs by the uniporter, OCT2, (Pelis and Wright, 2011).

Efforts to predict pharmacokinetic profiles of drug clearance increasingly rely on physiologically-based pharmacokinetic (PBPK) models that take into account the contribution of the individual metabolic events that influence drug clearance (Harwood et al., 2013), including renal transport (Posada et al., 2015; Burt et al., 2016). Mechanistically-based PBPK models require knowledge of the kinetic parameters of the underlying mediated transport processes (Harwood et al., 2013), including the Michaelis-Menten parameters, J_{max} (maximal rate of transport) and K_t (concentration of substrate resulting in half-maximal transport). Importantly, the scaling of transport activity determined *in vitro* to that occurring *in vivo* often uses the ratio J_{max}/K_t to provide an estimate of the intrinsic clearance (Cl_{int}) supported by a particular

transporter (Sjogren et al., 2009; Harwood et al., 2013). But despite insights into the contribution of a transport process to clearance of a target drug that are provided by the relationship between J_{max} and K_t, little attention has been given to the basis for different rates of transport that a multidrug transporter, such as OCT2, can exhibit for its structurally diverse suite of substrates.

Here we determined the kinetics of OCT2-mediated transport for a structurally diverse set of known substrates and found a strong positive correlation between J_{max} and apparent K_t; in other words, substrates for which the transporter had low apparent affinity (*i.e.*, high K_{tapp}) systematically displayed the highest maximal rates of transport. We also noted a similar correlation between IC_{50} values for inhibition of OCT2-mediated transport and the degree of *trans*-stimulation arising from pre-incubating cells in the inhibitory test agent; whereas preloading cells with low affinity substrates resulted in high levels of *trans*-stimulation, high affinity ligands (*i.e.* low IC_{50} values) produced no stimulation or even a *trans*-inhibition of transport activity. In light of these results we suggest that dissociation of a substrate from OCT2 is likely the predominant influence in defining its maximal rate of flux.

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Materials and Methods

Chemicals and Reagents: CHO cells containing the Flp-In target site, as well as hygromyocin, and Zeocin, were acquired from Invitrogen (Carlsbad, CA). [^{3}H]Tetraethylammonium ([^{3}H]TEA; 54 Ci/mmol) and [^{3}H]cimetidine (80 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP; 80 Ci/mmol), [^{3}H]N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium ([^{3}H]NBD-MTMA; 80 Ci/mmol; (Belzer et al., 2013)) and [^{3}H]choline (66.7 Ci/mmol), as well as unlabeled MPP and NBD-MTMA (Aavula et al., 2006), were prepared by the Synthesis Core of the Southwest Environmental Health Sciences Center/Department of Chemistry of the University of Arizona (Tucson, AZ). Additional [^{3}H]MPP (80 Ci/mmol) was purchased from Perkin-Elmer (Waltham, MA). [^{14}C]Metformin was purchased from Moravek Biochemicals (Brea, CA). Diphenidol was purchased from Santa Cruz Biotechnology (Dallas, TX), and all other chemicals were purchased from Sigma-Aldrich unless otherwise specified (St. Louis, MO).

Cell Culture: CHO cells were stably transfected at a single Flp-In recombinase site with the open reading frame for OCT2 in pcDNA5/FRT/V5-His TOPO (Pelis et al., 2007). Cells were cultured at 37°C with 5% CO_2. Culture medium contained Ham's F-12 Nutrient Mixture with 10% fetal bovine serum (Fisher Scientific, Pittsburg, PA). Hygromyocin (100 µg/ml) or Zeocin (100 µg/ml) was added to the culture medium of CHO-OCT2 cells or CHO-wild type (WT) cells, respectively, to maintain cell line expression profiles. Cells were passed every 2-4 days. When seeded into 96-well plates (Greiner; VWR Intl., Arlington Heights, IL) for transport assays, they were grown to confluence in antibiotic-free media.

Transport Assays: Wild type CHO cells and cells expressing hOCT2 were typically plated at densities sufficient to reach confluence within 24 hours (50,000 cells in 96-well cell culture

plates), after which they were used in transport experiments. For time course and kinetics experiments plates containing culture media were placed in an automatic fluid aspirator/dispenser (Model 406, BioTek, Winooski, VT) and automatically rinsed/aspirated three times with Waymouth's buffer (WB, in mM: 135 NaCl, 13 HEPES-NaOH, 28 D-glucose, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, and 0.8 $MgSO_4$) pH 7.4 at room temperature, after which transport buffer (60 µl) containing substrate and any additional required test agents were automatically introduced into each well. Following the experimental incubation the transport reaction was stopped by the rapid addition (and simultaneous aspiration) of ~750 µl of cold (4°C) WB. Following final aspiration of the cold stop, 200 µl of scintillation cocktail (Microscint 20, Perkin-Elmer, Waltham, MA) was added to each well and the plates were sealed (Topseal-A; Perkin-Elmer) and allowed to sit for at least 2 hrs before radioactivity was assessed in a 12 channel, multiwell scintillation counter (Wallac Trilux 1450 Microbeta, Perkin-Elmer). Accumulated substrate is expressed as picomoles per cm^2 of nominal cell surface or, in some cases, as a clearance in µl cm^{-2}. For the purpose of comparison to literature values that express rates of transport per mg of cell protein we find the conversion factor of 0.035 mg protein cm^{-2} to be reasonably accurate (Schomig et al., 2006).

To determine the effect on rates of substrate uptake of preloading cells with a test agent, cells were rinsed and then exposed for 20 minutes to WB containing the test agent (or just WB as a control). Following this preincubation, uptake of radiolabeled substrate was measured (in the absence of the preloaded compound) as outlined above.

Data Analysis: Uptake in OCT2-expressing cells was corrected for transport measured in wild-type CHO cells determined under the same experimental conditions and reported as a mean $\pm$ SE or mean $\pm$ SD as indicated for each data set. The significance of observed differences used

simple t-tests or ANOVA (with Tukey's multiple comparisons test), as appropriate, with differences at the 0.05 level considered to be significant. Analyses used either Excel 2007 (Microsoft, Redmond, WA) or Prism 6 (GraphPad, La Jolla, CA).

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Results

Figure 1 shows the time course of uptake (expressed as clearance) of three 'probe substrates' for OCT2 (MPP, TEA and NBD-MTMA), two commonly used drugs (cimetidine and metformin) and one endogenous OC (choline). Over the course of two minutes, uptake of all six substrates was adequately described as an exponential increase toward a constant, *i.e.*, steady state accumulation. Extrapolated rates of transport at time zero for each compound were calculated from their first order rate constants and plateau ('steady state') values. For five of the six substrates these estimates of the 'initial rate' were within 30% of the rates of transport estimated from net accumulation at 30 seconds, whereas the rate of MPP transport was within 45%. Consequently, 30 sec uptakes were used for subsequent kinetic experiments.

Figure 2 shows representative examples of the kinetics of OCT2-mediated transport of the six test substrates. Mediated transport of each was a saturable function of increasing substrate concentration that was adequately described by the Michaelis-Menten equation:

$$J = \frac{J_{max}[S]}{K_{tapp} + [S]} \qquad \text{Eq. 1}$$

where J is the rate of mediated transport from a substrate concentration of [S], J_{max} is the maximal rate of mediated substrate transport, and K_{tapp} is the apparent Michaelis constant, *i.e.*, the substrate concentration in the bulk medium that resulted in half maximal mediated transport. The kinetic values determined in five to nine separate experiments for each substrate are summarized in Table 1. J_{max} values ranged from ~30 pmol cm^{-2} min^{-1} (for MPP, cimetidine and NBD-MTMA) to as high as ~1000 pmol cm^{-2} min^{-1} (for metformin).

In addition to reflecting Cl_{int} (Sjogren et al., 2009), the ratio of J_{max} to K_{tapp} has been used as a measure of Transport Efficiency (TE; Schomig et al., 2006) because it is roughly analogous to enzymatic catalytic efficiency (k_{cat}/K_m) and, as such, it provides a useful means to compare the

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relative influence of a transporter to the flux of its structurally distinct substrates (Eisenthal et al., 2007). Cimetidine had the lowest TE at 1.3 x 10^{-3} cm min^{-1} (or expressed as a 'clearance,' ~40 µl mg^{-1} min^{-1}), whereas MPP had the highest at 6.5 x 10^{-3} cm min^{-1} (~200 µl mg^{-1} min^{-1}) (Table 1). Indeed, Schomig et al. (2006) found that OCT1, OCT2 and OCT3/EMT all display higher TEs for MPP transport than the other substrates they reviewed. But it was noteworthy that of the six substrates studied here, the only TE values found to be different from one another were those for MPP and cimetidine ($P < 0.05$); the other values were neither different from one another nor from the other two substrates. In other words, in our cells the average transport efficiency of OCT2 for its substrates was about ~3.2 (±0.8) x 10^{-3} cm min^{-1}.

The similarity of transport efficiency over a broad range of substrate structure and absolute rates of transport is evident in the relationship between increases in J_{max} and corresponding increases in the apparent K_t for transport, shown in Figure 3. For the six test substrates, there was a strong correlation ($r = 0.93$) between a decrease in the apparent affinity of OCT2 for a substrate and the maximal rate of transport of that compound (expressed as the log of each parameter). At one end of the range, the J_{max} for cimetidine transport of ~28 pmol cm^{-2} min^{-1} was associated with a K_{tapp} of ~21 µM, whereas at the other extreme, metformin's J_{max} of ~1000 pmol cm^{-2} min^{-1} was associated with a K_{tapp} of 500 µM.

The correlation between low affinity of substrate for OCT2 and high rate of substrate uptake proved to be true for efflux, as well. As a uniporter, OCT2 can support an electrogenic flux of organic cations (its normal mode of operation) or, alternatively, an electroneutral exchange of these charged substrates (Budiman et al., 2000). When operating as an exchanger, *trans*-oriented substrate gradients can stimulate transport from the *cis* compartment, resulting in classical *trans*-stimulation, or counterflow (*e.g.*, Stein, 1986). In light of the correlation between

maximum transport rate and apparent affinity of OCT2 for its substrates evident in Figure 3, we reasoned that oppositely oriented substrate gradients would stimulate transport of a probe substrate to a degree that was proportional to the *trans* compound's affinity for the transporter. To test this, we assembled eight known substrates of OCT2, and four inhibitors of OCT2-mediated transport, and assessed their relative affinity for the transporter by determining their IC_{50} values for inhibition of MPP transport. As expected, increasing concentrations of each compound resulted in increasing inhibition of OCT2-mediated MPP transport (Fig. 4), which was described by the relationship:

$$J^{MPP^*} = \frac{J_{app}^{MPP^*}[MPP^*]}{IC_{50} + [I]} \qquad \text{eq. 2}$$

where [I] is the concentration of inhibitor, J^{MPP*} is the rate of OCT2-mediated transport of [^{3}H]MPP from a concentration equal to [MPP*] (~10 nM); IC_{50} is the concentration of inhibitor that reduces mediated (*i.e.*, inhibitable) substrate transport by 50% (note: MPP uptake at each inhibitor concentration was corrected for uptake measured in wild type CHO cells); and $J_{app}^{MPP^*}$ is a constant that includes the maximal rate of MPP transport times the ratio of the inhibitor IC_{50} and the K_{tapp} for transport of MPP (Groves et al., 1994). Because the nM concentration of [^{3}H]MPP was much less than its K_{tapp} (6.5 μM), the IC_{50} values represent a reasonable approximation of each inhibitor's K_i. Resulting IC_{50} values were as low as 0.8 μM (for ethidium) to as large as 435 μM (for choline) (Table 2).

We then determined the effect on [^{3}H]MPP uptake of preincubating the cells in concentrations of each compound at least 2-fold, and generally >5-fold, larger than its IC_{50} (Fig. 5). There was a significant correlation between apparent affinity for OCT2 (*i.e.*, IC_{50}) and the

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rate of [^{3}H]MPP uptake produced by preloading the cells with the six test substrates; the degree of *trans* stimulation increased as apparent affinity for the transporter decreased (black circles in Fig. 5). This correlation was extended by the influence on MPP uptake of preloading the cells with two other known OCT substrates: ethidium and TMA. Whereas the high affinity substrate ethidium (IC_{50} of 0.8 μM and K_{tapp} of ~2 μM; Lee et al., 2009) had comparatively little effect on the rate of MPP uptake, the low affinity substrate TMA (Dresser et al., 2000) supported a marked stimulation of MPP transport (gray filled circles in Fig. 5). Two compounds shown previously to be non-transported inhibitors of OCT2, quinidine (Arndt et al., 2001) and diphenidol (Harper and Wright, 2012), and the known OCT2 inhibitor imipramine (Zolk et al., 2009), *trans*-inhibited MPP uptake (open circles in Fig. 5). Preincubating cells with tramadol, a substrate for OCT1 (Stamer et al., 2016), resulted in a modest, though not significant, stimulation of MPP transport.

Discussion

The 'multisubstrate specificity' of renal organic cation secretion was established by Karl Ullrich (1994), and since his seminal studies (Ullrich et al., 1991; Ullrich et al., 1992; David et al., 1995; Ullrich and Rumrich, 1996) the ability of the underlying molecular processes, *i.e.*, the OCTs and MATEs, to transport compounds of broadly diverse structure has been clearly established (Nies et al., 2011; Pelis and Wright, 2011). The first step in renal secretion – substrate uptake from blood into proximal tubule cells – is mediated by OCTs, and in humans OCT2 is the dominant process. Over the last 20 years, the kinetics of OCT2-mediated transport have been reported for a structurally diverse range of compounds (Nies et al., 2011). But interstudy comparisons of kinetic parameters are rife with *caveats*. Indeed, marked differences in kinetic values obtained by different lab groups (that often use different experimental and analytical methodologies) have plagued the study of membrane transport (*e.g.*, Bentz et al., 2013). Nevertheless, the comparatively large database for such studies (see Nies et al., 2011, and Supplemental Table 1) invites examination of the relationship between reported values for J_{max} for different OCT2 substrates and their associated apparent K_t values. As shown in Figure 6, these data suggest that there is an inverse correlation between the apparent affinity of OCT2 for a substrate and its maximal rate of transport. The results of the current study confirmed this general observation (the gray shaded data points in Fig. 6) and support the hypothesis that there is a systematic relationship between the affinity of OCT2 for a substrate and the maximal rate of that substrate's transport.

However, it should be noted that increases in apparent K_t values are an expected consequence of increases in J_{max} simply owing to the influence of unstirred waters layers (UWLs; Winne, 1973; Barry and Diamond, 1984). For carrier-mediated uptake into cells,

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transport activity reduces the concentration of substrate in the layer of effectively static fluid immediately adjacent to the extracellular face of the membrane, the UWL, thereby establishing a gradient of substrate concentration between the surface of the membrane and the (well-stirred) bulk medium. Consequently, the concentration of substrate in the experimental solution is greater than that actually available to the transporter, so 'apparent' K_t values (K_{tapp}) determined experimentally overestimate the actual concentration of substrate required to half-saturate the transporter. The 'bias' that an UWL introduces into measured values of K_{tapp} is directly proportional to the J_{max} of the transport process (Winne, 1973). We recently showed that simply changing the number of transporters expressed in the plasma membrane, thereby changing J_{max}, can result in 5 to 10-fold changes in the measured K_{tapp} values for OCT2-mediated transport of TEA and MPP (Shibayama et al., 2015). At steady state, the influence of UWLs and J_{max} on an experimentally determined K_t value is given by:

$$K_{tapp} = K_t + J_{max}\left(\frac{\delta}{2D}\right) \qquad \text{eq. 3}$$

where K_{tapp} is the experimentally determined Michaelis constant, K_t is the 'true' Michaelis constant of the transporter, J_{max} is the maximal rate of mediated transport, D is the diffusion coefficient of the transported substrate (approx. 6×10^{-6} cm^2 sec^{-1} for molecules the size of MPP or other small organic cations; Avdeef et al., 2004), and δ is the thickness of the UWL. The effective thickness of UWLs in unstirred multi-well plates has been measured using a variety of techniques with average values being on the order of 1500 microns (Korjamo et al., 2009). Using that value and a J_{max} of 1000 pmol cm^{-2} min^{-1} (the value we measured for metformin transport and the upper end of J_{max} values reported in the literature for OCT2-mediated transport; Fig. 6), the 'bias' introduced to steady state estimates of K_t is on the order of 200 µM. By this

reasoning, our measured value of ~500 μM for the K_{tapp} for metformin transport could reflect a 'true' K_t of ~300 μM. By similar logic, the smaller J_{max} for MPP transport (~35 pmol cm^{-2} min^{-1}) suggests that measured K_t values should overestimate the true value by ~7 μM. However, these calculations assume the concentration of substrate at the membrane is at steady state during the entire course of measurement, reflecting the impact of the UWL on establishing a constant gradient in concentration between the membrane surface and the bulk medium. In fact, this is not the case. The substrate concentration at the membrane immediately following addition of an experimental solution is actually zero, owing to the presence of substrate-free medium at the membrane surface that remains even after thorough aspiration of pre-incubation media. Although substrate quickly diffuses through this layer, the ensuing activity of the transporter begins to influence the concentration. The steady state condition was assumed in all previous assessments of the impact of UWLs on transport kinetics (Winne, 1973; Thomson and Dietschy, 1977; Balakrishnan et al., 2007), but it takes several minutes of unabated transport activity before the 'steady state' condition reflected in the relationship derived by Winne (1973) is established. Our modeling of this effect indicates that, during the course of the 30 sec uptakes we used in our studies, the concentration of substrate at the membrane was both constantly changing and, on average, higher than expected at steady state, thereby blunting the influence of UWLs on the measured K_t (unpublished observations). Consequently, at the low end of the J_{max} range (*e.g.*, MPP, cimetidine), K_t values may have been overestimated by 1-3 μM (rather than 7 μM), whereas at the high end (metformin, choline), the overestimate was likely on the order of 100 μM (rather than 200 μM). Thus, although the ubiquitous presence of UWLs will contribute to the relationship between J_{max} and K_{tapp} apparent in Figures 3 and 6, it cannot account for the bulk of this correlative effect. Supporting this conclusion we to point to the *trans* stimulation of

MPP uptake produced by preloading cells with OCT2 substrates. Despite the fact that there would have been no substantive UWL at the cytoplasmic face of the protein to influence the interaction of the preloaded substrate with the transporter, the correlation between maximal rate of transport and the apparent affinity of substrate for the transporter was observed.

It is also worth commenting on the influence of preloading cells with non-transported inhibitors of OCT2 on the rate of substrate uptake. Diphenidol was an effective inhibitor of OCT2 (IC_{50} of 34 μM), but is not itself transported by OCT2 (Harper and Wright, 2012). As a weak base (pK_a of 9.2), a modest rate of diffusion of the uncharged species would, however, be expected to occur, leading to its gradual intracellular accumulation during the 20 min preincubation in 500 μM diphenidol. Intracellular diphenidol would likely bind to the cytoplasmic face of the transporter, and its failure to support the conformational changes required for translocation would be expected to result in a *trans* inhibition of MPP uptake, as noted in Figure 5. Although the transportability of quinidine by OCT2 has not been clearly established, its enantiomer, quinine, is a non-transported inhibitor of OCT2 (Arndt et al., 2001), so the *trans* inhibition produced by preincubating cells in a 500 μM concentration this weak base was also expected.

The J_{max} of substrate transport into a cell is the product of the number of functional transporters expressed at the membrane and the turnover number (TN) of each transporter (the number of substrate molecules it can translocate from one side of the membrane to the other per unit time under saturating conditions). Assuming that the transport process is adequately described by the 'alternating access' model (Kaback et al., 2011), the TN is influenced by the rate of interconversion of the outward- and inward-facing forms of the carrier and/or the rate of dissociation of substrate from the carrier (Stein, 1986). Our observation that the line of stably

expressing CHO cells that supported OCT2-mediated transport of different substrates at maximal rates that varied by almost 40-fold suggests that the TN of OCT2 can vary by that amount, depending on substrate identity. That TNs varied inversely with apparent affinity of OCT2 for substrate invites the suggestion that substrate dissociation may be rate-limiting to the rate of transport. But identifying the basis for transporter TN is difficult because we lack information on rates of substrate association (k_{on}; M^{-1} sec^{-1}) with, and dissociation (k_{off}; sec^{-1}) from, the carrier. However, Stein (1986) described a method that permits a tentative test of the hypothesis that substrate dissociation is the rate-limiting element of transporter turnover. The argument makes the temporary assumption that TN is given by k_{off}. Given that the dissociation constant for substrate binding to the transporter, K_D, is equal to k_{off}/k_{on}, one calculates an estimate for k_{on} from k_{off}/K_D, identified tentatively as TN/K_t, and then compares the estimated k_{on} to a range of 'likely' values for that parameter. This comparison takes advantage of the existing database for small ligand interactions with their binding partners. Rates of association for ligands can range from 10^4 to 10^9 M^{-1} sec^{-1}. However, the applicable range can be narrowed markedly by considering a range of measured k_{on} values for low molecular weight ligands with their protein binding partners; from about 1 x 10^5 M^{-1} sec^{-1} to about 1 x 10^7 M^{-1} sec^{-1} (*e.g.*, Stein, 1986; Bloom et al., 1987; Wright et al., 1992; Kapur and Seeman, 2000). Moreover, there is evidence that large differences in K_D values observed for a range of structurally distinct ligands generally reflect changes in k_{off}, rather than k_{on}, which tend to be relatively constant (Tummino and Copeland, 2008). For the current purpose, we use 10^5 to 10^7 M^{-1} sec^{-1} for the range of likely k_{on} values for OCT2 substrates. The TN of OCT2 for transport of atenolol at 37^{o}C has been estimated from the kinetics of its transport and measurement of the number of (total) OCT2 transporters expressed in the HEK 293 expression system used in the study (Yin et al., 2015); the

resulting TN was ~3 sec^{-1}. The range of TN values for transporters is generally reported as 10 – 2000 sec^{-1} (Stein and Litman, 2015), and for the current purpose we will use the range 1 to 1000 sec^{-1}. As shown in Figure 6, Michaelis constants reported in the literature for OCT2-mediated transport range from ~1 μM to ~1000 μM, and we will use this range. Figure 7 shows a matrix of estimated k_{on} values for physiologically relevant pairs of TN and K_t values, and values lying within the 'likely' range of association rates are highlighted. In fact, those highlighted values represent the majority of those in the matrix and, as expected, show a clear correlation between high TN (*i.e.*, high J_{max}) and high K_t (*i.e.*, low affinity), consistent with the observed correlation between those parameters and supporting the hypothesis that dissociation of substrate from the carrier is rate-limiting for establishing maximal rates of OCT2-mediated transport.

J_{max} and K_t values for drug transporters determined from *in vitro* experiments are playing an increasingly important role in physiologically-based pharmacokinetic modeling (PBPK; Harwood et al., 2013; Burt et al., 2016). When interpreted as a measure of Cl_{int}, the ratio of J_{max} to K_t is an element of the scaling factors used for the *in vitro-in vivo* extrapolation (IVIVE) required for mechanistic PBPK (Harwood et al., 2013). If future work confirms the rate-limiting impact of apparent affinity of OCT2 for substrate on the maximal rate of transport, then establishing Cl_{int} of an *in vitro* system for several substrates may provide scaling factors that are common for a wide range of OCT2 substrates for which direct transport data are not available.

In summary, maximal rates of OCT2-mediated uptake for an array of structurally distinct substrates were inversely correlated with the apparent affinity of the transporter for these compounds. Similarly, the rate of *trans* stimulation of transport activity produced by preloading cells with a structurally diverse range of known OCT2 substrates was inversely correlated with the apparent affinity of the transporter for the preloaded compound. The correlation between

affinity of OCT2 for a substrate and the maximal rate of transport of that compound was consistent with the hypothesis that the rate limiting step in OCT2-mediated transport is dissociation of substrate from the transporter (rather than differences in the rate of conformational changes associated with translocation of structurally distinct substrates). These observations may provide useful insights for applying scaling factors for OCT2 activity required for mechanistic PBPK modeling of novel compounds for which direct transport data is lacking.

Authorship Contributions

Participated in research design: AC Severance and SH Wright

Conducted experiments: AC Severance and PJ Sandoval

Performed data analysis: AC Severance, Pj Sandoval and SH Wright

Wrote or contributed to writing of the manuscript: AC Severance, PJ Sandoval and SH Wright

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Footnote

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Figure Legends

Figure 1. Time course of OCT2-mediated transport of six structurally diverse substrates, expressed as clearance. Each point is the mean (±SE) of two separate experiments each performed in quintuplicate. The substrate concentrations of [^{3}H]labeled MPP, TEA, cimetidine, NBD-MTMA and choline ranged from 10 to 12 nM; [^{14}C]metformin concentration was ~12 µM. Each uptake was corrected for substrate accumulation measured in non OCT2-expressing cells. Lines reflect best fits of first order association (Prism, GraphPad, San Diego, CA).

Figure 2. Kinetics of OCT2-mediated uptake of the six structurally distinct test substrates. Each plot shows data from a representative experiment (see Table 1 for compiled data). The data points reflect mean uptake (±SE) from 5 to 6 replicate wells. The lines reflect best fits of the Michaelis-Menten equation (Prism).

Figure 3. Relationship between J_{max} and apparent K_t for the six test substrates. Each point is the mean value (±SE) for 5 to 9 separate determinations of the kinetics of OCT2-mediated transport.

Figure 4. Determination of IC_{50} values for inhibition of OCT2-mediated MPP transport produced by eight OCT2 substrates and four inhibitors of OCT2-mediated transport. Each figure shows inhibition of [^{3}H]MPP transport (~10 nM; 30 sec uptake), expressed as % of control uptake, averaged for 2 to 4 separate experiments for each inhibitor (see Table 2). The listed IC_{50} values reflect the average of the values obtained in the individual experiments with each test agent.

Figure 5. Relationship between IC_{50} values for inhibition of OCT2-mediated MPP transport and the degree of stimulation of MPP uptake resulting from preloading cells with test compound. Each point is the average (±SE) of *trans*-stimulation measured in 4 to 7 separate experiments with the test compounds, plotted as a function of their respective IC_{50} values (from Table 2). * indicates a difference (relative to control uptake) significant at $P < 0.05$.

Figure 6. Relationship between J_{max} and apparent K_t values for OCT2-mediated transport of 23 distinct substrates. Solid black circles show values reported in the literature (see Supplemental Table 1 for a list of these values and the corresponding references). Solid red circles show the values for the six substrates used in the present study. The line is simple linear regression of these data (Prism).

Figure 7. Calculated association rates (k_{on}; M^{-1} sec^{-1}) for a range of likely K_t (μM) and Turnover Number (sec^{-1}) values for substrate interaction with OCT2. The bold/highlighted values represent k_{on} values considered most likely relevant for low molecular weight interactions of the type anticipated to occur between OCT2 and its substrates. (See text for discussion)

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Table 1.

Kinetics of OCT2-mediated transport of the indicated substrates. Transport efficiency reflects the ratio J_{max}/K_{tapp}.

Substrate	**J_{max} (±SE)** **(pmol cm^{-2} min^{-1})**	**K_{tapp} (±SE)** **(μM)**	**Transport Efficiency** **(10^{-3} cm min^{-1})**	**n**
MPP	35.2 ±9.1	5.1 ± 0.8	6.5 ± 1.4	9
NBD-MTMA	36.2 ± 5.5	14.5 ± 4.0	3.0 ± 0.3	9
Cimetidine	27.7 ± 7.5	20.8 ± 2.8	1.3 ± 0.3	6
TEA	276 ± 125	71.9 ± 9.9	4.7 ± 2.8	6
Choline	673 ± 116	465 ± 116	1.6 ± 0.1	5
Metformin	1046 ± 171	518 ± 45	2.0 ± 0.2	8

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Table 2.

IC_{50} values for inhibition of OCT2-mediated transport of MPP. In each experiment rates of transport were based on 30 sec uptakes of 10-20 nM ^{3}H]MPP in the presence of increasing concentrations (typically eight concentrations) of test inhibitor (n=4-6 wells of a 96 well plate for each concentration). 'n' refers to the number of separate experiments performed.

Inhibitor	**IC_{50} (μM)**	**n**
Ethidium	0.8 ± 0.1	2
MPP	7.	1
Imipramine	28.9 ± 15.7	3
Diphenidol	34.3 ± 7.9	5
NBD-MTMA	46 ± 20	2
TEA	77 ± 3	2
Quinidine	92.6 ± 29.1	6
Cimetidine	113 ± 53	2
Tramadol	140.1 ± 31.9	3
Metformin	223 ± 30	4
TMA	301 ± 15	2
Choline	435 ± 153	2

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Figure 1

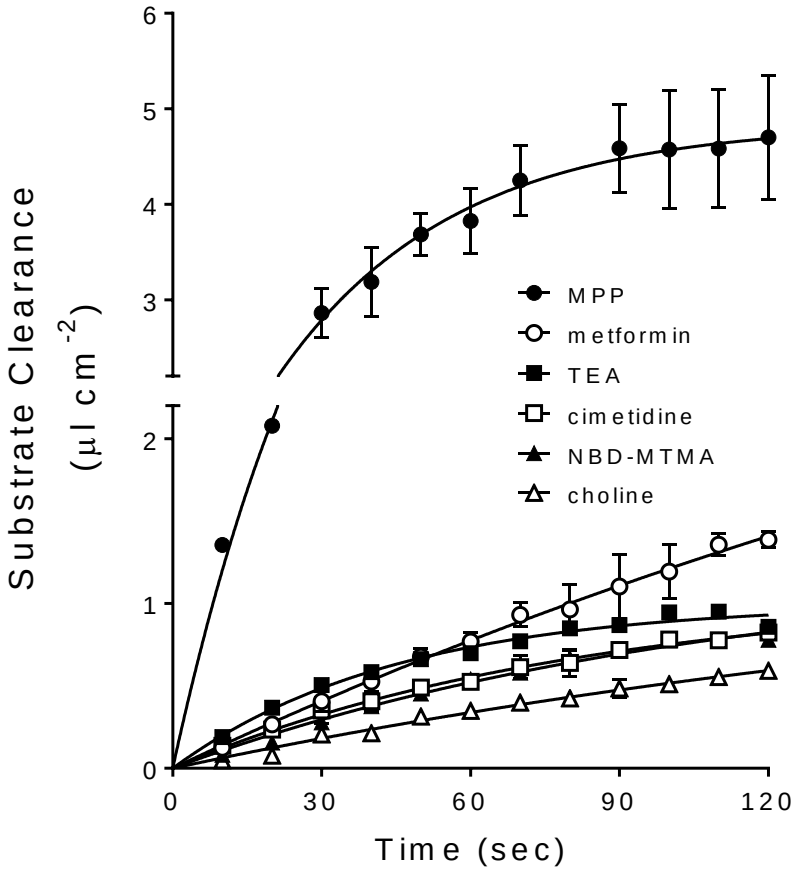

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Figure 2

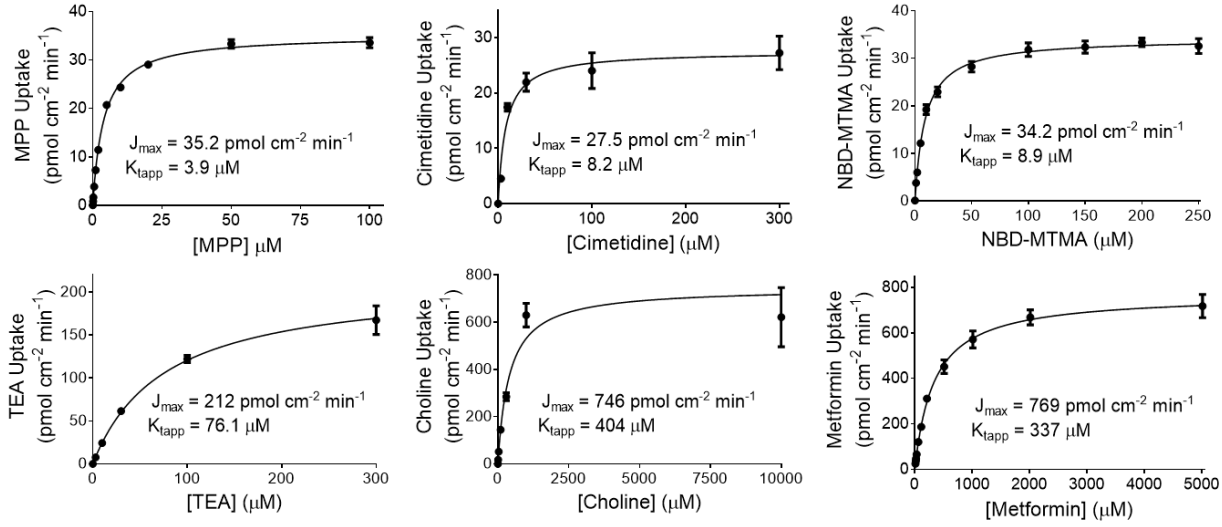

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Figure 3

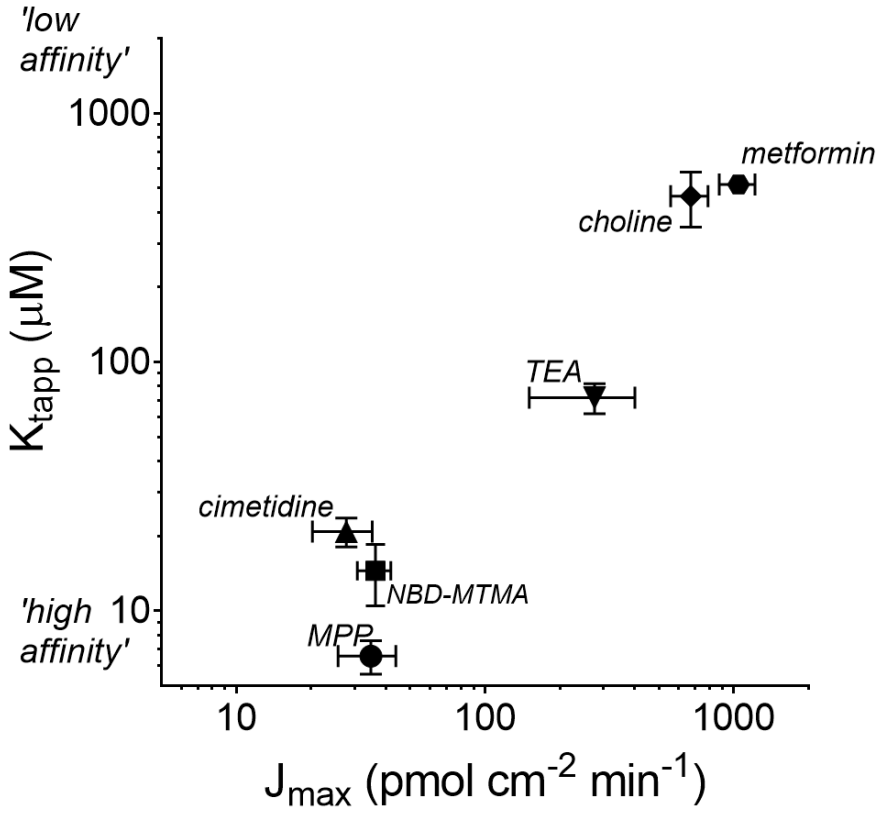

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Figure 4

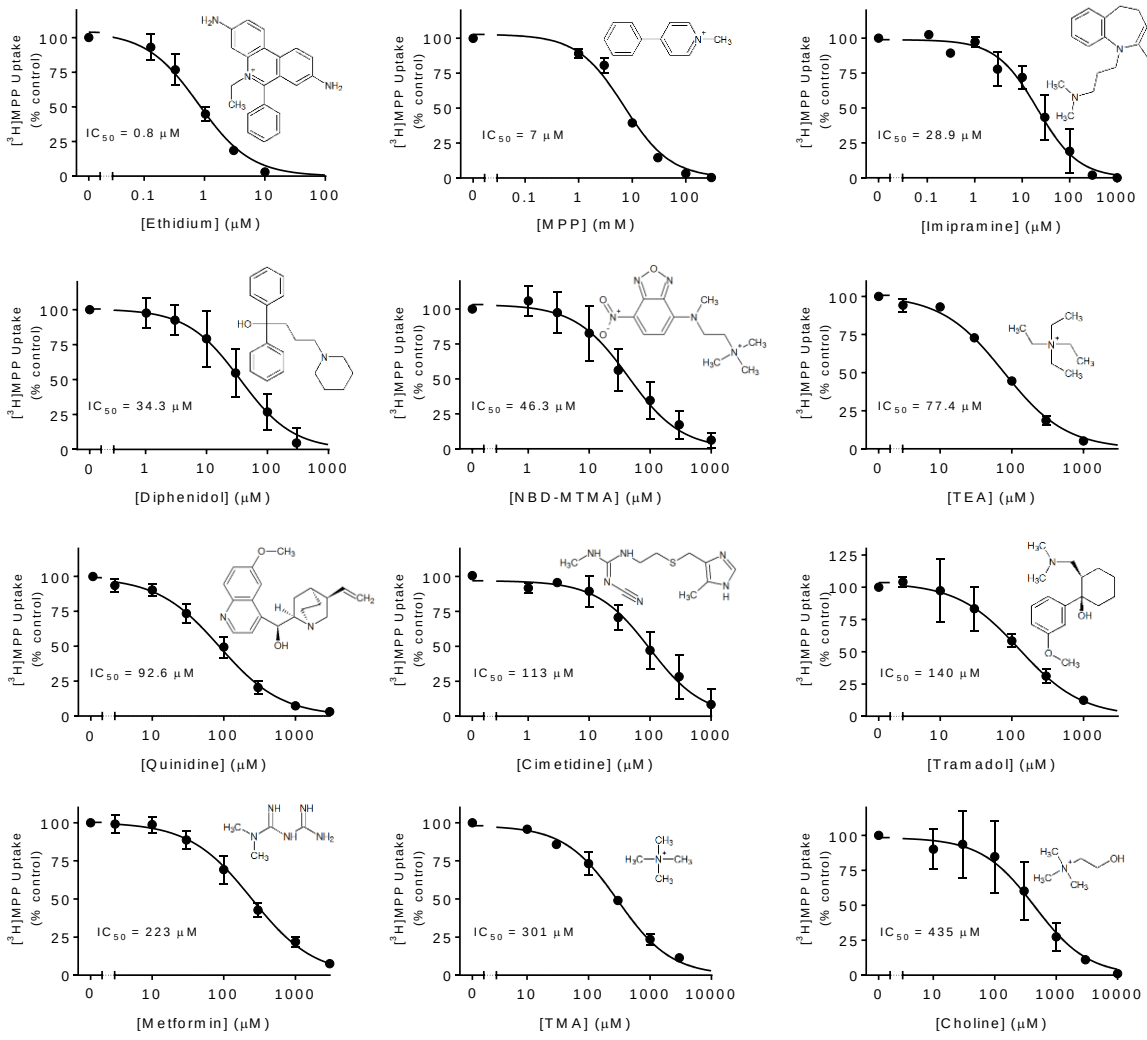

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Figure 5

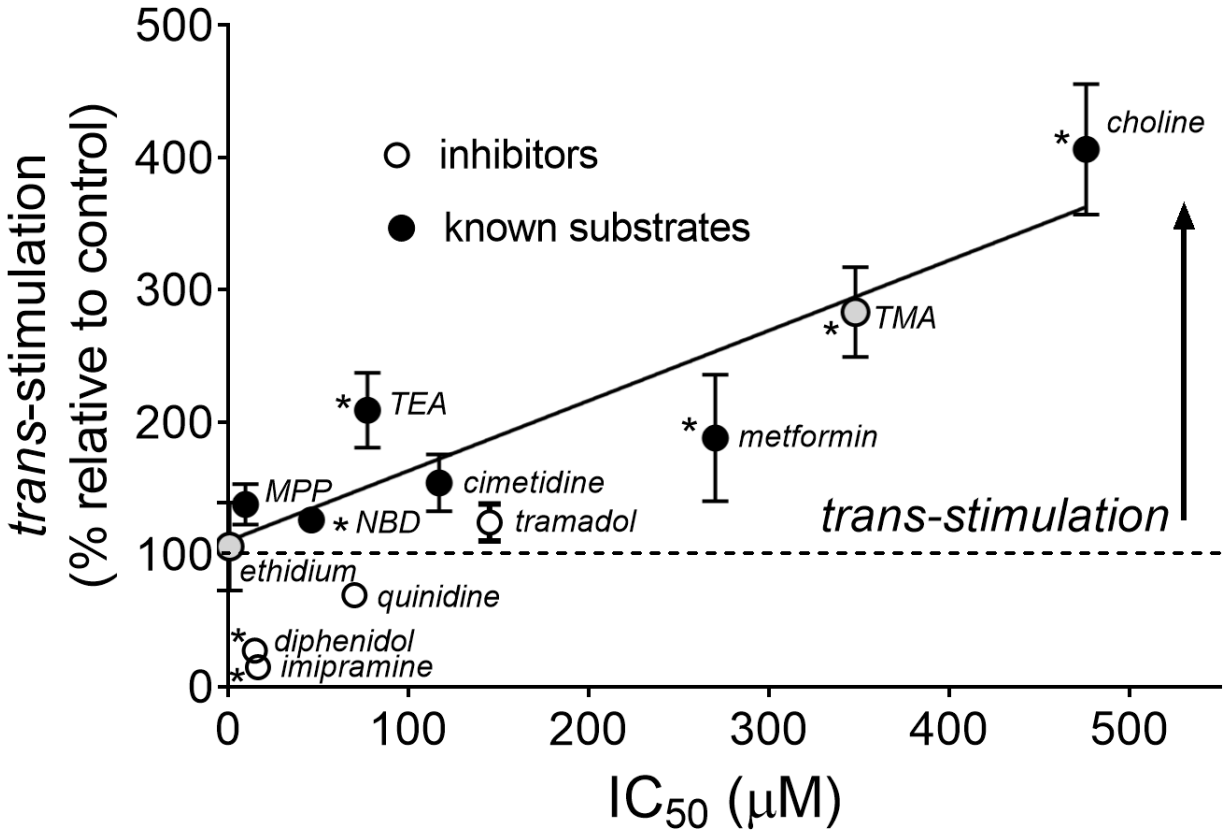

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Figure 6

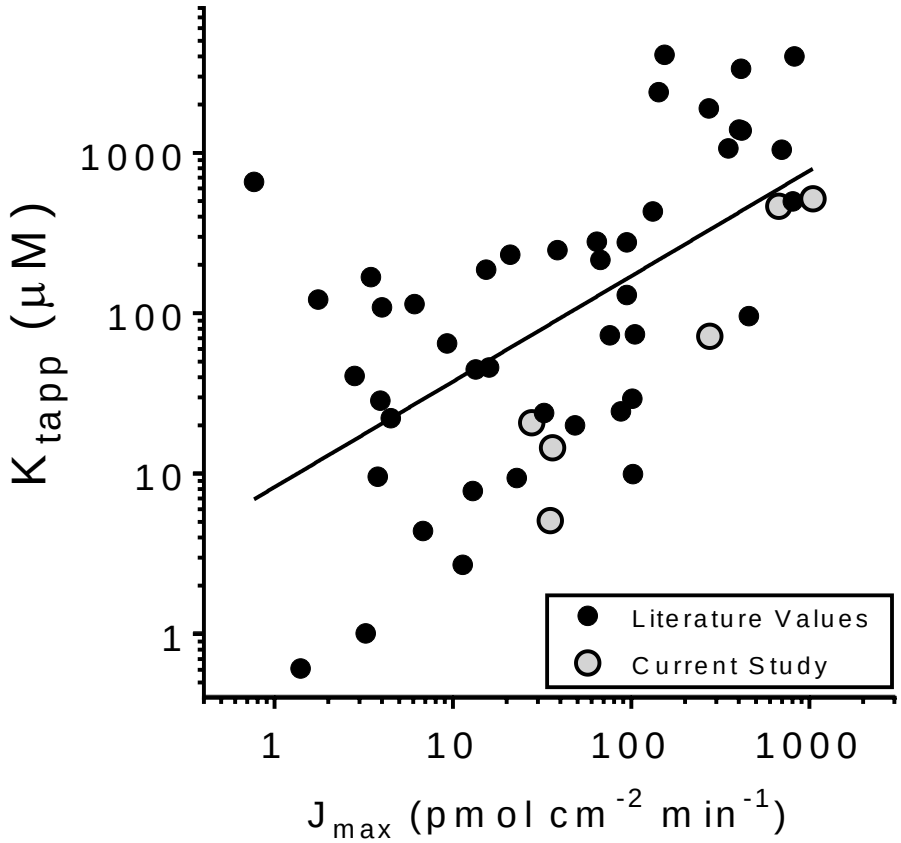

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Figure 7