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Title Page

Impact of Experimental Conditions on the Evaluation of Interactions between Multidrug and Toxin Extrusion Proteins and Candidate Drugs

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Running Title Page

Running title:

Impact of experimental conditions on MATE assessment

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Abbreviations:

DDI, drug-drug interaction;

E3S, estron-3-sulfate;

HEK293, human embryonic kidney 293,

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MATE, multidrug and toxin extrusion;

MES, 4-morpholineethanesulfonic acid

MPP^+, 1-methyl-4-phenylpyridinium;

NBuPy-Cl , N-butylpyridinium chloride

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Abstract

Multidrug and toxin extrusion transporters (MATEs) have a determining influence on the pharmacokinetic profiles of many drugs and are involved in several clinical drug-drug interactions (DDIs). Cellular uptake assays with recombinant cells expressing human MATE1 or MATE2-K are widely used to investigate MATE-mediated transport for DDI assessment. However, experimental conditions and used test substrates vary among laboratories. We therefore initially examined the impact of three assay conditions that have been applied for MATE substrate and inhibitor profiling in the literature. One of the tested conditions resulted in significantly higher uptake rates of the three test substrates [^{14}C]metformin, [^{3}H]thiamine, and [^{3}H]MPP^{+}, but IC_{50} values of four tested MATE inhibitors varied only slightly among the three conditions (<2.5-fold difference). Subsequently, we investigated the uptake characteristics of the five MATE substrates [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]estrone-3-sulfate (E3S), and rhodamine 123 as well as the impact of the utilized test substrate on the inhibition profiles of ten MATE inhibitors at one selected assay condition. [^{3}H]E3S showed atypical uptake characteristics compared to those observed with the other four substrates. IC_{50} values of the tested inhibitors were in a similar range (<4-fold difference) when [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+} or [^{3}H]E3S were used as substrates but were considerably higher with rhodamine 123 (9.8-fold and 4.1-fold differences compared to [^{14}C]metformin with MATE1 and MATE2-K, respectively). This study demonstrated for the first time that the impact of assay conditions on IC_{50} determination is negligible, that kinetic characteristics differ among used test substrates and that substrate-dependent inhibition exists for MATE1 and MATE2-K. This will give a valuable insight into the assessment of clinically relevant MATE-mediated DDIs *in vitro*.

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Introduction

The renal tubular secretion of cationic drugs is mediated by specific sets of transporters in the basolateral and apical membranes of the proximal tubule cells. The first step of the renal secretion process is the basolateral uptake of organic cations from the circulation into the proximal tubule cells. The main responsible transporter for this process is organic cation transporter 2 (OCT2) (SLC22A2) (Inui *et al.*, 2000; Fujita *et al.*, 2006). In contrast to the comprehensive knowledge about this OCT2-mediated basolateral uptake of organic compounds, the understanding of the molecular mechanisms underlying the subsequent apical secretion into the tubular lumen and the identification of the involved transporters has only recently begun. Growing evidence was found that this process is presumably mediated by the two human MATE isoforms MATE1 (SLC47A1) and its paralog MATE2-K (SLC47A2), which are abundantly expressed in the apical membrane of proximal tubule cells and work as H^+/organic ion antiporters, driven by an inwardly directed H^+ gradient (Yonezawa and Inui, 2011; Motohashi and Inui, 2013). Both MATE isoforms share a partially overlapping substrate specificity and it has been shown that they transport a wide range of cationic, zwitterionic and anionic compounds *in vitro*, including several renally secreted drugs such as metformin, cimetidine and others (Masuda, 2006; Tanihara *et al.*, 2007; Chen *et al.*, 2009).

A number of subsequent *in vitro* and *in vivo* studies demonstrated the clinical importance of MATE transporters including their role as determinants of the pharmacokinetic profiles of various drugs and their direct involvement in several clinical DDIs (Tsuda *et al.*, 2009; Kusuhara *et al.*, 2011; Ito *et al.*, 2012). As a consequence, MATEs are now perceived as transporters of emerging importance by the International Transporter Consortium (Hillgren *et al.*, 2013) and regulatory authorities such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have included MATE *in vitro* evaluation into

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their guidelines for drug interaction studies (European Medicines Agency, 2012; U.S. Food and Drug Administration, 2012).

The most commonly used *in vitro* tool to investigate MATE-mediated transport activities are cell-based transport assays with recombinant epithelial cell lines expressing the human MATE1 or MATE2-K isoform. Numerous studies utilizing such cell systems have helped understanding the molecular function, driving force and substrate specificity of MATE transporters and allowed for the identification of a wide range of compounds as substrates or inhibitors of MATE1 and/or MATE2-K (Tanihara *et al.*, 2007; Tsuda *et al.*, 2007; He *et al.*, 2010). However, the applied experimental conditions such as buffer compositions and extra- and intracellular pH vary considerably among different laboratories and to the best of our knowledge, it has not yet been investigated whether these varying conditions can affect *in vitro* assay outputs. In addition,, varying test substrates such as metformin, tetraethylammonium (TEA), 1-methyl-4-phenylpyridinium (MPP^+), or 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+) are being used in different laboratories and it has been demonstrated for a number of SLC drug transporter isoforms such as OCT2 and the organic anion transporting polypeptides (OATPs) 2B1 and 1B1 that the utilized substrate can severely affect the inhibitory effects of tested compounds (Shirasaka *et al.*, 2012; Belzer *et al.*, 2013; Izumi *et al.*, 2013; Hacker *et al.*, 2015). First evidence of a suchlike substrate-dependent inhibition has also been reported for MATE1 (Martínez-Guerrero and Wright, 2013).

In order to investigate the possible effect of varying assay conditions, we initially examined the impact of three different conditions on substrate and inhibitor profiling of compounds using MATE1- and MATE2-K-expressing cells. Based on the results, a suitable condition was selected under which MATE-mediated uptake characteristics of five different

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test substrates and the impact of the utilized test substrate on the inhibition profiles of ten different MATE inhibitors were investigated.

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Materials and Methods

Chemicals and reagents

[^{3}H]Thiamine (20 Ci/mmol) and [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP$^+$; 80 Ci/mmol) were purchased from American Radiolabeled Chemicals (Saint Louis, MO, USA), [^{3}H]estrone-3-sulfate ([^{3}H]E3S; 45 Ci/mmol) was purchased from PerkinElmer (Waltham, MA, USA) and [^{14}C]metformin (90 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Unlabeled thiamine, MPP$^+$, E3S and rhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and unlabeled metformin was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were of analytical grade and are commercially available.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in low glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Life Technologies, Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. For the generation of MATE1- and MATE2-K-expressing cells, parental HEK293 cells were initially seeded onto poly-D-lysine-coated 24-well plates at a density of 0.75×10^5 cells/well. On the next day, the cells were transfected with pcDNA3.1(-)/MATE1 (accession number NM_018242.2), pcDNA3.1(-)/MATE2-K (accession number AB250364.1) or control vector using FuGENE 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately 24 hours after transfection, the medium was changed to culture medium supplemented with 5 mM sodium butyrate and the cells were incubated for additional 24 hours to induce transporter gene expression.

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Uptake experiments using transiently transfected HEK293 cells

Uptake experiments with transiently transfected HEK293 cells were conducted approximately 48 h after transfection using three different experimental conditions (conditions A-C, Table 1). For intracellular acidification used in condition A, cells were initially washed twice and pre-incubated with K^+-based transport buffer (pH 7.4) supplemented with 20 mM NH_4Cl for 10 min at 37°C and subsequently incubated for additional 5 min with NH_4Cl-free K^+-based transport buffer. In uptake experiments performed under condition B and C, cells were washed twice and pre-incubated with respective transport buffer (pH 7.4) for 10 min at 37°C. For the investigation of pH-dependent uptake at an extracellular pH between 6.0 and 7.0, 20 mM HEPES in the transport buffer was replaced with 20 mM MES.

Uptake assays were initiated by aspiration of the pre-incubation buffer and addition of transport buffer (pH 7.4) containing radiolabeled [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^+, [^{3}H]E3S or unlabeled rhodamine 123 and test inhibitors where applicable. Uptake was terminated at the designated incubation times by washing the cells three times with ice-cold transport buffer. To determine the uptake of radiolabeled substrates, cells were solubilized with NaOH for 1 h at 37°C followed by the addition of an equal amount of HCl to neutralize the cell lysates. Aliquots of the lysates were transferred to scintillation vials containing scintillation cocktail (Hionic Fluor; PerkinElmer Waltham, MA, USA) and radioactivity was measured in a liquid scintillation counter (TRI-CARB 3110 TR, PerkinElmer, Waltham, MA, USA). The remaining cell lysates were used to determine the protein concentration using the Lowry method with bovine serum albumin as standard.

To ensure reproducibility of the inhibition studies, more than 10% of all IC_{50} values were re-determined in a separate experiment and the observed differences between repetitions were within a 2-fold range.

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Rhodamine 123 measurement by fluorescence detection

Rhodamine 123 concentration was analyzed by transferring aliquots of the lysates to black 96-well plates (PerkinElmer, Waltham, MA, USA) and determining the fluorescence intensity (485 nm excitation, 535 nm emission) with a fluorescence plate reader (EnVision 2102, PerkinElmer, Waltham, MA, USA). The calibration curve in a range from 0.1 – 100 μM was linear ($R^2 > 0.9994$) and 1 μM rhodamine 123 solution was used as control. The obtained inter-assay coefficient of variation (plate-to-plate variation) from the control samples was 17% (n = 11).

Data analysis

Cellular uptake was normalized to the amount of radioactivity in the buffer and protein concentration in each well and was calculated as given in the following equation:

$$Uptake = \frac{C_{cell}}{C_{buffer}}$$

where *Uptake* is the mean uptake (μl/designated time/mg protein), C_{cell} is the radioactivity associated with the cell specimens (dpm/designated time/mg protein), and C_{buffer} is the radioactivity concentration in the buffer (dpm/μl). For uptake experiments with rhodamine 123, mean uptake was calculated using fluorescence intensity instead of radioactivity. Transporter-mediated uptake was calculated by subtracting the uptake in empty-vector transfected cells from that in transporter expressing cells.

Kinetic parameters were obtained using the following equation:

$$v = \frac{V_{max} \times S}{K_m + S}$$

where v is the uptake rate of the substrate (pmol/min/mg protein), S is the substrate concentration in the buffer (μM), K_m is the Michaelis-Menten constant (μM), and V_{max} is the

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maximum uptake rate (pmol/min/mg protein). Fitting was performed by the nonlinear least-squares method using the GraphPad PRISM software (Version 6.04, GraphPad Software, La Jolla, CA).

In case statistical significant inhibition was observed, we determined the half-inhibitory concentrations (IC_{50}) of test inhibitors using the GraphPad PRISM software based on the following four-parameter logistic equation:

$$CL = CL_{min} + \left(\frac{(CL_{max} - CL_{min})}{1 + 10^{((logIC_{50} - I) \times Hill\ slope)}} \right)$$

where *CL*, represents the uptake clearance, *I* is the inhibitor concentration and *Hill slope* is the slope factor.

Statistical analysis

Statistically significant differences in this study were determined using Student's two-tailed unpaired *t*-tests. $p < 0.05$ and $p < 0.01$ were considered to be significant.

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Results

Impact of different assay conditions on substrate profiling and inhibitor profiling

There are various experimental conditions available for *in vitro* MATE evaluation such as the use of an intracellular acidification technique to generate an outwardly-directed H^+ gradient or the use of different buffer systems to change the extracellular concentration of H^+ and other ions. Among these various experimental conditions, three conditions (A, B and C, see Table 1) were tested to check their impact on the substrate and inhibition profiles of known MATE substrates and inhibitors. In order to do this, we conducted uptake studies with three *in vitro* probe substrates ([^{14}C]metformin, [^{3}H]thiamine and [^{3}H]MPP$^+$) and examined the inhibitory effects of four inhibitors (pyrimethamine, quinidine, ondansetron and N-butylpyridinium chloride (NBuPy-Cl)) on the MATE1- and MATE2-K-mediated uptake of the three *in vitro* probe substrates at three different assay conditions.

Experiments conducted at condition A generally resulted in significantly higher uptake rates of all test substrates than experiments conducted at condition B and C (Fig. 1). In HEK293-MATE1 cells, the average substrate uptake after 1 min under condition A was 2.3-fold ([^{14}C]metformin), 2.3-fold ([^{3}H]thiamine), and 2.6-fold ([^{3}H]MPP$^+$) higher than under condition B and 2.4-fold ([^{14}C]metformin), 2.3-fold ([^{3}H]thiamine), and 2.7-fold ([^{3}H]MPP$^+$) higher than under condition C. Similar observations were made in HEK293-MATE2-K cells, where the average uptake after 1 min under condition A was 2.1-fold ([^{14}C]metformin), 2.6-fold ([^{3}H]thiamine), and 4.0-fold ([^{3}H]MPP$^+$) higher than under condition B and 2.8-fold ([^{14}C]metformin), 3.6-fold ([^{3}H]thiamine), and 5.9-fold ([^{3}H]MPP$^+$) higher than under condition C.

Under all three conditions, uptake of [^{14}C]metformin, [^{3}H]thiamine and [^{3}H]MPP$^+$ was decreased along with an increasing concentration of each of the four tested inhibitors except for NBuPy-Cl (Supplemental Fig. 1-4). As shown in Table 2 and Fig. 2, IC_{50} values of all

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inhibitors for MATE1- and MATE2-K-mediated uptake of the three *in vitro* probe substrates varied only slightly among the three conditions. In HEK293-MATE1 cells, all observed differences were within a 2.5-fold range with an average variation of 0.9-fold between condition A and condition B, 1.0-fold between condition A and condition C and 1.1-fold between condition B and condition C, respectively. The correlation coefficients were 0.979, 0.986, and 0.997. In HEK293-MATE2-K cells, the average variations were 1.1-fold between condition A and condition B, 1.3-fold between condition A and condition C and 1.3-fold between condition B and condition C and correlation coefficients of 0.996, 0.993, and 0.997, respectively.

Uptake characteristics of five substrates

MATE transporter isoforms accept a large variety of substrates from organic cations to organic anions and from compounds with low to high molecular weights. In order to know whether uptake profiles differ among MATE substrates, [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]E3S and rhodamine 123 were nominated as test substrates based on selection criteria such as clinical relevance, frequency of use in the literature, and physicochemical properties. All substrate profiling studies using these compounds, i.e. time-, concentration-, and pH-dependent studies, were conducted at condition A which was previously selected as experimental condition for further characterization.

Uptake of all substrates into HEK293-MATE1- and HEK293-MATE2-K cells increased with time and was significantly higher than in the vector-transfected control cells (Supplemental Fig. 5). [^{14}C]metformin, [^{3}H]thiamine, and [^{3}H]MPP^{+} uptake was linear over the first 2 min while uptake of [^{3}H]E3S and rhodamine 123 was linear over the first 5 min. Based on these findings, we selected an incubation time for subsequent studies at which MATE1- and MATE2-K-mediated transport activities of each substrate were within the initial

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linear phase (1 min for [^{14}C]metformin, [^{3}H]thiamine, and [^{3}H]MPP^{+} and 2 min for [^{3}H]E3S and rhodamine 123). Subsequently, transport activities in HEK293-MATE1 and HEK293-MATE2-K cells were assessed at increasing concentrations of the five test substrates in order to determine their kinetic profiles. The results are shown as Eadie-Hofstee plots in Fig. 3 and in Supplemental Fig. 6 and kinetic parameters (K_m and V_{max}) obtained from the concentration-dependency studies are given in Table 3. MATE1- and MATE2-K-mediated uptake of [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+} and rhodamine 123 was saturable while atypical kinetic characteristics were observed with [^{3}H]E3S. K_m values of [^{14}C]metformin, [^{3}H]MPP^{+}, and rhodamine 123 were lower in HEK293-MATE1 than in HEK293-MATE2-K cells, indicating higher affinities of all tested compounds for MATE1 than for MATE2-K. Rhodamine 123 had the lowest K_m values of all compounds in both HEK293-MATE1 (K_m = 0.793 μM) and HEK293-MATE2-K cells (K_m = 10.2 μM) while the highest K_m values were found for [^{14}C]metformin transport (K_m = 208 μM and K_m = 2.28 mM in HEK293-MATE1 and HEK293-MATE2-K cells, respectively).

Fig. 4 shows the results of the investigation of uptake of the test substrates at different pH conditions in a range from pH 6.0 to pH 8.0. The pH-dependent uptake of [^{14}C]metformin, [^{3}H]thiamine, and [^{3}H]MPP^{+} showed a peak at an extracellular pH of 7.5 for MATE1 and increased until pH 8.0 for MATE2-K. The pH-dependent uptake properties of rhodamine 123 were comparable to those observed by the above-mentioned three substrates, although the peak of MATE1-mediated transport was observed at an extracellular pH of 7.0. In contrast to this, a distinct pH-dependency was found with [^{3}H]E3S for MATE1 and MATE2-K, which both showed a continuous decrease of uptake activities along with an increasing extracellular pH.

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Impact of the utilized test substrate on IC_{50} determination

For the clarification of the question whether IC_{50} determination is affected by the utilized substrate, the inhibitory effects of ten compounds (pyrimethamine, cimetidine, trimethoprim, zosuquidar, valspodar, quinidine, ondansetron, famotidine, topotecan and NBuPy-Cl) on MATE1- and MATE2-K-mediated uptake of [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]E3S and rhodamine 123 were examined. Uptake of all test substrates was decreased in the presence of an increasing concentration of each of the tested inhibitors except zosuquidar for which no inhibition was observed. The calculated IC_{50} values are summarized in Table 4 and the comparisons of the calculated IC_{50} values between [^{14}C]metformin and the remaining four substrates [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]E3S, and rhodamine 123 are shown in Fig. 5.

Compared to [^{14}C]metformin, the IC_{50} values for the uptake of [^{3}H]thiamine, [^{3}H]E3S and [^{3}H]MPP^{+} were all within a 4-fold range (Fig. 5A-C). Low differences and a good correlation (correlation coefficients of 0.974 for MATE1 and 0.998 for MATE2-K) were found between [^{14}C]metformin and [^{3}H]thiamine. All observed differences were within a 2-fold (MATE1) and 3-fold (MATE2-K) range and the average variation was 1.2-fold and 1.7-fold, respectively. The observed differences between [^{3}H]E3S and [^{14}C]metformin were all within a 3-fold (MATE1 and MATE2-K) range with an average variation of 0.7-fold and 1.6-fold and correlation coefficients of 0.998 and 0.995 for MATE1 and MATE2-K, respectively. The observed differences between [^{3}H]MPP^{+} and [^{14}C]metformin were within a 3-fold (MATE1) and 4-fold (MATE2-K) range with an average variation of 1.7-fold and 2.6-fold and a correlation coefficient of 0.981 and 0.985, respectively. IC_{50} values of all inhibitors against rhodamine 123 uptake were considerably higher than those against the other four test substrates (Fig. 5D). The average variation of IC_{50} values compared to [^{14}C]metformin was 9.8-fold for MATE1. Inhibitors showing >4-fold variation for MATE1 are trimethoprim (IC_{50} >100 μM, >24-fold difference), quinidine (IC_{50} >100 μM, >17-fold difference),

cimetidine (IC_{50} = 36.8 μM, 14-fold difference), ondansetron (IC_{50} = 3.96 μM, 9-fold difference), famotidine (IC_{50} = 6.72 μM, 7-fold difference), valspodar (IC_{50} >15 μM, 6-fold difference) and topotecan (IC_{50} = 26.9 μM, 5-fold difference). The corresponding correlation coefficient for MATE1 was only 0.468. A similar observation was made for the inhibition of MATE2-K-mediated uptake with and average variation of 4.1-fold and >4-fold differences observed with famotidine (IC_{50} = 24.1 μM, 8-fold difference), ondansetron (IC_{50} = 1.74 μM, 7-fold difference), cimetidine (IC_{50} = 30.5 μM, 6-fold difference) and pyrimethamine (IC_{50} = 0.833 μM, 5-fold difference).

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Discussion

IC_{50} values of investigational drugs for drug transporters are determinant for the magnitude of DDIs at clinical settings. Thus, there are great concerns on the standardization of *in vitro* experimental conditions to determine robust and reliable IC_{50} values in the pharmaceutical industry. The present study focused on MATE transporters, the importance of which is recently perceived by the regulatory authorities and the International Transporter Consortium. The latter has included an overview of available methodologies for the *in vitro* evaluation of MATEs in their latest white paper on emerging transporters of clinical importance (Hillgren *et al.*, 2013). However, the possibility of different results with different experimental conditions was not discussed, which is why the present study is an important contribution to future white papers.

In order to obtain higher activities in MATE uptake studies, an artificial pH gradient is often generated by using an alkaline buffer system in the extracellular compartment or by pre-acidification of the intracellular compartment using an NH_4Cl pre-pulse (Hillgren *et al.*, 2013). Currently, there is little information whether these varying experimental conditions affect the assessment of the interactions of test compounds with MATEs. We therefore compared the uptake of three typical *in vitro* probe substrates ([^{14}C]metformin, [^{3}H]thiamine, and [^{3}H]MPP^+) and the inhibitory profiles of four inhibitors with particular characteristics (pyrimethamine as potent, quinidine as moderate, ondansetron as MATE1-preferring, NBuPy-Cl as MATE2-K-preferring) at three different assay conditions.

Condition A (with NH_4Cl pre-pulse) resulted in significantly higher uptake rates of all three test compounds than conditions B and C (both without NH_4Cl pre-pulse). It was therefore assumed that the increased MATE activity was caused by the intracellular pre-acidification through the NH_4Cl pre-pulse used in condition A. In contrast, no significant difference was detected between condition B (K^+-based buffer) and condition C (Na^+-based

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buffer), representing a depolarized or a polarized state of the cell membrane, respectively. This finding implicates that the membrane potential was not a determining factor for the MATE-mediated uptake of the tested substrates and is well in line with previous reports which have shown that a valinomycin-induced depolarization of the membrane had no effect on MATE1- and MATE2-K-mediated uptake of the prototypical MATE substrate TEA (Otsuka et al., 2005; Tsuda et al., 2009).

As shown in Fig. 2 and Table 2, only slight differences in the IC_{50} values among the three tested conditions were found for each combination of substrate and inhibitor (<2.5-fold difference). These results suggest that the selected condition does not substantially affect the IC_{50} determination of test compounds. Because DDI risks of drug candidates are directly extrapolated from the IC_{50} values, a robust and reliable IC_{50} determination is essential during drug development. From this perspective, the use of condition A is preferable as it results in higher uptake ratios, is therefore less susceptible to small changes of experimental conditions and will deliver more robust results.

It is well known that MATEs accept a large variety of substrate but by now, no study has directly compared the transport profiles of test substrates with diverse chemical and functional characteristics (such as their frequency of use, clinical relevance or physicochemical properties). Based on this, we chose five literature-reported MATE substrates with a range of different attributes and assessed their transport characteristics using the priorly selected experimental condition A. The selected compounds were MPP^+ as prototypical, metformin as clinically relevant, thiamine as physiological, and E3S as atypical (i.e. anionic) substrate. In addition, rhodamine 123 was selected since it has a relatively high molecular weight and the ability to interact with P-gp, both unusual features among MATE substrates.

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MATE1- and MATE2-K-mediated uptake of all test compounds was pH-dependent (Fig. 4). The observed pH-dependencies of [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+} and rhodamine 123 were similar to those reported previously for MPP^{+} and TEA (Tanihara *et al*., 2007; Tsuda *et al*., 2007; Dangprapai and Wright, 2011; Astorga *et al*., 2012). As opposed to this, uptake of [^{3}H]E3S continuously decreased with increased pH and showed atypical saturation kinetics in both HEK293-MATE1 and HEK293-MATE2-K cells (Fig. 3). Such pH-dependency has also been observed for norfloxacine (Ohta *et al.*, 2009) and cephalexin (Watanabe *et al.*, 2010). Since these compounds are weak acids (pKa of 4.5 and 5.7, respectively), the extracellular pH affects the percentage of the neutral and zwitterionic forms at the examined range. However, E3S is a fairly strong acid with a pK_a value of -3 and it can be assumed that it predominantly exists in its anionic form over the complete extracellular pH range from pH 6.0 to 8.0. Consequently, the reasons for the unusual uptake characteristics of [^{3}H]E3S remain unknown but might be a general feature of MATE-mediated transport of anionic drugs.

Recently, it was gradually recognized that substrate-dependent differences in IC_{50} values exist in transporters such as OATP1B1, OATP2B1 and OCT2 (Shirasaka *et al*., 2012; Belzer *et al.*, 2013; Izumi *et al.*, 2013; Hacker *et al.*, 2015) as well as in the cytochrome P450 enzyme CYP3A4 (Kenworthy *et al*., 1999; Obach *et al*., 2006). As for MATEs, Martínez-Guerrero and Wright (2013) could show a substrate-dependent inhibition of MATE1 with a set of ionic liquids. We therefore determined the IC_{50} values of a set of ten selected inhibitors using the five aforementioned test substrates to further investigate the substrate-dependency of IC_{50} valuesfor MATE1 and MATE2-K. The inhibitors were selected based on clinical relevance (drugs with known DDI), high inhibition potency (low IC_{50}), and selectivity (for either MATE1 or MATE2-K) as reported in the literature. Additionally, three known P-gp inhibitors (zosuquidar, valspodar and quinidine) were included to take account of the reported

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overlapping substrate specificity between P-gp and MATEs (Tanihara et al., 2007). No pronounced substrate-dependency was found in IC_{50} values among [^{14}C]metformin, [^{3}H]MPP^{+}, [^{3}H]thiamine, and [^{3}H]E3S. In contrast, markedly higher values (>4-fold) were determined with rhodamine 123. These substantial substrate-dependent changes of IC_{50} values with rhodamine 123 have to be taken into account when considering it as a potential test substrate in a fluorescent assay system. The use of rhodamine 123 as the only test substrate will likely lead to an underestimation of the DDI risks of candidate drugs, possibly resulting in severe clinical safety issues, and is therefore not advised.

When selecting an appropriate test substrate, one of the determining factors is a good transferability of the results to *in vivo* results from clinical studies. A clinically relevant MATE substrate, i.e. a therapeutic drug or a test substrate with transport characteristics similar to a typical test drug, can contribute to a good *in vitro-in vivo* correlation (IVIVC). In this regard, the clinically used antidiabetic metformin would be well-suited. Several studies have reported clinical DDIs with known MATE inhibitors (cimetidine, cephalexin or pyrimethamine) that significantly changed pharmacokinetic parameters of concomitantly administered metformin (Somogyi *et al.*, 1987; Jayasagar *et al.*, 2002; Kusuhara *et al.*, 2011). IC_{50} determination in our study identified [^{14}C]metformin as the most conservative test substrate for the detection of interactions with MATE2-K and, in most cases, with MATE1. [^{14}C]metformin is hence unlikely to underestimate DDI risks and therefore rated as an appropriate substrate for MATE *in vitro* studies.

Kato et al. recently identified thiamine as an endogenous MATE substrate that could be a useful biomarker for the detection of DDIs involving MATEs (Kato et al., 2014). By monitoring the urinary excretion of thiamine in clinical studies, MATE-mediated DDIs of drug candidates could be evaluated without the need to administer exogenous probe drugs. However, this requires that the inhibition profiles of MATE inhibitors are similar when

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thiamine and other typical MATE probe substrates such as MPP^+ and metformin are used as substrates. Here, we have shown that [^{3}H]thiamine has comparable *in vitro* substrate characteristics to [^{3}H]MPP^+ and that the IC_{50} values of ten tested inhibitors are similar to those obtained with [^{3}H]MPP^+ and [^{14}C]metformin. Therefore, [^{3}H]thiamine could be a valuable probe substrate for the *in vitro* prediction of MATE-mediated DDIs, particularly if it is also used as biomarker in clinical studies. In this case, the *in vitro* data reflects an effectively used combination of test compounds during clinical phase 1 studies. MPP^+ and E3S are commonly used as a prototypical *in vitro* test substrate for several other transporters but they have a low clinical relevance since they are either exogenous and neurotoxic (MPP^+) or since there is no information about the possible use as biomarker for the assessment of MATE-mediated DDIs in the literature (E3S).

In conclusion, our study has investigated the impact of the experimental conditions and the choice of the test substrate on the determination of IC_{50} values of known inhibitors against MATE1 and MATE2-K. We demonstrated that the IC_{50} values of four selected inhibitors did not significantly change with the used assay condition and therefore conclude that all of the three tested *in vitro* assay conditions are applicable. Furthermore, we recommend to use [^{14}C]metformin, [^{3}H]thiamine, or [^{3}H]MPP^+ as test substrates based on the comparable IC_{50} values of ten test inhibitors. Taken together, we believe that our findings will contribute to the establishment of a robust and reliable standard assay system for the *in vitro* assessment of MATE-mediated DDIs.

Authorship Contributions

Participated in research design: Lechner, Ishiguro, Fukuhara, Washio, Yamamura, and Kusuhara

Conducted experiments: Lechner, Shimizu, Ohtsu, Nishiyama, and Takatani

Performed data analysis: Lechner, Ishiguro, Fukuhara, Washio, Shimizu, Otsu, Nishiyama, Takatani, and Kusuhara

Wrote or contributed to the writing of the manuscript: Lechner, Ishiguro, and Kusuhara

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Footnotes

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Figure Legends

Fig 1. Uptake of [^{14}C]metformin (A), [^{3}H]thiamine (B), and [^{3}H]MPP^{+} (C) at three different assay conditions. Uptake of [^{14}C]metformin (10 μM), [^{3}H]thiamine (1 μM), and [^{3}H]MPP^{+} (1 μM) was determined for 1 min in HEK293-MATE1, HEK293-MATE2-K and HEK293-mock cells at three different assay conditions (conditions A-C, see Table 1). Each bar represents the mean value ± S.E of triplicate measures from at least 4 separate experiments. Asterisks (*) represent significantly different uptake compared to condition A ($p < 0.01$).

Fig. 2. Comparison of IC_{50} values between different assay conditions. Uptake of [^{14}C]metformin (10 μM, 1 min), [^{3}H]thiamine (1 μM, 1 min), and [^{3}H]MPP^{+} (1 μM, 1 min) was determined in the absence and presence of various concentrations of inhibitors as shown in Supplemental Fig. 1-4 and IC_{50} values were estimated by nonlinear regression analysis (see Table 2). All experiments were conducted with HEK293-MATE1 (closed squares) and HEK293-MATE2-K cells (open squares) and at three different assay conditions (condition A-C, see Table 1). (A) condition A vs. condition B, (B) condition A vs. condition C, (C) condition B vs. condition C. Each point represents the mean value ± relative error from one experiment.

Fig. 3. Concentration-dependent uptake of [^{14}C]metformin (A), [^{3}H]thiamine (B), [^{3}H]MPP^{+} (C), [^{3}H]E3S (D) and rhodamine 123 (E) by HEK293 cells expressing MATE1 or MATE2-K. Uptake of [^{14}C]metformin (10 - 10000 μM, 1 min), [^{3}H]thiamine (0.1 - 1000 μM, 1 min), [^{3}H]MPP^{+} (0.1 - 1000 μM, 1 min), [^{3}H]E3S (0.1 - 300 μM, 2 min) and rhodamine 123 (0.1 - 100 μM, 2 min) was determined in HEK293-MATE1 (closed circles) and HEK293-MATE2-K (closed squares) at condition A (see Table 1). Data are shown as Eadie-Hofstee plots.

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Transporter-mediated uptake was calculated by subtracting the uptake in HEK293-mock cells from that in transporter expressing cells. Each point represents the mean value ± S.E. of triplicate measures from one experiment in case of [^{14}C]metformin and [^{3}H]MPP^{+} and from one representative experiment out of at least two separate experiments in case of the other substrates.

Fig. 4. pH-dependent uptake of [^{14}C]metformin (A), [^{3}H]thiamine (B), [^{3}H]MPP^{+} (C), [^{3}H]E3S (D) and rhodamine 123 (E) by HEK293 cells expressing MATE1 or MATE2-K and control cells. Uptake of [^{14}C]metformin (10 μM, 1 min), [^{3}H]thiamine (1 μM, 1 min), [^{3}H]MPP^{+} (1 μM, 1 min), [^{3}H]E3S (10 μM, 2 min) and rhodamine 123 (1 μM, 2 min) was determined in HEK293-MATE1 (closed circles), HEK293-MATE2-K (closed squares) and HEK293-mock cells (open circles) at condition A (see Table 1). Each point represents the mean value ± S.E. of triplicate measures from one experiment in case of [^{14}C]metformin and from one representative experiment out of at least two separate experiments in case of the other substrates.

Fig. 5. Comparison of IC_{50} values between different test substrates. Uptake of [^{14}C]metformin (10 μM, 1 min), [^{3}H]thiamine (1 μM, 1 min), [^{3}H]MPP^{+} (1 μM, 1 min), [^{3}H]E3S (10 μM, 2 min) and rhodamine 123 (1 μM, 2 min) was determined in the absence and presence of various concentrations of inhibitors and IC_{50} values were estimated by nonlinear regression analysis (see Table 4). All experiments were conducted with HEK293-MATE1 (closed squares) and HEK293-MATE2-K cells (open squares) at condition A (see Table 1). (A) [^{14}C]metformin vs. [^{3}H]thiamine, (B) [^{14}C]metformin vs. MPP^{+}, (C) [^{14}C]metformin vs. [^{3}H]E3S, and (D) [^{14}C]metformin vs. Rhodamine 123. Each point represents the mean value ± relative error from one experiment.

Tables

Table 1. Experimental conditions

Condition	pH	NH_4Cl pre-pulse	Transport buffer	Transport buffer composition (mM)						
				NaCl	KCl	KH_2PO_4	$MgSO_4$	$CaCl_2$	HEPES	Glucose
A	7.4	yes	K^+-based	-	130	2.0	1.2	1.0	20	5.0
B	7.4	no	K^+-based	-	130	2.0	1.2	1.0	20	5.0
C	7.4	no	Na^+-based	118	4.7	1.2	1.2	1.5	25	11

Table 2. IC_{50} values for MATE1- and MATE2-K-mediated uptake of [^{3}H]MPP$^+$, [^{3}H]thiamine and [^{14}C]metformin at different assay conditions. Uptake of [^{14}C]metformin (10 μM, 1 min), [^{3}H]thiamine (1 μM, 1 min), and [^{3}H]MPP$^+$ (1 μM, 1 min) was determined in the absence and presence of various concentrations of inhibitors at three different assay conditions (condition A-C, see Table 1) as shown in Supplemental Fig. 1-4. IC_{50} values were estimated by nonlinear regression analysis and are given as mean ± S.D. from one experiment.

		Substrate		
Inhibitor	Condition	[^{14}C]metformin	[^{3}H]thiamine	[^{3}H]MPP$^+$
		IC_{50} (μM), MATE1		
Pyrimethamine	A	0.313 ± 0.052	0.330 ± 0.052	0.492 ± 0.039
	B	0.337 ± 0.065	0.268 ± 0.020	0.353 ± 0.028
	C	0.583 ± 0.043	0.397 ± 0.072	0.443 ± 0.031
Quinidine	A	5.82 ± 0.06	6.77 ± 0.05	6.77 ± 0.08
	B	5.77 ± 0.05	5.81 ± 0.05	6.26 ± 0.04
	C	2.48 ± 0.70	3.64 ± 0.08	4.34 ± 0.07
Ondansetron	A	0.436 ± 0.083	0.493 ± 0.041	0.797 ± 0.025
	B	0.570 ± 0.040	0.344 ± 0.043	0.475 ± 0.068
	C	0.499 ± 0.040	0.430 ± 0.028	0.619 ± 0.033

NBuPy-Cl	A	55.2 ± 0.1	83.5 ± 0.1	76.8 ± 0.1
	B	69.8 ± 0.1	71.0 ± 0.1	65.8 ± 0.1
	C	74.5 ± 0.1	78.9 ± 0.1	81.1 ± 0.1
		IC_{50} (μM), MATE2-K		
Pyrimethamine	A	0.180 ± 0.083	0.372 ± 0.068	0.650 ± 0.063
	B	0.281 ± 0.073	0.170 ± 0.053	0.615 ± 0.048
	C	0.298 ± 0.068	0.319 ± 0.113	0.703 ± 0.065
Quinidine	A	6.44 ± 0.06	11.7 ± 0.1	17.6 ± 0.1
	B	9.79 ± 0.05	11.4 ± 0.0	20.6 ± 0.1
	C	12.3 ± 0.1	13.9 ± 0.1	31.8 ± 0.1
Ondansetron	A	0.259 ± 0.086	0.597 ± 0.071	0.725 ± 0.055
	B	0.388 ± 0.042	0.251 ± 0.106	0.869 ± 0.080
	C	0.527 ± 0.064	0.438 ± 0.040	0.979 ± 0.083
NBuPy-Cl	A	>100	>100	86.5 ± 0.1
	B	>100	>100	>100
	C	>100	>100	>100

Table 3. Saturation kinetics of MATE1- and MATE2-K-mediated uptake of [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]E3S and rhodamine 123. Uptake of [^{14}C]metformin (10 - 10000 μM, 1 min), [^{3}H]thiamine (0.1 - 1000 μM, 1 min), [^{3}H]MPP^{+} (0.1 - 1000 μM, 1 min), [^{3}H]E3S (0.1 - 300 μM, 2 min) and rhodamine 123 (0.1 - 100 μM, 2 min) was determined at condition A (see Table 1). Kinetic parameters were estimated by nonlinear regression analysis and are given as mean ± S.D from one experiment in case of [^{14}C]metformin and [^{3}H]MPP^{+} and from one representative experiment out of at least two separate experiments in case of the other substrates.

Substrate	K_m (μM)		V_{max} (pmol/min/mg protein)	
	MATE1	MATE2-K	MATE1	MATE2-K
[^{14}C]metformin	208 ± 29	2275 ± 284	8890 ± 282	13779 ± 652
[^{3}H]thiamine	31.0 ± 4.5	23 ± 2.4	1584 ± 63	674 ± 25
[^{3}H]MPP^{+}	47.6 ± 2.1	81.2 ± 7.7	5199 ± 66	4038 ± 117
[^{3}H]E3S[a]	n.d.	n.d.	n.d.	n.d.
Rhodamine 123	0.793 ± 0.395	10.2 ± 1.2	372 ± 41	1796 ± 64

n.d.: not determined

[a]Kinetic parameters of [^{3}H]E3S were not determined due to its atypical saturation kinetics.

Table 4. IC_{50} values for MATE1- and MATE2-K-mediated uptake of [^{14}C]metformin, [^{3}H]thiamine, [^{3}H]MPP^{+}, [^{3}H]E3S and rhodamine 123. Uptake of [^{14}C]metformin (10 μM, 1 min), [^{3}H]thiamine (1 μM, 1 min), [^{3}H]MPP^{+} (1 μM, 1 min), [^{3}H]E3S (10 μM, 2 min) and rhodamine 123 (1 μM, 2 min) was determined in the absence and presence of various concentrations of inhibitors. IC_{50} values were estimated by nonlinear regression analysis and are given as mean ± S.D. from one experiment.

Inhibitor	Category	Substrate			
		[^{14}C]metformin	[^{3}H]thiamine	[^{3}H]MPP^{+}	[^{3}H]E3S	Rhodamine 123
		IC_{50} (μM), MATE1				
Pyrimethamine	Drug with known DDI	0.313 ± 0.052	0.330 ± 0.052	0.492 ± 0.039	0.150 ± 0.057	1.16 ± 0.13
Cimetidine	Drug with known DDI	2.56 ± 0.04	3.19 ± 0.04	4.43 ± 0.05	1.30 ± 0.06	36.8 ± 0.2
Trimethoprim	Drug with known DDI	4.13 ± 0.09	4.19 ± 0.05	8.16 ± 0.06	2.69 ± 0.03	>100
Zosuquidar	P-gp inhibitor	>50	>50	>50	>50	>50
Valspodar	P-gp inhibitor	2.67 ± 0.05	4.83 ± 0.06	5.89 ± 0.05	2.02 ± 0.07	>15
Quinidine	P-gp inhibitor	5.82 ± 0.06	6.77 ± 0.05	6.77 ± 0.08	6.96 ± 0.06	>100
Ondansetron	High inhibition potency	0.436 ± 0.083	0.493 ± 0.041	0.797 ± 0.025	0.287 ± 0.029	3.96 ± 0.07
Famotidine	Selectivity (MATE1 > MATE2-K)	0.905 ± 0.046	1.20 ± 0.03	1.67 ± 0.03	0.456 ± 0.036	6.72 ± 0.16

Topotecan	Selectivity (MATE1 > MATE2-K)	5.34	±	0.03	4.49	±	0.03	8.25	±	0.05	2.66	±	0.03	26.9	±	0.1
NBuPy-Cl	Selectivity (MATE2-K > MATE1)	55.2	±	0.1	83.5	±	0.1	76.8	±	0.1	51.8	±	0.1	>100		
		IC_{50} (μM), MATE2-K														
Pyrimethamine	Drug with known DDI	0.180	±	0.083	0.372	±	0.068	0.650	±	0.063	0.372	±	0.112	0.833	±	0.130
Cimetidine	Drug with known DDI	5.47	±	0.06	11.2	±	0.0	13.4	±	0.1	11.2	±	0.1	30.5	±	0.1
Trimethoprim	Drug with known DDI	0.421	±	0.052	0.440	±	0.027	1.22	±	0.03	0.440	±	0.047	1.05	±	0.12
Zosuquidar	P-gp inhibitor	>50			>50			>50			>50			>50		
Valspodar	P-gp inhibitor	0.118	±	0.039	0.169	±	0.025	0.238	±	0.034	0.109	±	0.031	0.219	±	0.050
Quinidine	P-gp inhibitor	6.44	±	0.06	11.7	±	0.1	17.6	±	0.1	14.7	±	0.2	28.5	±	0.1
Ondansetron	High inhibition potency	0.259	±	0.086	0.597	±	0.071	0.725	±	0.055	0.430	±	0.086	1.74	±	0.07
Famotidine	Selectivity (MATE1 > MATE2-K)	3.10	±	0.09	5.30	±	0.07	8.88	±	0.05	6.66	±	0.10	24.1	±	0.1
Topotecan	Selectivity (MATE1 > MATE2-K)	5.43	±	0.04	9.15	±	0.04	15.8	±	0.0	6.11	±	0.08	14.7	±	0.1

NBuPy-Cl	Selectivity (MATE2-K > MATE1)	>100		>100	86.5	±	0.1	>100		>100

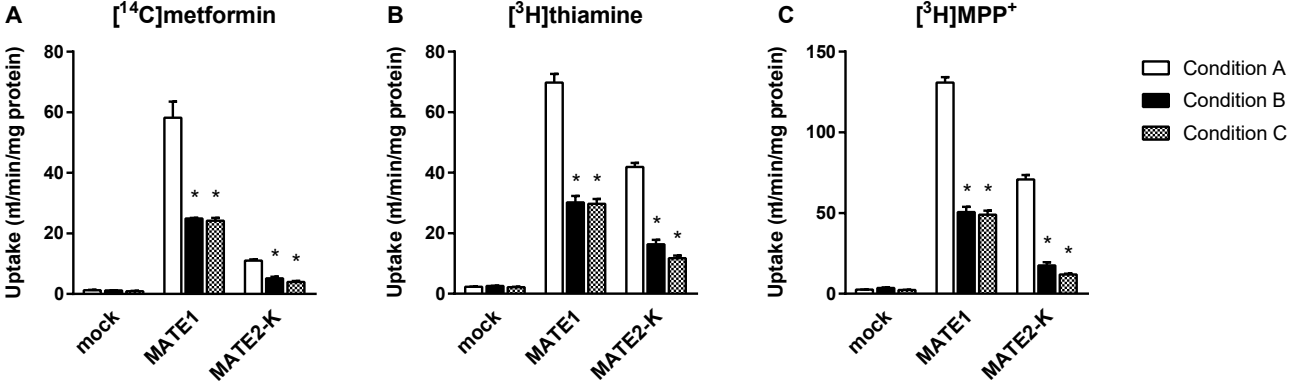
A
[14C]metformin
B
[3H]thiamine
C
[3H]MPP+
Uptake (nl/min/mg protein)
80
60
40
20
0
150
100
50
mock
MATE1
MATE2-K
*
Condition A
Condition B
Condition C

Figure 1

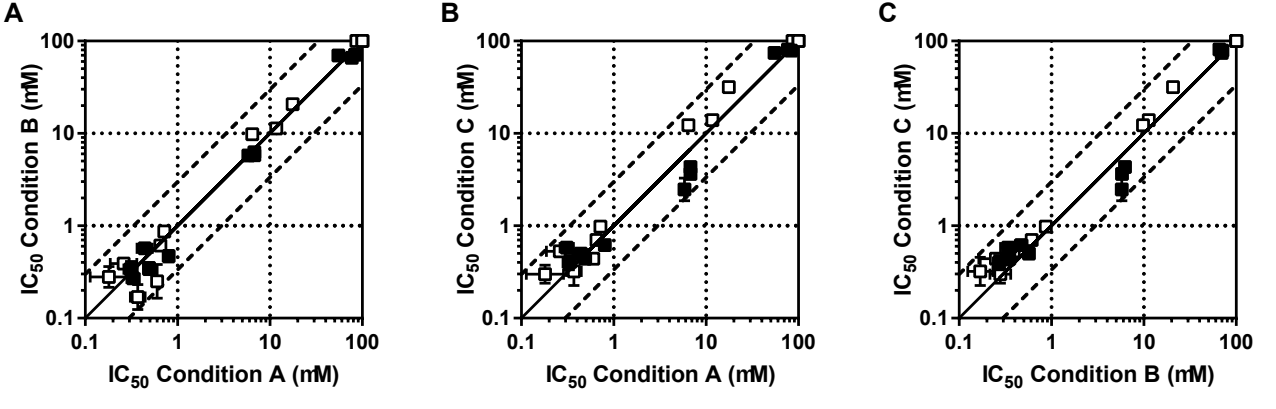
A
B
C
100
10
1
0.1
0.1
1
10
100
IC50 Condition B (mM)
IC50 Condition A (mM)
IC50 Condition C (mM)
IC50 Condition A (mM)
IC50 Condition C (mM)
IC50 Condition B (mM)

Figure 2

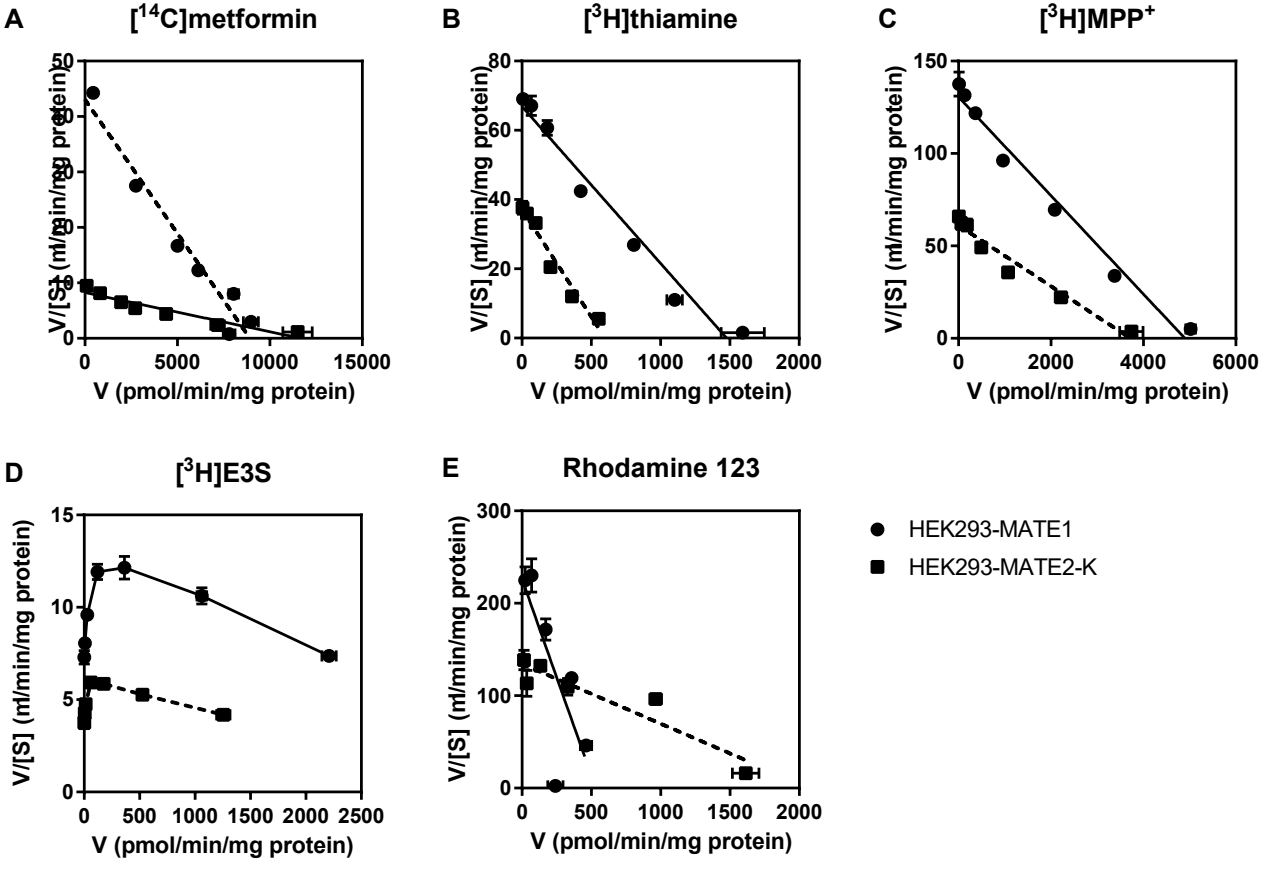
A
[14C]metformin
V/[S] (ml/min/mg protein)
V (pmol/min/mg protein)
B
[3H]thiamine
V/[S] (ml/min/mg protein)
V (pmol/min/mg protein)
C
[3H]MPP+
V/[S] (ml/min/mg protein)
V (pmol/min/mg protein)
D
[3H]E3S
V/[S] (ml/min/mg protein)
V (pmol/min/mg protein)
E
Rhodamine 123
V/[S] (ml/min/mg protein)
V (pmol/min/mg protein)
HEK293-MATE1
HEK293-MATE2-K

Figure 3

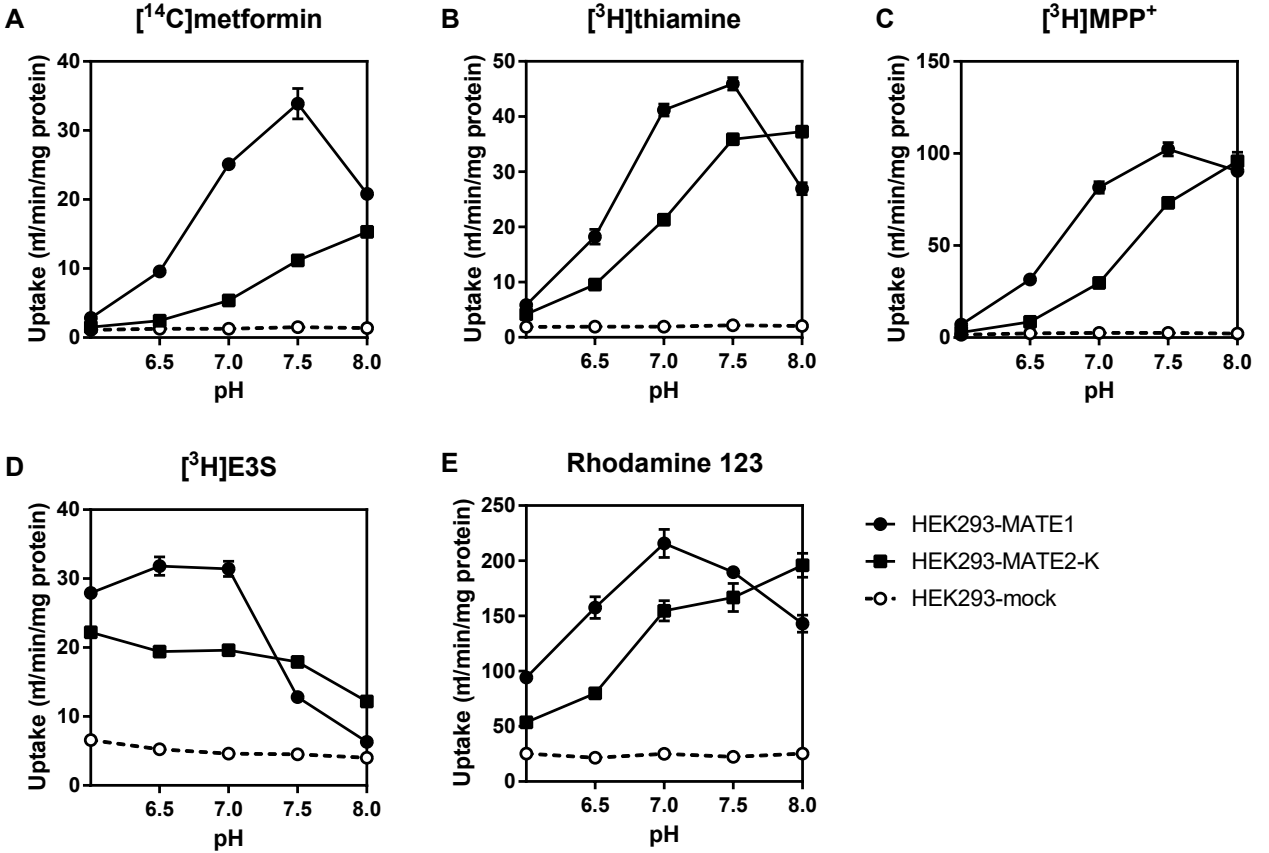
A
[14C]metformin
B
[3H]thiamine
C
[3H]MPP+
D
[3H]E3S
E
Rhodamine 123
Uptake (nl/min/mg protein)
pH
6.5
7.0
7.5
8.0
HEK293-MATE1
HEK293-MATE2-K
HEK293-mock

Figure 4

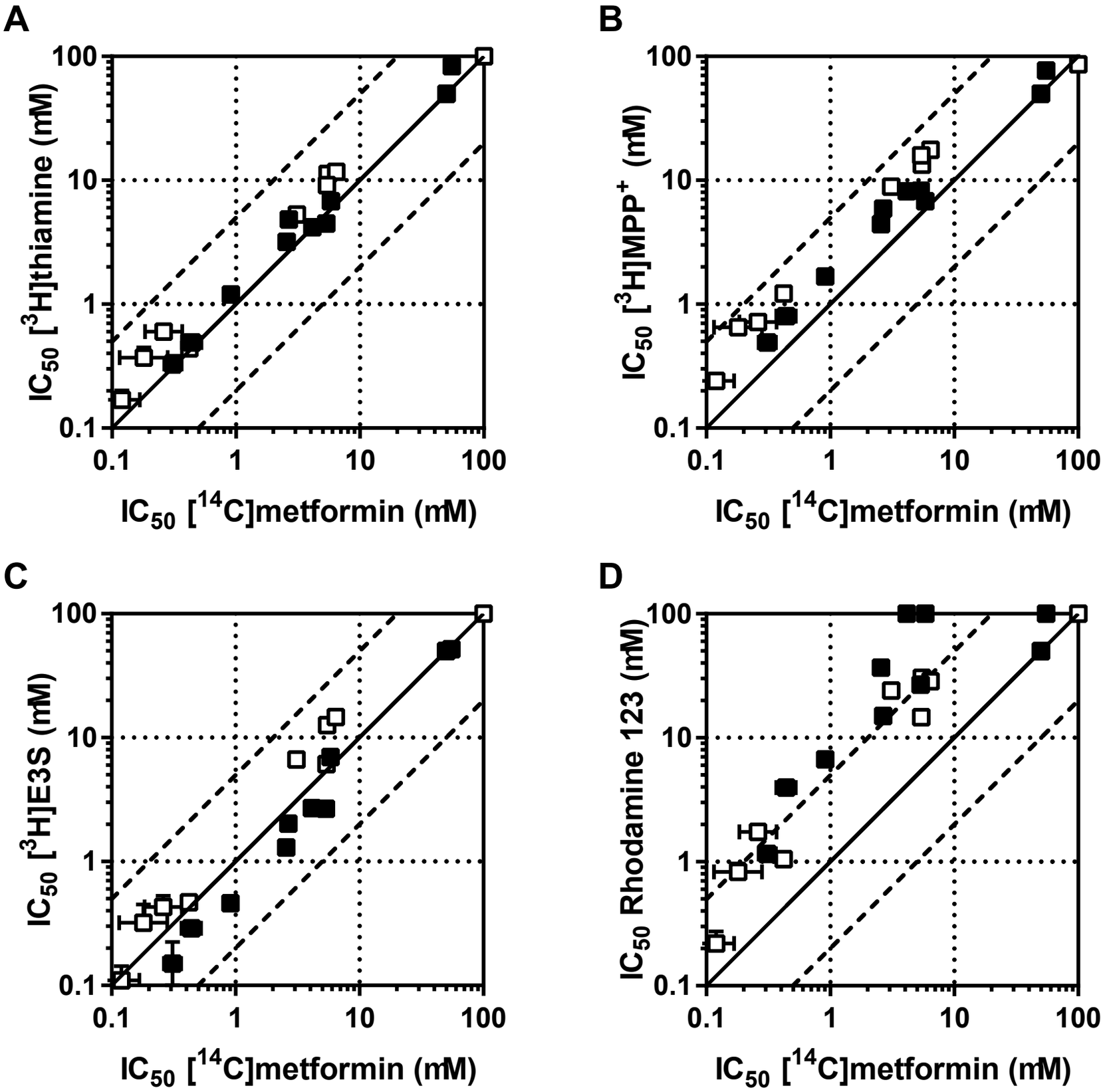
A
B
C
D
100
10
1
0.1
0.1
1
10
100
IC50 [3H]thiamine (mM)
IC50 [3H]MPP+ (mM)
IC50 [3H]E3S (mM)
IC50 Rhodamine 123 (mM)
IC50 [14C]metformin (mM)

Figure 5