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Title

Quantitation of plasma membrane drug transporters in kidney tissue and cell lines using a novel proteomic approach enabled a prospective prediction of metformin disposition

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Running Title

Novel approach for plasma membrane transporter quantitation

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Abbreviations:

AUC, area under the curve; EV, empty vector; HEK, human embryonic kidney; GGT1, γ-glutamyl transpeptidase 1; IVIVE, in vitro-in vivo extrapolation; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MATE, multidrug and toxin exclusion protein; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PBPK, physiologically-based pharmacokinetic; PTC, proximal tubule cells; REF, relative expression factor; Na^+/K^+-ATPase, sodium-potassium adenosine triphosphatase; TFA, trifluoroacetic acid.

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Abstract

The successful prospective incorporation of in vitro transporter kinetics in physiologically based pharmacokinetic (PBPK) models to describe drug disposition remains challenging. While determination of scaling factors to extrapolate in vitro to in vivo transporter kinetics has been facilitated by quantitative proteomics, no robust assessment comparing membrane recoveries between different cells/tissues has been made. HEK293 cells overexpressing OCT2, MATE1 and MATE2K or human kidney cortex were homogenized and centrifuged to obtain the total membrane fractions, which were subsequently subjected to liquid-liquid extraction followed by centrifugation and precipitation to isolate plasma membrane fractions. Plasma membrane recoveries determined by quantitation of the marker Na^+/K^+-ATPase in lysate and plasma membrane fractions were ≤20% but within three-fold across different cells and tissues. A separate study demonstrated that recoveries are comparable between basolateral and apical membranes of renal proximal tubules, as measured by Na^+/K^+-ATPase and γ-glutamyl transpeptidase 1, respectively. The plasma membrane expression of OCT2, MATE1 and MATE2K was quantified and relative expression factors (REFs) were determined as the ratio between the tissue and cell concentrations. Corrections using plasma membrane recovery had minimal impact on REF values (<two-fold). In vitro transporter kinetics of metformin were extrapolated to in vivo using the corresponding REFs in a PBPK model. The simulated metformin exposures were within two-fold of clinical exposure. These results demonstrate that transporter REFs based on plasma membrane expression enable a prediction of transporter-mediated drug disposition. Such REFs may be estimated without the correction of plasma membrane recovery when the same procedure is applied between different matrices.

Significance Statement

Transporter REFs based on plasma membrane expression enable in vitro-in vivo extrapolation of transporter kinetics. Plasma membrane recoveries as determined by the quantification of Na^+/K^+-ATPase were comparable between the in vitro and in vivo systems used in the present study, and therefore had minimal impact on the transporter REF values.

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Introduction

PBPK models are used widely within the pharmaceutical industry and are also recommended by regulatory agencies to aid in the prediction of drug disposition and drug-drug interactions in humans (Zhao et al., 2011; EMA, 2012; FDA, 2012; Jones et al., 2015). These models have been most predictive when compounds are highly permeable and predominantly metabolized by cytochrome P450 enzymes. However, with the current industry focus on advancing metabolically stable compounds to the clinic, the drugs being developed now often have significant transporter-mediated clearance, for which building a reliable PBPK model has limitations and challenges (Jones et al., 2015; Taskar et al., 2020).

To effectively incorporate transporter kinetic data into a PBPK model, transporter abundance differences between in vitro and in vivo systems must be considered. Among a host of methodologies for transporter quantification, quantitative proteomics is considered to have the fewest limitations while offering the highest accuracy when compared to other approaches such as mRNA quantification and Western blot (Ohtsuki et al., 2011; Prasad and Unadkat, 2014; Qiu et al., 2014). Meta-analysis of quantitative proteomics data, however, has revealed inter-study variability in transporter abundances. For example, the hepatic expression of organic anion transporting polypeptide (OATP) 1B1, OATP1B3 and OATP2B1 showed statistically significant differences between studies (Badee et al., 2015). Variability may be due to differences in sample preparation methodologies, impacting recovery of target proteins (Harwood et al., 2014). However, no robust cross-system assessment has been conducted to compare the membrane protein recovery between different systems. Indeed, a recent study comparing six different methodologies of quantitative proteomics revealed nearly 100-fold variability in the measured transporter abundance starting from the same tissue samples (Wegler et al., 2017).

As a result, there have been both successes and challenges for the in vitro-in vivo extrapolation (IVIVE) of transporter kinetics using quantitative proteomics. For example, the intrinsic hepatic clearance of rosuvastatin in vivo was accurately predicted by extrapolating the uptake clearance measured in human embryonic kidney (HEK) 293 cells stably expressing OATP1B1, OATP1B3 or OATP2B1 (major transporters involved in rosuvastatin hepatic uptake) to whole liver using a PBPK model after accounting for the difference in transporter abundance on plasma membrane (Bosgra et al., 2014). Similarly, the correction with protein expression on

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plasma membrane in vitro and consideration of membrane potential difference between in vitro and in vivo enabled a successful IVIVE of metformin uptake clearance via organic cation transporter (OCT) 2 (Kumar et al., 2018). Conversely, an average empirical scaling factor of 58 was required to explain the clinical exposure of seven OATP substrates when the uptake clearance determined in sandwich culture human hepatocytes was used in a PBPK model (Jones et al., 2012). This empirical scalar is not fully explained by human liver to sandwich culture hepatocyte abundance ratios based on quantification of total membrane transporter proteins; 1.7-fold (OATP1B1), 7.5-fold (OATP1B3) and 3.6-fold (OATP2B1) (Kimoto et al., 2012). These observations are consistent with the hypothesis that transporters expressed on plasma membrane, not total membranes, would determine functional activity, and may provide a better scalar for IVIVE.

Recently, an optimized biotinylation method was developed and applied to quantify the plasma membrane expression of transporters in vitro (Kumar et al., 2017). This methodology demonstrated that the fraction of transporters expressed on the plasma membrane can vary between different transporters and different host cells, further emphasizing the importance of considering transporter abundance on plasma membrane in IVIVE. However, this biotinylation method can only be applied to in vitro cells and does not allow the quantification of transporter expression on plasma membranes from whole tissue samples.

The aim of this study was 1) to establish a novel methodology to quantify the transporter protein expression on plasma membranes, and 2) to investigate if there are differences in plasma membrane recovery between in vitro and in vivo systems and if corrections are warranted in transporter IVIVE using REFs. Sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) is a well-characterized plasma membrane protein marker (Rajasekaran et al., 2010; Imai et al., 2013), and in this study, its abundance was used to assess plasma membrane recovery. Plasma membrane recovery was determined in HEK293 cells overexpressing the renal transporters OCT2, multidrug and toxin exclusion protein (MATE) 1 and MATE2K, and human kidney cortex. A potential difference in the recovery between basolateral and apical membranes was also explored. The plasma membrane expression of these transporters was quantified using proteomics, and REFs determined with or without the correction for plasma membrane recovery.

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Materials and Methods

Materials

HEK293 cell lines stably expressing OCT2, MATE1 or MATE2K (HEK-OCT2, HEK-MATE1 or HEK-MATE2K), and the corresponding mock-transfected cell line (HEK-EV) were obtained from Prof. Kathleen M. Giacomini (Department of Biopharmaceutical sciences, School of Pharmacy, University of California San Francisco). These cells were cultured as described previously (Kikuchi et al., 2013). Snap frozen human kidney cortexes from 9 individuals were obtained from Amsbio (Cambridge, MA). The demographics of these kidney tissues are summarized in Supplemental Table 1. All other materials were purchased from a commercial source unless stated otherwise.

Plasma membrane purification

Plasma membrane fractions were prepared from transporter expressing HEK293 cells and human kidney cortex using a plasma membrane protein extraction kit (ab65400; Abcam, Cambridge, MA) according to the manufacturer's instructions. Briefly, cells or tissue were resuspended in the homogenization buffer containing a protease inhibitor cocktail included in the kit and homogenized using either Dounce homogenizer (cells) or OMNI-YHO electric homogenizer (tissues). Centrifugation steps were then performed (10,000 × g for 30 minutes at 4°C) to separate the cytosolic fraction from the total membrane fraction, which consists of plasma membranes and cellular organelle membranes. To isolate plasma membrane proteins, the total membrane fraction was resuspended in upper and lower phase solution from the kit (liquid-liquid extraction). This mixture was then centrifuged (1,000 × g for 10 minutes), with the upper phase collected and extracted twice to maximize the yield of plasma membrane proteins. The pooled upper phase was combined, and 5 volumes of cold water was added and then it was stored at 4°C for overnight. The plasma membrane fraction was then pelleted by centrifugation (17,000 × g for 30 minutes at 4°C).

Western blot

Total and plasma membrane fractions were subject to Western blot analysis to confirm the enrichment of plasma membrane using Na^+/K^+-ATPase and calreticulin as markers for plasma

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and endoplasmic reticulum membranes, respectively. Proteins (10 μg) from each fraction were electrophoresed on 4-12% SDS-polyacrylamide gel and transfered to a polyvinylidene difluoride membrane. Anti-Na^+/K^+-ATPase monoclonal (1:1000; Abcam) and anti-calreticulin polyclonal antibodies (1:1000; Rockland Immu, Limeerick, PA) were used as the primary antibodies with anti-mouse IgG AlexaFluor 680 and anti-rabbit IgG IRDye 800 used as secondary antibodies, repectively. The signals were detected using an LLI-COR OSYDDEY system.

Trypsin digestion

Plasma membrane fractions were suspended in 0.2% of RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate and the protein concentrations were determined by the Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA) with bovine serum albumin as a standard according to the manufacturer's instructions. Protein fractions were digested using an In-Solution Tryptic Digestion Kit (Thermo, Rockford, IL) according to the manufacturer's suggestions. Briefly, the lysate or plasma membrane proteins (5-24 μg from transporter-expressing HEK293 cells and 20-500 μg from human kidney cortex) were diluted in 50 mM ammonium bicarbonate and heat-denatured (5 mM dithiothreitol; 5 minutes at 95°C) followed by alkylation (10 mM iodoacetamide; 20 minutes in the dark at room temperature). The incubation was then digested with trypsin (37°C for 3 hours), followed by an additional trypsinization at the same trypsin/protein ratio at 30°C overnight. In a preliminary experiment, various ratios of trypsin/protein were evaluated, with the condition yielding the highest overall peptide amounts resulting from a trypsin/protein ratio of 1:20. The digestion was terminated by the addition of trifluoroacetic acid (TFA, final concentration of 0.1%) and the samples were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described below. In a separate experiment conducted to compare the plasma membrane recovery between basolateral and apical membranes as measured by Na^+/K^+-ATPase or γ-glutamyl transpeptidase 1 (GGT1), respectively, trypsin digestion was performed on acetone precipitated protein pellets using the iST kit (PREOMICS GMBH).

Assumptions in quantitative proteomics

The following key assumptions were made in the quantitative proteomics and data analysis in this study:

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1. Na^+/K^+-ATPase and GGT1 are predominantly expressed on the basolateral and apical membranes of renal proximal tubules, respectively, as supported by immunohistochemistry (Katz, 1982; Hanigan and Frierson, 1996; Wetzel and Sweadner, 2001; Peti-Peterdi et al., 2002; Hanigan et al., 2015).
2. The recovery of membrane proteins is the same whether looking at the transporter of interest or plasma membrane markers.
3. Only transporters expressed on the plasma membrane are involved in drug transport into or out of the cells.
4. OCT2, MATE1 and MATE2K are equally distributed through proximal tubules in human kidney cortex.
5. The value for the number of proximal tubule cells in human kidney cortex reported by McLaren et al. (16×10^6 PTC/g of kidney cortex) (McLaren et al., 1995) was used in this study to convert the transporter expression in human kidney cortex to the level at proximal tubule cells.

Quantitative proteomics for plasma membrane markers and transporters

Surrogate peptides for each protein were selected as described previously (Prasad and Unadkat, 2014). The sequences and multiple reaction monitoring transitions of surrogate peptides, native and stable isotope label (SIL), are shown in Supplemental Table 2. Na^+/K^+-ATPase was quantified as the average of two different surrogate peptides (<25% difference between peptides, indicating similar digestion efficiency), except in the relative quantification in human kidney cortex (subject #7-9) where a single peptide was used, while GGT1, OCT2, MATE1 and MATE2K were quantified using the surrogate peptide which yielded the highest signal (representing best digestion efficiency, extraction and/or MS ionization) among multiple peptides evaluated in the preliminary assessment. All analyses were performed using a Sciex QTRAP-6500 while the liquid chromatography (LC) conditions used three methods (Supplemental Table 3). OCT2, MATE1 and MATE2K peptides were quantified in three separate preparations of plasma membrane fractions from transporter-expressing HEK293 cells and plasma membrane fractions of human kidney cortex from six subjects (#1-6). Na^+/K^+-ATPase and GGT1 were quantified in kidney cortex from additional three subjects (#7-9) to compare the recovery of basolateral and apical membranes.

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Determination of plasma membrane recovery

Abundances of the basolateral membrane marker Na^+/K^+-ATPase in transporter expressing HEK293 cells and human kidney cortex, as well as the apical membrane marker GGT1 in human kidney cortex, were determined by LC-MS/MS in lysate and plasma membrane fractions prepared from each cell and tissue as described above. The recovery of plasma membranes (PM recovery, %) relative to cellular lysate during the sample preparation was calculated using the equation below, assuming that Na^+/K^+-ATPase and GGT1 are exclusively expressed on basolateral and apical plasma membranes, respectively:

$$PM\ Recovery\ (\%) = \frac{[Na^+/K^+\text{-}ATPase\ or\ GGT1]_{PM}}{[Na^+/K^+\text{-}ATPase\ or\ GGT1]_{Lysate}} \times 100$$

where the numerator and denominator represent the abundance of Na^+/K^+-ATPase or GGT1 in the plasma membrane and lysate fractions, respectively.

Determination of transporter relative expression factors

The expression of OCT2, MATE1 and MATE2K was corrected for the plasma membrane recovery as follows:

$$[Transporter]_{PM,normalized} = \frac{[Transporter]_{PM,apparent}}{PM\ recovery}$$

where $[Transporter]_{PM,apparent}$ and $[Transporter]_{PM,normalized}$ represent the plasma membrane expression of transporters before and after the correction for plasma membrane recovery, respectively. The transporter abundance in human kidney cortex was further converted to the proximal tubule cells (PTC) level using the number of proximal tubule cells per gram of kidney cortex (16×10^6 PTC/g of kidney cortex) (McLaren et al., 1995). REFs were calculated as the ratio of transporter abundance between proximal tubule cells and in vitro cell lines prior to or following the correction by plasma membrane recovery.

Uptake assays

Uptake studies using HEK-OCT2, HEK-MATE1, HEK-MATE2K or HEK-EV were conducted as described previously (Kikuchi et al., 2013). The uptake of metformin by HEK-OCT2 was linear up to 4 minutes (data not shown). The uptake linearity by HEK-MATE1 and HEK-MATE2K couldn't be accurately determined due to the known overshoot of uptake under intracellular acidification condition (increase followed by decrease due to shortage of driving

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force) (Kikuchi et al., 2013); the uptake of metformin reached its maximum at 1.5 to 3 minutes (data not shown). The uptake of metformin for 2 minutes (OCT2) or 1 minute (MATE1 and MATE2K) was determined at various concentrations (13.7-30,000 μM). Transporter-specific uptake was obtained by subtracting the uptake into mock-transfected cells from that into transporter-expressing cells. K_m and V_{max} values were obtained using the following equation:

$$v = V_{max} \times S/(K_m + S)$$

where v is the initial uptake velocity of the substrate and S is the substrate concentration in the medium. Fitting was performed by the nonlinear least-squares method using GraphPad Prism. V_{max} values were normalized to account for number of cells (nmol/min/10^6 cells) using a cellular protein concentration (mg protein/10^6 cells) determined in a separate experiment, where the protein abundance in one million cells was determined by Pierce BCA Protein Assay after being precipitated in acetonitrile: water (1:1, v/v) then dissolved in 0.1N NaOH/0.5% SDS.

In vitro to in vivo extrapolation of metformin transport kinetics in the Simcyp PBPK model

In vitro transport kinetics of metformin by OCT2, MATE1 and MATE2K were extrapolated to in vivo using experimentally determined K_m and V_{max} values and the corresponding REFs (following plasma membrane recovery correction) in the Simcyp metformin PBPK model (mechanistic kidney model; version16; Simcyp Ltd, Sheffield, UK). Except for the in vitro transport kinetics and REF values, the default input parameters were used in the simulation. The electrochemical gradient model was activated for OCT2 as described previously (Burt et al., 2016). A sensitivity analysis for REFs was conducted in the metformin PBPK model to confirm the model's utility in evaluating the experimentally determined REFs for the basolateral uptake (OCT2) and apical efflux transporters (MATE1 and MATE2K; MATE1 was selected only for the purpose of sensitivity analysis). The metformin exposure-time profile was simulated at two different doses (250 and 500 mg) across 70 (10 trials × 7 subjects) and 60 (10 trials × 6 subjects) healthy volunteers for the 250 mg and 500 mg dose, respectively, using the experimentally determined in vitro transporter kinetics and REF values for OCT2, MATE1 and MATE2K. Model performance was assessed by comparing the simulated profiles to the clinically observed exposures (Somogyi et al., 1987; Wang et al., 2008).

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Results

Enrichment of plasma membranes and recovery

The total and plasma membrane fractions obtained from HEK293 cells transfected with either an empty vector (EV) or OCT2 were analyzed by Western blot to verify the enrichment of plasma membranes (Figure 1A). In both cell lines, the plasma membrane marker Na^+/K^+-ATPase was significantly enriched in the plasma membrane fraction compared with the total membrane fraction. The levels of calreticulin, an intracellular membrane marker associated with endoplasmic reticulum, were substantially lower in plasma membrane fractions than total membrane fractions. These results demonstrate the effectiveness in decreasing intracellular membrane proteins while retaining plasma membrane proteins in the plasma membrane fractions. The recovery of plasma membranes from transporter expressing HEK293 cells and human kidney cortex is shown in Figure 1B and Supplemental Table 4. While higher variability was seen in HEK-OCT2 cells, average recoveries were comparable (<3-fold) between different cells and tissues; 20%, 13%, 7.4% and 12% in HEK-OCT2, -MATE1, -MATE2K cells and human kidney cortex, respectively.

Comparison of basolateral and apical membrane recovery

The basolateral membrane marker Na^+/K^+-ATPase and apical membrane marker GGT1 were quantified in the lysate and plasma membrane fractions of kidney cortex from three additional subjects to compare the plasma membrane recoveries between the two sides of renal proximal tubule epithelial cells (Supplemental Table 5). The recovery values of Na^+/K^+-ATPase were comparable between a) when the absolute quantification values (reference standard of known concentration; fmol/10^6 cells) are used in calculation and b) when the relative quantification values (reference standard of approximate concentration; arbitrary unit/10^6 cells) are used, indicating that the relative quantification approach is sufficient to determine the membrane recovery. The recovery of GGT1 determined by relative quantification was comparable (<1.5-fold) to that of Na^+/K^+-ATPase. Thus, the plasma membrane recovery determined by the quantification of Na^+/K^+-ATPase in individual kidney cortex samples was used to normalize the transporter expression in subsequent analysis regardless of the localization of transporters.

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Quantification of transporters and determination of relative expression factors

The mean abundance of OCT2, MATE1 and MATE2K on the plasma membrane of over-expressing cells was 771, 471 and 391 fmol/10^6 cells, respectively, before plasma membrane recovery correction (Table 1). Values were corrected using the plasma membrane recovery from the individual experiments, resulting in 4064, 4276 and 5351 fmol/10^6 cells, respectively (Table 1). The mean abundance in human kidney cortex before and after plasma membrane recovery (determined by Na^+/K^+-ATPase) correction was 4233, 6774 and 789 fmol/10^6 PTC , and 37355, 58982 and 6947 fmol/10^6 PTC for OCT2, MATE1 and MATE2K, respectively (Table 2). Accordingly, the relative expression factors for OCT2, MATE1 and MATE2K were 5.5, 14 and 2.0 prior to correction and 9.2, 14 and 1.3 following plasma membrane recovery correction, respectively (Table 3).

In vitro transporter kinetics of metformin by OCT2, MATE1 and MATE2K

Metformin uptake was saturable with mean K_m and V_{max} values of 1872 μM and 7.29 nmol/min/10^6 cells (OCT2), 454 μM and 2.93 nmol/min/10^6 cells (MATE1) and 1840 μM and 3.40 nmol/min/10^6 cells (MATE2K) (Table 4). A representative data and individual values are shown in Figure 2 and Supplemental Table 6, respectively.

IVIVE of metformin transport kinetics

A sensitivity analysis for REFs was conducted in the metformin PBPK model to confirm the model's utility in evaluating the experimentally determined REFs for the basolateral uptake (OCT2) and apical efflux transporters (MATE1 and MATE2K). Although both MATE transporters exist on the apical membrane of proximal tubule cells; the sensitivity analysis here focused on MATE1 as it likely plays a more predominant role in metformin transport; ~8.5-fold higher plasma membrane expression assuming the same relative digestion, extraction and ionization efficiencies between the two surrogate peptides (Table 2) and 3.5-fold higher metformin uptake clearance (V_{max}/K_m) (Table 4) than MATE2K. The metformin renal clearance and plasma area under the curve (AUC) were sensitive to both OCT2 and MATE1 REFs; a corresponding increase in renal clearance and decrease in AUC were seen as REF values for OCT2 and MATE1 were increased (Figure 3). In the subsequent PBPK simulation of metformin exposure, all three metformin transporters (OCT2, MATE1 and MATE2K) were included using the corresponding in vitro transport kinetics and REF values. The simulated exposures-time

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profiles of metformin at two doses (250 and 500 mg) based on the experimental parameters were within two-fold of the clinically observed exposure (Figure 4 and Table 5).

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Discussion

The IVIVE of transporter kinetics coupled with transporter quantification is an emerging area of interest that has shown promise in the prediction of drug disposition for transporter substrates, but with varying degrees of success. One complicating factor is that transporter proteins are not only found on the plasma membrane but also in the intracellular compartment, and only transporters on the plasma membrane surface can contribute to compound uptake to or efflux from the cell (Bow et al., 2008; Hayashi and Sugiyama, 2013). This indicates that the extrapolation of in vitro kinetics data to in vivo outcomes should consider differences in transporter abundance on plasma membranes, rather than on whole cell lysate or total membranes. Furthermore, a correction for plasma membrane recovery may be required to determine the absolute abundance of transporters due to the different matrices involved. In the present study, a simple and reliable method using quantitative proteomics was developed to accurately determine the plasma membrane expression of target proteins.

Enrichment of the plasma membrane fraction is advantageous when quantifying low abundance proteins such as transporters, which are often difficult to detect in the lysate or total membrane fractions from cells or tissues. These enrichment methods can increase the concentration of plasma membrane proteins while reducing background protein levels. This results in a large increase in overall LC-MS/MS signal from equivalent amounts of starting total protein. In this study, plasma membrane fractions were successfully enriched from both transporter overexpressing cells and whole tissue samples (Figure 1, Supplemental Table 4). In addition to enabling the successful protein quantification of OCT2 and MATE1, the quantitation of MATE2K was also possible, even though it had a much lower abundance in the plasma membrane fractions from human kidney cortex (assuming the same digestion, extraction and ionization efficiencies between different surrogate peptides) (Table 2). This is in contrast to observations by Prasad et al. who were able to detect, but not quantify, MATE2K (below the lower limit of quantification) in the total membrane fraction from human kidney cortex (Prasad et al., 2016). In the same study, OCT2 and MATE1 were successfully quantified, which is consistent with the higher abundance of these transporters compared with MATE2K on the plasma membrane fraction from human kidney cortex determined in the present study (Table 2).

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OCT2 is localized on the basolateral membrane of renal proximal tubules, while MATE1 and MATE2K are on the apical membrane (Hillgren et al., 2013). A separate experiment demonstrated that the recovery is comparable between basolateral and apical membranes from human kidney cortex using the current methodology (Supplemental Table 5), thus supporting the use of a unified plasma membrane recovery number, as measured by Na^+/K^+-ATPase, to normalize the transporter expression regardless of cellular localizations. Mean plasma membrane recovery was comparable between different cells and tissues, and the transporter REF values did not change substantially (<2-fold change) when recovery was considered (Table 3). While there was no significant impact in this study, correction may normalize variability when different matrices or methods are used. In addition, it is important to note that plasma membrane recovery was generally low (ranged from 6.5-20% except one replicate from HEK-OCT2, 41%; Supplemental Table 4), and correction would be necessary if the purpose is to determine absolute transporter abundance. Higher data variability is also likely if different methods are used to enrich the membrane proteins and recovery is not considered. These factors may contribute to the notable cross-laboratory differences (up to nearly 100-fold) observed for transporter quantitation (Harwood et al., 2014; Harwood et al., 2016; Wegler et al., 2017).

In vitro kinetic parameters of metformin for OCT2, MATE1 and MATE2K were determined (Figure 2, Table 4) and K_m values in the present study are similar to those previously reported (present study vs literature range, μM); OCT2 (1872 vs 1465-3356), MATE1 (454 vs 228-780) and MATE2K (1840 vs 819-1980) (Tanihara et al., 2007; Song et al., 2008; Zolk et al., 2009; Shen et al., 2016). The V_{max} values (nmol/min/mg protein) obtained do highlight experimental system-dependent differences, particularly for MATE1 and MATE2K; OCT2 (37.0 vs 12-153), MATE1 (15.6 vs 2.2-4.5) and MATE2K (16.9 vs 0.85-7.1). These observations emphasize the importance of determining REFs in the same experimental systems used for transport characterization. The differences in V_{max} values are likely attributed to the difference in transporter abundance on the plasma membrane. As a result, caution should be exercised when using REF values reported in the literature for IVIVE.

In vitro kinetics of metformin for OCT2, MATE1 and MATE2K were translated to in vivo clearance using the corresponding REFs (corrected with plasma membrane recovery) in a PBPK model. The simulated exposure-time profiles of metformin at two doses (250 and 500 mg) correctly captured the elimination phase of metformin pharmacokinetics (Figure 4).

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Furthermore, the predicted exposures were within 2-fold of clinically observed exposures (Table 5) (Somogyi et al., 1987; Wang et al., 2008). These results demonstrate that in vitro transporter kinetics can be accurately extrapolated to in vivo by using REFs derived from the plasma membrane expression of transporters.

Metformin is predominantly cleared from the systemic circulation by urinary excretion of unchanged drug via glomerular filtration and tubular secretion (Graham et al., 2011). Considering the lack of binding to plasma proteins (Tucker et al., 1981) and low lipid solubility, which likely leads to negligible passive reabsorption (Graham et al., 2011), the highest achievable renal clearance of metformin when the activities of secretory transporters are very significant can be approximated by the sum of glomerular filtration rate (7.2 L/hr) and renal blood flow (60 L/hr) (Neuhoff et al., 2013). It is worthwhile to note that the simulated renal clearance of metformin is close to the theoretically highest value (67.2 L/hr) when the REFs for both OCT2 and MATE1 are greater than 3 (Figure 3). The experimentally determined REFs for these transporters (5.5 and 14 before and 9.2 and 14 after the correction for plasma membrane recovery, respectively; Table 3) are in this range, and therefore, a 3- to 4-fold change in REF values is not expected to change the predicted renal clearance and resulting exposure of metformin.

The number of proximal tubule cells in human kidney cortex has not been well established (Scotcher et al., 2016). In the present study, 16×10^6 PTC/g of kidney cortex (average of four kidney preparations) (McLaren et al., 1995) was used to convert the transporter expression in human kidney cortex to the level at proximal tubule cells and calculate the REF values comparing to the expression in corresponding in vitro cell lines. Another literature reports that approximately 70×10^6 kidney cells primarily of proximal tubule origin were obtained from 1 g of cortical tissue (Cummings and Lash, 2000). Using this higher value in the analysis results in the REF values of 2.1, 3.2 and 0.30 for OCT2, MATE1 and MATE2K after the correction for plasma membrane recovery, respectively, while those were originally 9.2 (OCT2), 14 (MATE1) and 1.3 (MATE2K) (Table 3). The predicted AUCs of metformin using these REF values are also comparable to the clinical AUCs (data not shown), which is in line with the sensitivity analysis where metformin AUC is not sensitive to REFs in that range because of its already high renal clearance (Figure 3). A compound with lower renal clearance mediated by these transporters would be required for additional validation of the experimental REFs. The

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current work does show that the quantification of renal drug transporters allows for the prediction of metformin disposition. Additional work will be required to determine whether this approach will be successful for other renally cleared drugs.

In conclusion, a novel approach was established to reproducibly quantify the transporter protein expression on plasma membranes in both cells and tissue. The plasma membrane recoveries determined by the quantification of Na^+/K^+-ATPase as the marker protein were comparable between HEK293 cells overexpressing OCT2, MATE1 and MATE2K and human kidney cortex in this study, suggesting that transporter REFs may be able to be estimated without the correction of plasma membrane recovery as long as the same procedure is applied between different matrices. Further studies are warranted to expand this observation to other transporters and in vitro/in vivo systems. In vitro transporter kinetics of metformin was successfully extrapolated to in vivo using the REFs for OCT2, MATE1 and MATE2K based on the plasma membrane transporter expression. These results demonstrate that the careful quantification of transporter proteins on plasma membrane enables prediction of human drug disposition using PBPK modeling.

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Author Contributions

Participated in research design: Kikuchi, Chiou, Durbin, Savaryn, de Morais, Jenkins, Bow

Conducted experiments: Chiou, Ma, Emami Riedmaier, Savaryn

Performed data analysis: Kikuchi, Chiou, Durbin, Savaryn, Ma, Emami Riedmaier

Wrote or contributed to the writing of the manuscript: Kikuchi, Chiou, Durbin, Savaryn, Ma, Emami Riedmaier, de Morais, Jenkins, Bow

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Footnotes

AbbVie sponsored and funded the study; contributed to the design; participated in collection, analysis, and interpretation of data; and in writing, reviewing, and approval of the final version. All authors are employees, former employees or retirees of AbbVie and may own AbbVie stock. KD is currently an employee at Proteinaceous and has no additional conflicts of interest to disclose. AER is currently an employee at Bristol Myers Squibb; Drug Development and Preclinical Studies, DMPK; Research and Early Development.

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Legend for figures

Figure 1

Plasma membrane preparation and recovery. (A) The levels of Na^+/K^+-ATPase and calreticulin were measured by Western blot for both the total membrane and plasma membrane fractions from HEK-EV and HEK-OCT2 cells. 10 μg of protein from each fraction were electrophoresed and detected as described in Materials and Methods. (B) Plasma membrane recovery was determined as described in Materials and Methods in transporter-expressing cells (three separate preparations) and human kidney cortex (six subjects). Results are shown as mean ± standard deviation. ATPase, Na^+/K^+-ATPase; Calr, calreticulin; TM, total membrane; PM, plasma membrane.

Figure 2

Concentration-dependent uptake of metformin. The concentration-dependent transporter-specific uptake of metformin by OCT2 (A), MATE1 (B) and MATE2K (C) is shown as a Michaelis-Menten plot. Solid lines represent the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± SEM of triplicate experiments. Where error bars are not visible, the SEM values are within the limits of the symbols. Representative data are shown from two independent experiments each run in triplicates.

Figure 3

Sensitivity analysis of OCT2 and MATE1 REFs in metformin PBPK model. The renal clearance (A) and AUC (B) of metformin (250 mg) was simulated by varying the REFs for OCT2 and MATE1 between 0.03 and 14. Population representative was used for these simulations in Simcyp version 16.

Figure 4

Simulated and observed exposure-time profiles of metformin. Metformin exposure-time profiles were simulated at two different doses (A, 250 and B, 500 mg) and compared to the clinically observed exposures. Dots, solid lines and dashed lines represent the observed exposure, median and $5^{th}/95^{th}$ percentile of simulated exposures, respectively.

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Tables

Table 1

Quantification of transporters in transfected cells

Cells	Replicate #	Apparent expression (fmol/10^6 cells)	PM recovery (%)	Normalized expression (fmol/10^6 cells)
	1	348	6.8	5080
	2	1542	41	3800
	3	424	13	3312
	Mean	771	-	4064
HEK-OCT2	**SD**	669	-	913
	1	528	10	5202
	2	465	8.5	5486
	3	419	20	2141
	Mean	471	-	4276
HEK-MATE1	**SD**	55	-	1855
	1	413	6.5	6334
	2	307	7.8	3958
	3	454	7.9	5761
	Mean	391	-	5351
HEK-MATE2K	**SD**	76	-	1240

PM, plasma membrane

Table 2

Quantification of transporters in human kidney cortex

Tissues	Subject #	Apparent expression (fmol/10^6 PTC)			PM recovery (%)	Normalized expression (fmol/10^6 PTC)		
		OCT2	MATE1	MATE2K		OCT2	MATE1	MATE2K
Human kidney cortex	1	2302	5809	907	13	17672	44584	6960
	2	7165	10680	1081	17	43391	64677	6550
	3	4224	5805	549	10	42398	58263	5512
	4	3989	5220	749	8.1	49538	64828	9296
	5	4406	8610	861	11	41440	80991	8099
	6	3310	4520	587	11	29690	40546	5265
	Mean	4233	6774	789	-	37355	58982	6947
	SD	1629	2366	202	-	11603	14824	1542

PTC, proximal tubule cells

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Table 3

Transporter abundance and relative expression factors

	Prior to correction			Following correction		
Transporter	**Transfected cells**	**Human kidney cortex**	**REF**	**Transfected cells**	**Human kidney cortex**	**REF**
OCT2	771	4233	5.5	4064	37355	9.2
MATE1	471	6774	14	4276	58982	14
MATE2K	391	789	2.0	5351	6947	1.3

Mean transporter abundance values were used to calculate REFs prior to or following the correction by plasma membrane recovery.

Units: transfected cells (fmol/10^6 cells); human kidney cortex (fmol/10^6 PTC); REF (relative expression factor, no unit); PTC, proximal tubule cells.

Table 4

Protein content and in vitro transport kinetics of metformin by OCT2, MATE1 and MATE2K

			V_{max}		
Transporter	**Protein content (mg/10^6 cells)**	**K_m (μM)**	**(nmol/min/mg protein)**	**(nmol/min/10^6 cells)**	**V_{max}/K_m (μL/min/10^6 cells)**
OCT2	0.197	1872	37.0	7.29	3.90
MATE1	0.188	454	15.6	2.93	6.46
MATE2K	0.201	1840	16.9	3.40	1.85

Mean K_m and V_{max} values were calculated from two independent experiments each run in triplicate.

Table 5

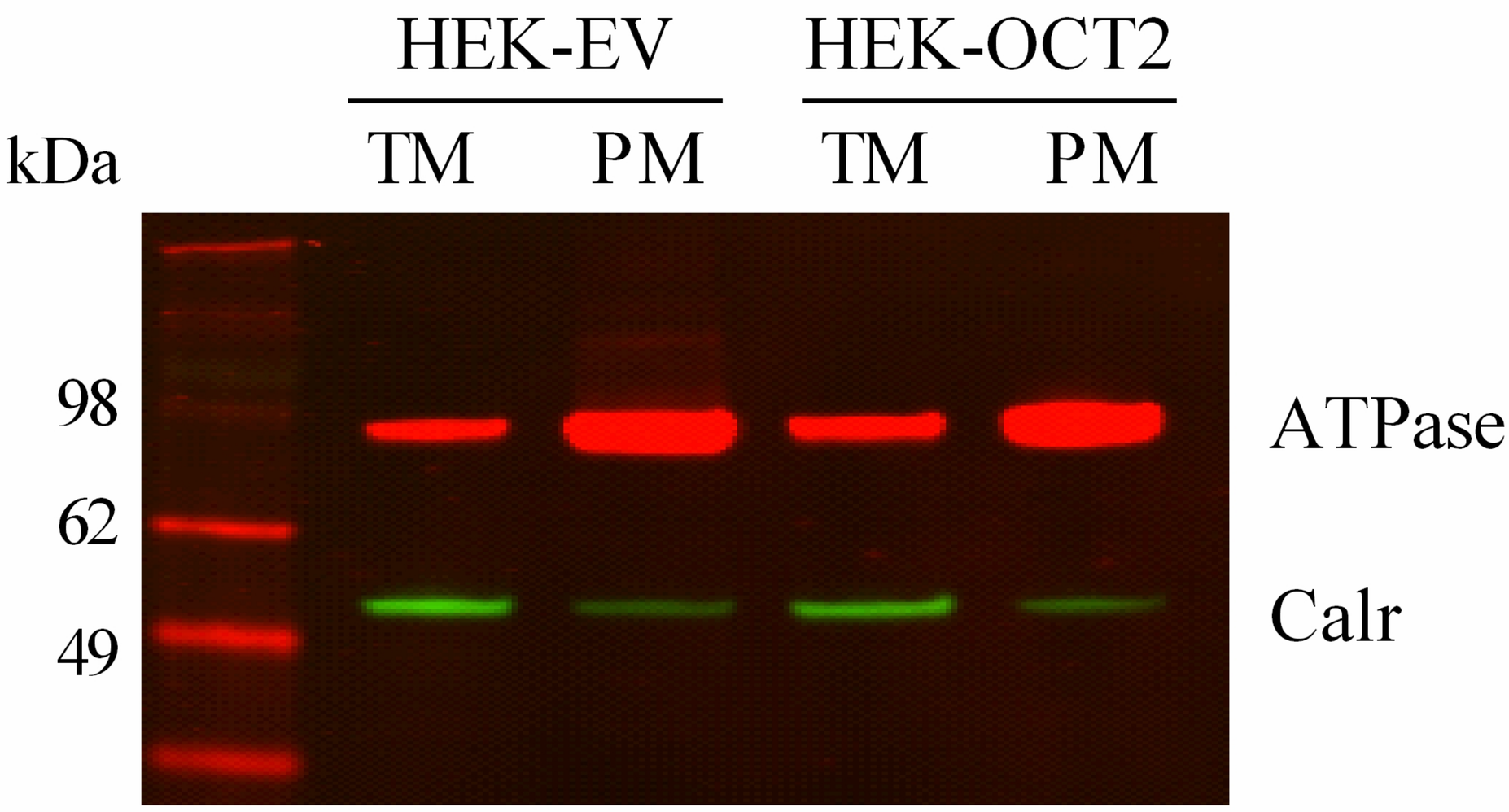
Predicted and observed exposure of metformin

Dose (mg)	**250**			**500**		
Parameters	**Predicted**	**Observed[1]**	**Ratio (Obs/Pred)**	**Predicted**	**Observed[2]**	**Ratio (Obs/Pred)**
C_{max} (μg/mL)	0.36	0.59	1.7	0.75	1.19	1.6
$AUC_{0\text{-}24}$ (μg*hr/mL)	2.42	4.26	1.8	5.03	6.58	1.3

[1]Somogyi et al., 1987; [2]Wang et al., 2008

Figure 1

(A)



(B)

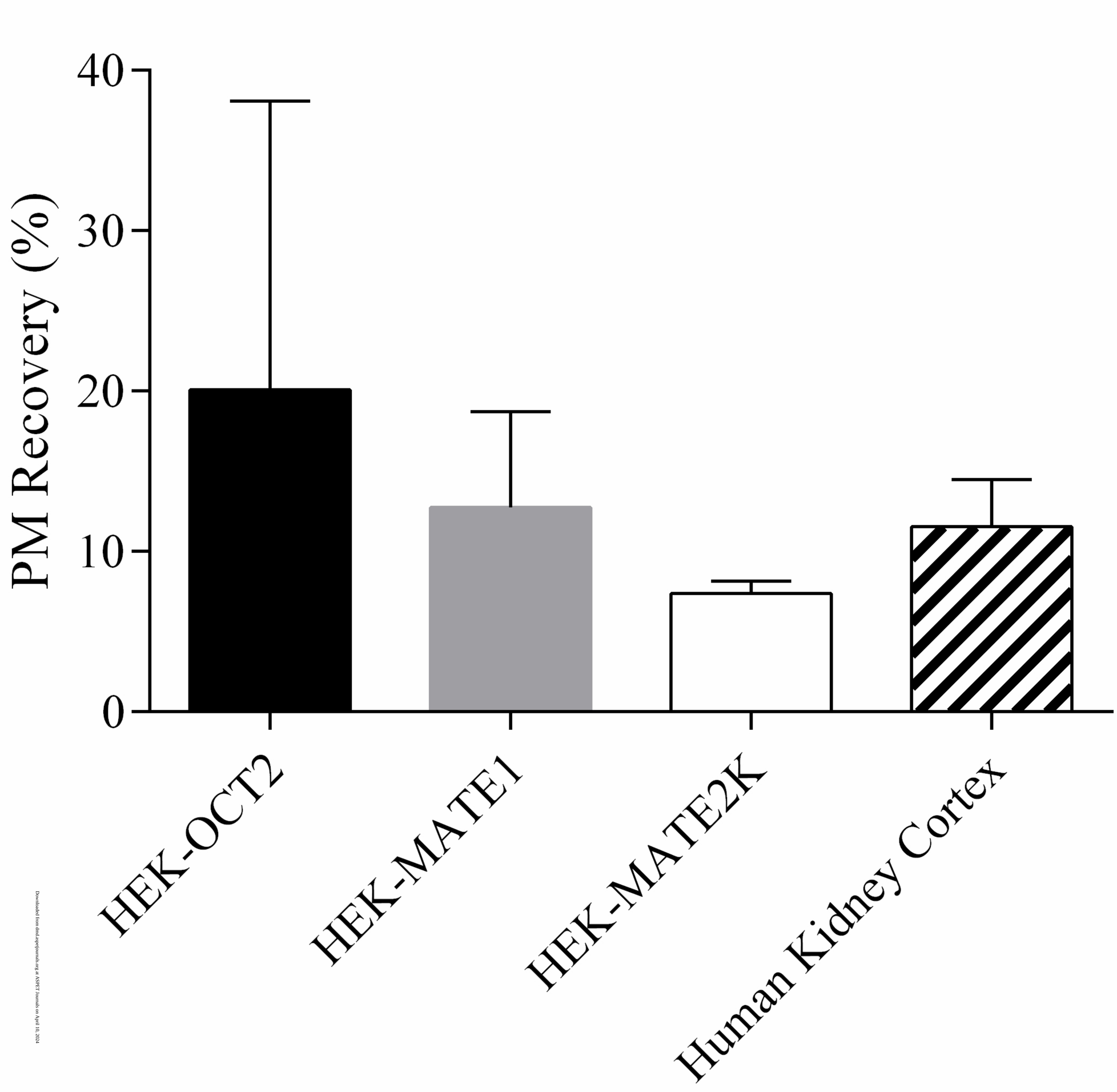


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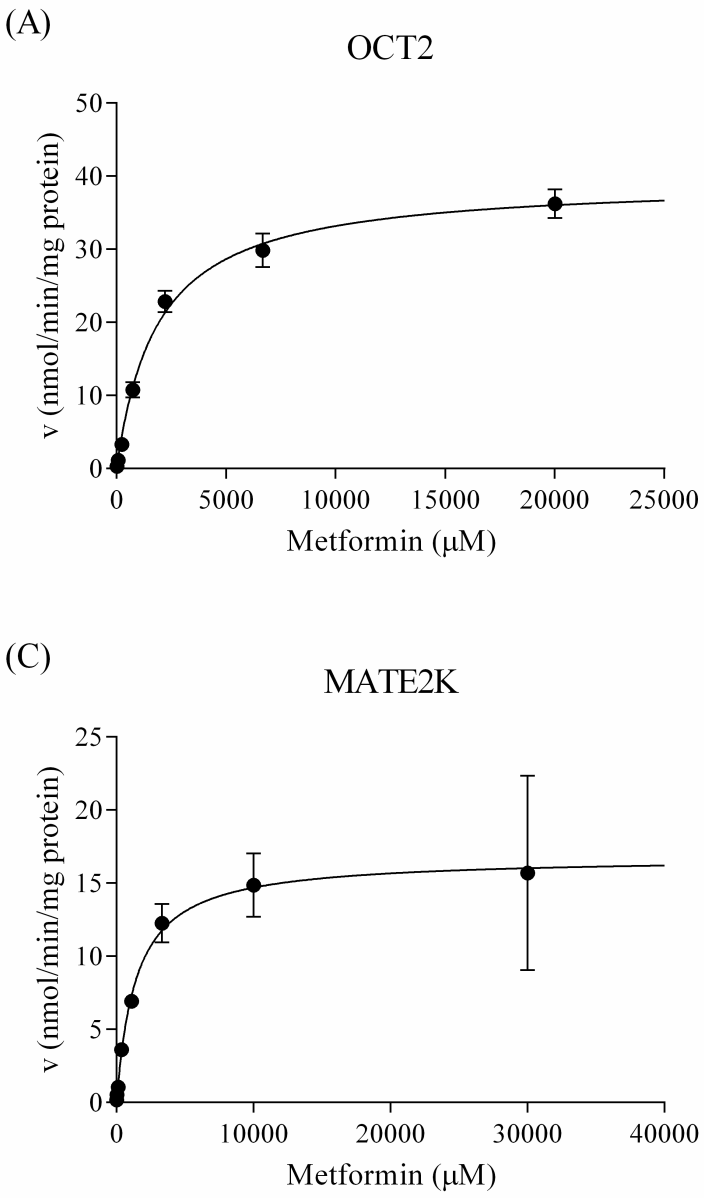
Figure 2



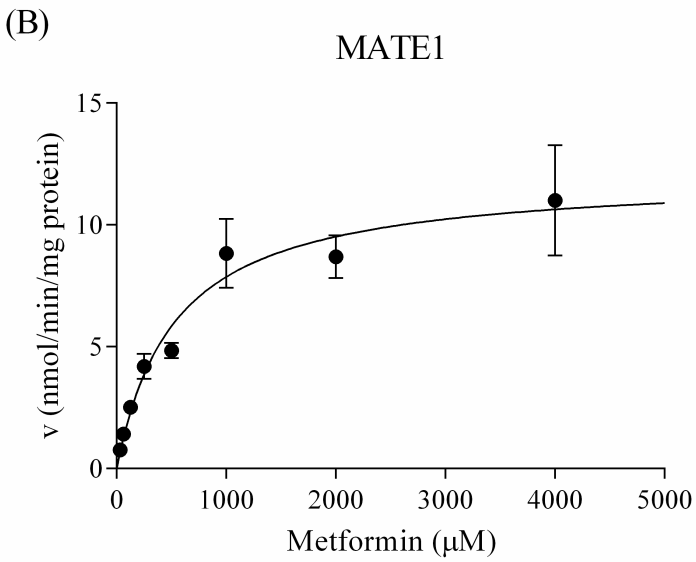


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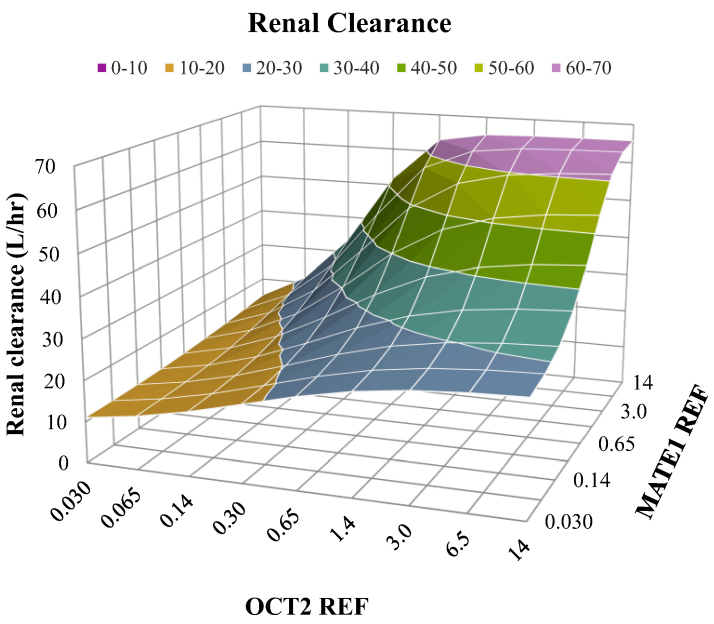
Figure 3

(A)



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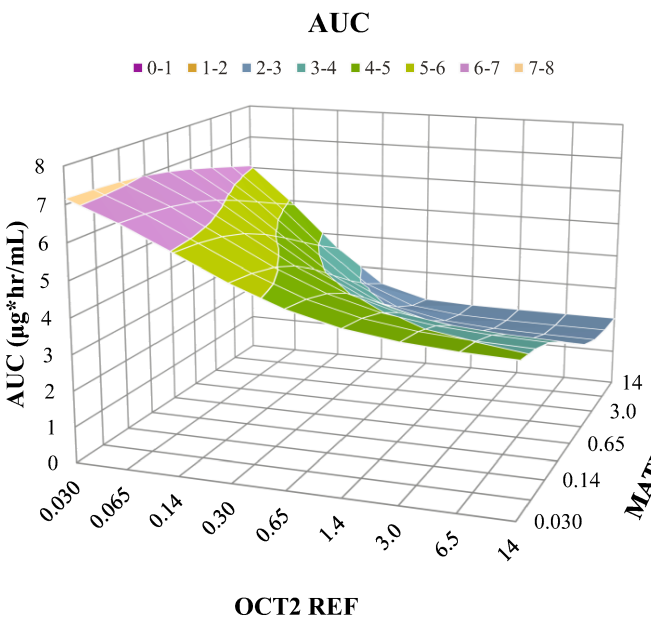

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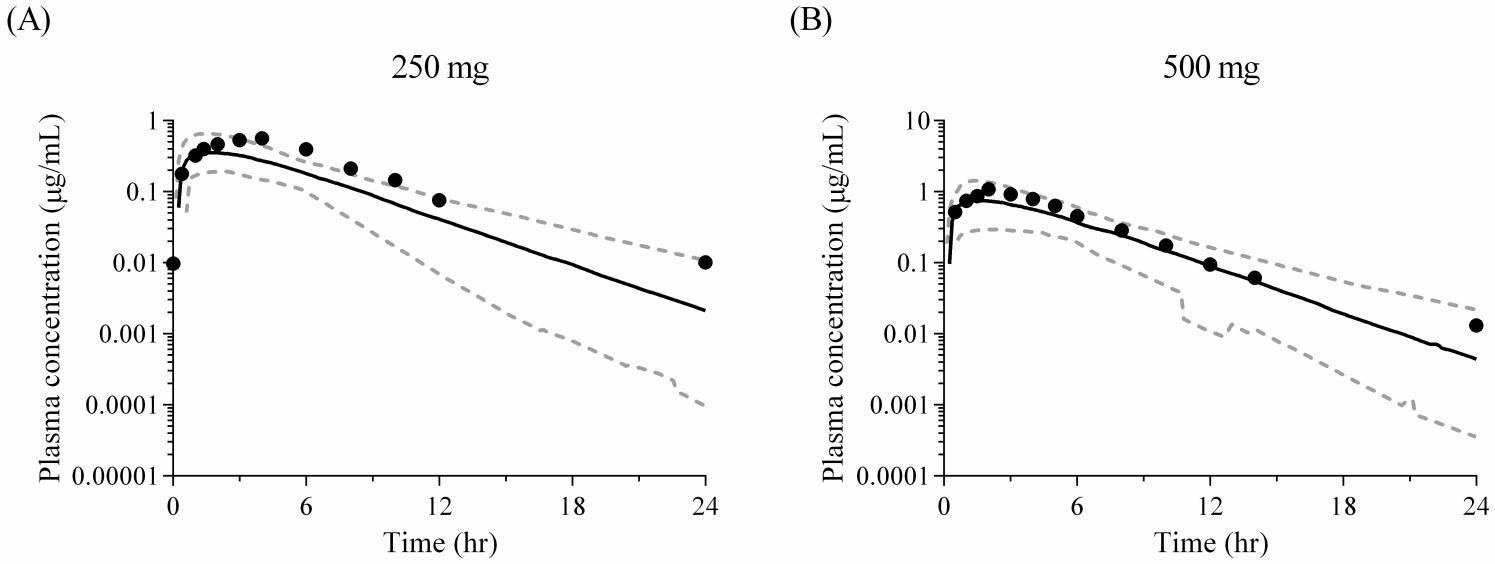
Figure 4



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Title

Quantitation of plasma membrane drug transporters in kidney tissue and cell lines using a novel proteomic approach enabled a prospective prediction of metformin disposition

Ryota Kikuchi, William J. Chiou, Kenneth R. Durbin, John P. Savaryn, Junli Ma, Arian Emami Riedmaier, Sonia M. de Morais, Gary J. Jenkins and Daniel A.J. Bow.

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Supplemental Tables

Supplemental Table 1

Tissue Demographics

Donor	Subject ID	Anatomical Site	Tissue Specification	Ethnicity (Race)	Age	Sex	Procurement Type	Cause of death	PMI (hrs)
1	AMS-12-11-S1-31	Kidney, cortex	Normal	Caucasian (White)	38	Male	Autopsy	Traumatic Injury	3
2	AMS-12-11-S2-31	Kidney, cortex	Normal	Caucasian (White)	52	Female	Autopsy	Traumatic Injury	4
3	AMS-12-11-S3-31	Kidney, cortex	Normal	Caucasian (White)	30	Male	Autopsy	Traumatic Injury	4
4	AMS-24-11-S4-31	Kidney, cortex	Normal	Caucasian (White)	30	Male	Autopsy	Traumatic Injury	4
5	AMS-09 11-S6-29	Kidney, cortex	Normal	Caucasian (White)	46	Male	Autopsy	Traumatic Injury	3
6	AMS-09 11-S7-29	Kidney, cortex	Normal	Caucasian (White)	47	Male	Autopsy	Traumatic Injury	3
7	113AFJF185	Kidney, cortex	Normal	Caucasian (White)	60	Male	Autopsy	CVA Stroke; Organ recovery	1
8	113AGET292	Kidney, cortex	Normal	Caucasian (White)	41	Male	Organ recovery	Blunt head trauma	1
9	46-116068	Kidney, cortex	Normal	Caucasian (White)	43	Male	Autopsy	Pulmonary edema	8

PMI, Post-Mortem Interval

Supplemental Table 2

Sequences and multiple reaction monitoring transitions of the surrogate peptides for OCT2, MATE1, MATE2K, Na^+/K^+-ATPase and GGT1

Protein	Peptide type	Peptide sequence	MW	Q1	Q3	DP	CE
				436.1	671.6	30	18
				436.1	574.4	30	27
	Native	SLPASLQR	870.5	436.1	503.3	30	28
				439.6	678.2	41	23
				439.6	581.3	41	29
OCT2	SIL	SLPASL**Q**R	877.955	439.6	510.2	41	27
				514.8	688.5	34	30
	Native	GGPEATLEVR	1027.529	514.8	617.3	38	23
				517.2	693.3	50	29
MATE1	SIL	GGPEA**T**LEVR	1033.09	517.2	622.3	50	27
				733.7	802.5	101	45
				733.7	618.4	101	45
	Native	TPEEAHALSAPTSR	1466.56	733.7	460.2	101	45
				736.7	808.4	66	45
				736.7	624.4	66	43
MATE2K	SIL	TPEEAHALSA**P**TSR	1472.528	736.7	466.2	66	43
Na^+/K^+-ATPase	Native	VDNSSLTGESEPQTR	1619.65	540.8	501.3	60	23
	SIL	VDNSSLTGESE**P**QTR	1625.618	542.7	507.1	60	25
Na^+/K^+-ATPase	Native	AAVPDAVGK	826.94	414.2	586.3	60	26
	SIL	AAVPDA**V**GK	832.905	417.2	592.3	60	28
				516.8	717.3	69	28
				516.8	588.3	69	28
GGT1	Native	ESVESPEQK	1031.48	516.8	501.3	69	28

The bold faced letters indicate the location of stable-isotope-label (SIL). Two or three MRM transitions are monitored for each peptide (OCT2, MATE1, MATE2K and GGT1) and the concentrations were calculated using the average. MRM, multiple reaction monitoring; MW, molecular weight; DP, declustering potential; CE, collision energy.

Supplemental Table 3

Liquid chromatography conditions

Samples	Autosampler	Pump	Column	Column Temp	MP A	MP B	Flow rate (µL/min)	Example Gradients		
								Time (min)	MP A %	MP B %
Absolute quantitation (transfected cells and kidney cortex subject #1-6)	Gerstel MultiPurpose Sampler MPS 3C	Eksigent Ekspert microLC 200 UHPLC	3C18-EP120 (50 × 0.5 mm, 3 µm) or HALO C18 (50 × 0.5 mm, 2.7 µm)	40°C	0.1% FA, 0.025% TFA in water	0.1% FA in acetonitrile	35	0	100	0
								0.5	95	5
								4	70	30
								4.5	100	0
Absolute quantitation (kidney cortex subject #7-9)	Gerstel MultiPurpose Sampler MPS 3C	Agilent Technologies 1290 Infinity pump	Acquity UPLC® CSH™ C18 (2.1 × 150 mm, 1.7 µm)	60°C	0.1% FA in water	0.1% FA in acetonitrile	250	0	95	5
								10	50	50
								10.1	5	95
								12.1	5	95
								12.2	95	5
Relative quantitation (kidney cortex subject #7-9)	Gerstel MultiPurpose Sampler MPS 3C	Agilent Technologies 1290 Infinity pump	Kinetex C18 (2.1 × 30 mm, 5 µm)	Room Temp	0.1% FA in water	0.1% FA in acetonitrile	1500	0	97	3
								0.2	97	3
								0.75	50	50
								0.76	5	95
								0.86	5	95
								0.87	97	3

Temp, temperature; MP, mobile phase; FA, formic acid; TFA, trifluoroacetic acid

Supplemental Table 4

Plasma membrane recovery from transfected cells and human kidney cortex

Cells	Replicate #/Subject #	Na^+/K^+-ATPase expression (fmol/10^6 cells)		PM recovery (%)	PM recovery (%)	
		Lysate	PM fraction		Mean	SD
HEK-OCT2	1	1671	114	6.8	20	18
	2	1208	490	41		
	3	1364	175	13		
HEK-MATE1	1	2884	293	10	13	6
	2	2614	222	8.5		
	3	1054	206	20		
HEK-MATE2K	1	2988	195	6.5	7.4	0.8
	2	1902	148	7.8		
	3	2488	196	7.9		
Human kidney cortex	1	123867	16138	13	12	3
	2	150991	24932	17		
	3	147435	14689	10		
	4	130267	10490	8.1		
	5	126211	13418	11		
	6	99343	11074	11		

PM recovery in transfected cells and human kidney cortex was determined using Na^+/K^+-ATPase. PM, plasma membrane

Supplemental Table 5

Comparison of basolateral and apical membrane recovery

Tissue	Subject #	Basolateral PM recovery (%)		Apical PM recovery (%)
		Absolute quantification	Relative quantification	Relative quantification
Human kidney cortex	7	12	9.1	20
	8	24	23	38
	9	20	20	23
	Mean	19	18	27
	SD	7	8	9
	CV (%)	35	43	35

Basolateral and apical PM recovery was determined using Na^+/K^+-ATPase and GGT1, respectively.

Supplemental Table 6

In vitro transport kinetics of metformin by OCT2, MATE1 and MATE2K in individual experiments

Transporter	Experiment	K_m (μM)	V_{max} (nmol/min/mg protein)
OCT2	Exp 1	1874	39.4
	Exp 2	1870	34.6
MATE1	Exp 1	376	19.2
	Exp 2	532	12.1
MATE2K	Exp 1	1418	16.8
	Exp 2	2261	17.1

Exp, experiment