SHORT COMMUNICATION

Kidney cortical transporter expression across species using quantitative proteomics

Abdul Basit[1], Zaher Radi[2], Vishal S. Vaidya[2], Mathew Karasu[1], Bhagwat Prasad[1]

[1] Department of Pharmaceutics, University of Washington, Seattle, P.O. Box 357610, WA 98195 USA

[2] Pfizer Worldwide Research Development, Drug Safety Research Development, One Portland Street, Cambridge, MA 02139 USA

Running Title: Quantitative profiling of kidney transporters across species

Corresponding author: Bhagwat Prasad, Ph.D., Department of Pharmaceutics, University of Washington, Seattle, P.O. Box 357610, WA 98195, USA. Phone: +1-206-221-2295. Fax: +1-206-543-3204. Email: bhagwat@uw.edu

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Abbreviations: ABB, ammonium bicarbonate buffer; BSA, bovine serum albumin; DTT, dithiothreitol; HSA, human serum albumin; IAA, iodoacetamide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PK, Pharmacokinetics; OAT, organic anion transporters; OATP, Organic anion transporter polypeptide; OCT, organic cation transporter; MATE1, Multidrug and toxin extrusion protein 1 ; MDR1, Multidrug resistance protein1; MRP, Multidrug resistance-associated protein; SGLT2, Sodium/glucose cotransporter 2; BCRP, Breast cancer resistance protein; OCTN1/2, Organic cation/carnitine transporter 1/2, SLC = Solute Carrier; SLCO = solute carrier organic anion transporter; ABCB = ATP-binding cassette sub-family B; ABCG = ATP-binding cassette super-family G; ABCC = ATP-binding cassette sub-family C

ABSTRACT

Limited understanding of species differences in kidney transporters is a critical knowledge gap for prediction of drug-induced acute kidney injury, drug interaction, and pharmacokinetics in humans. Here, we report protein abundance data of nineteen transporters in the kidney cortex across five species (human, monkey, dog, rat, and mouse). In general, the abundance of all the 19 membrane transporters was higher in preclinical species as compared to human except for MDR1, OCT3 and OCTN1. In nonhuman primate, the total abundance of 12 transporters for which absolute data were available, was 2.1-fold higher (p value = 0.025) relative to human but the % distribution of these transporters was identical in both species. MRP4, OCTN2, OAT2, Na^+K^+ATPase, MRP3, SGLT2, OAT1, MRP1, MDR1, OCT2 were expressed differently with cross-species variability of 8.2, 7.4, 6.1, 5.9, 5.4, 5.2, 4.1, 3.3 and 2.8-fold, respectively. Sex differences were only significant in rodents and dog. High protein-protein correlation was observed in OAT1 versus MRP2/MRP4 as well as OCT2 versus MATE1 in human and monkey. The cross-species and sex-dependent protein abundance data are important for animal to human scaling of drug clearance as well as for mechanistic understanding of kidney physiology and derisking of kidney toxicity for new therapeutic candidates in drug development.

INTRODUCTION

In the early toxicity assessment of candidate therapeutics in drug development, the data obtained from animal models need to be interpreted with caution for predicting human pharmacokinetics (PK) (Tang and Mayersohn, 2011), risk of organ injury as well as drug-drug interaction (DDI) (Fisel et al., 2014). Clinical and preclinical species differences in the mRNA expression, protein abundance and activity of transporters in organs relevant to drug disposition (i.e., intestine, liver, and kidney) remain a major reason for the poor allometric scaling (Wang et al., 2015). Although significant progress has been made towards the understanding of species and sex differences of drug transporters in the liver (Wang et al., 2015), limited data exist in the kidney and intestine. Particularly, kidney transporters can affect systemic drug clearance by regulating drug secretion and/or reabsorption and contribute to kidney toxicity by affecting intracellular drug concentration (Filipski et al., 2009). For example, kidney toxicity of tenofovir, methotrexate, cisplatin, ifosfamide, ciprofloxacin, and sitagliptin is associated with solute carrier (SLC) transporters such as organic anion transporters (OATs) or organic cation transporters (OCTs) (Fisel et al., 2014) (Figure 1). Allometric scaling methods are used for extrapolation of preclinical renal disposition data to human (Zou et al., 2012), however these methods are not always successful particularly when a drug undergoes kidney transport and metabolism. For example, tenofovir is taken up into proximal tubules mainly by OAT1 and effluxed into the urine by MRP4, and both of these transporters are shown to be associated with tenofovir kidney toxicity (Kohler et al., 2011). Further, examples exist where kidney toxicity is different in male and female, e.g., cisplatin, an OCT2 substrate, is more toxic to male rats (Nematbakhsh et al., 2013). In addition, rises in serum creatinine (sCr) is used as a surrogate of kidney function. However, as a fraction of sCr is secreted by active transporters, cross-species and sex-dependent variability in the sCr transport could lead to false positive or negative conclusions regarding the kidney function. Particularly, rise in sCr could be a result of inhibition of renal transporters without direct kidney injury (Chu et al., 2016).

Therefore, characterization of the cross-species and sex-dependent differences in protein abundances of kidney transporters is important for scaling and better prediction of renal secretion, reabsorption, and toxicity in humans. Accordingly, we hypothesized that mapping interspecies and sex-differences in the abundance of kidney cortical transporters would enable development of physiologically-based PK (PBPK) models that will improve the prediction of PK, kidney toxicity, and the potential risk of DDIs.

MATERIALS AND METHODS

Chemicals and reagents

LC-MS grade acetonitrile, methanol, chloroform and formic acid were purchased from Fisher scientific (Fair Lawn, NJ, USA) and formic acid was purchased from Sigma-Aldrich (St. Louis, MO). The ProteoExtract native membrane protein extraction kit was procured from Pierce Biotechnology (Rockford, IL). The protein quantification BCA kit and the in-solution trypsin digestion kit were purchased from Pierce Biotechnology (Rockford, IL). Iodoacetamide (IAA), dithiothreitol (DTT), and pierce trypsin protease (MS grade) were purchased from Thermo Fisher Scientific (Rockford, IL). Ammonium bicarbonate buffer (ABC, 98% purity) was purchased from Acros Organics (Geel, Belgium). Human serum albumin (HSA) and bovine serum albumin (BSA) were obtained from Calbiochem (Billerica, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. Surrogate light and heavy peptides were obtained from New England Peptides (Boston, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. OAT1 antibody was procured from Abcam (Cambridge, MA), anti-mouse IgG, HRP-linked secondary antibody was purchased from (Cell signaling technologies) and SDS gel (Mini-PROTEAN TGX) was from BIO-RAD (Hercules, CA).

Procurement of normal kidney cortices from experimental animals

Normal kidney cortical tissue (approximately 100-150 mg) was collected consistently at autopsy from the same kidney region from the preclinical species, i.e., Cynomolgus monkey (n = 11; 5 male and 6 female), Beagle dog (n = 12; 6 male and 6 female), Wistar Han rat (n = 20; 10 male and 10 female), and CD-1 mouse (n = 18; 8 male and 10 female). The autopsies were conducted at the Pfizer Worldwide R&D facility (Cambridge, MA), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. After collection, kidney tissue was flash frozen and stored at -80 ^{o}C before shipping to the University of Washington. The study was conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and in compliance with the Animal Welfare Act and its implementing regulations, under Institutional Animal Care and Use Committee approved protocols.

Membrane protein isolation from kidney tissue

Total membrane protein was extracted from the kidney cortex (~30-60 mg) of preclinical species using a previously described protocol (Xu et al., 2018). In brief, 30-60 mg of kidney cortical tissue were homogenized using hand-held rotary homogenizer with plastic probes. The homogenate was centrifuged at 16000 x g for 30 min at 4°C. The supernatant (cytosolic fraction) was transferred to a new tube and the remaining pellet (membrane fraction) was resuspended with 600 µL of solubilization buffer (Pierce Biotechnology, Rockford, IL) and incubated at 4°C for 30 min with continuous mixing. Membrane fraction was used for the transporters quantification.

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Peptide selection and LC-MS/MS protein quantification of kidney drug transporters

We applied an optimized LC-MS/MS methodology, which relies on selective quantification of surrogate peptides of drug transporters. Whenever applicable, conserved peptides across species were selected for precise comparison. When conserved peptides were not available, a novel matrix approach (supplemental Figure 1) was used.

Peptide selection for individual drug transporters (Supplemental Table 1) in kidney across species was performed using a previously discussed in silico approach (Bhatt and Prasad, 2018). Total protein in kidney samples was quantified using a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL) following the vendor protocol. Samples were digested as described previously (Bhatt et al., 2018). Briefly, 80 µL of the membrane sample (2 mg/mL total protein) was mixed with 30 µL ABC (100 mM), 10 µL of HSA (10 mg/mL) and 20 µL of BSA (0.02 mg/ml) in 1.5 mL micro-centrifuge tube. Proteins were denatured and reduced with 10 µL of 250 mM DTT at 95°C for 10 min with gentle shaking at 300 rpm. Sample was cooled at room temperature for 10 min, and the denatured protein was alkylated with 20 µL of 500 mM IAA; the reaction was carried out in the dark for 30 min. Ice-cold methanol-chloroform (600 µL), 5:1 v/v) and water (400 µL) were subsequently added to each sample. After vortex-mixing and centrifugation at 16,000 × g (4°C) for 5 min, the upper aqueous and lower organic layers were carefully removed without disturbing the protein pellet, using vacuum suction. The protein pellet was dried at room temperature for 10 min and then washed with 500 µL ice-cold methanol and followed centrifugation at 8000 × g (4°C) for 5 min. Supernatant was removed, pellet was dried at room temperature for 30 min and re-suspended in 60 µL ABC buffer (50 mM, pH 7.8). Finally, reconstituted protein sample was digested by adding 20 µL of trypsin (protein: trypsin ratio, approximately 50:1) and incubated at 37°C for 16 hours. The reaction was quenched by the addition of 20 µL of peptide internal standard cocktail (prepared in 80% acetonitrile in water containing 0.5% formic acid) and 10 µL 80% acetonitrile in water containing 0.5% formic acid. The sample was mixed by vortex mixing, and centrifuged at 4000 ×g for 5 min. The supernatant was collected in a LC-MS vial for analysis. LC-MS/MS data acquisition was performed on Waters Acquity UPLC system coupled with a SCIEX API-6500 triple quadrupole mass spectrometer using optimized parameters outlined in Supplemental Tables 2 and 3 as per validated method descried previously (Prasad et al., 2016). LC-MS/MS data analysis is discussed in the supplementary file. Previously published human transporter levels were used as reference data (Prasad et al., 2016), whereas an archived pooled (n=21) human kidney tissue sample from our previous study was analyzed along with the animal samples as a quality control.

To control for cross-species variability in the membrane isolation recovery, we have utilized total membrane protein per gram of kidney (TM-PPGK), which served as a scaling factor to express data in pmol/gram unit. This scaling was particularly important as TM-PPGK value for human (Supplemental Figure 2) was significantly lower among species.

To validate results of LC-MS/MS, Western blotting analysis of a representative protein, OAT1 was performed using established protocol (Neradugomma et al., 2017) (Supplementary file).

RESULTS

Cross-species kidney cortical transporter abundances

We quantified and investigated nineteen basolateral and apical membrane transporters in normal kidneys cortex obtained from four commonly used preclinical species in toxicity studies and drug safety evaluation and development, i.e., nonhuman primates (Cynomolgus monkeys), Beagle dogs, Wistar Han rats, and CD1 mice. These data were compared with our previously generated human kidney data (Prasad et al., 2016). We report absolute data for 12 transporters (pmol/gram of tissue) and relative abundance (normalized to total protein) data for seven transporters (Figure 2 and Supplemental Table 4). In general, the abundance of all the 19 membrane transporters were higher in preclinical species as compared to human except for MDR1, OCT3 and OCTN1 (Figure 2B). Human MDR1 abundance is similar to monkey, dog and rat, but it was higher (2.5 fold; p value <0.05) than mouse MDR1. OCT3 is only detectable in human and OCTN1 was similar in human and monkey. In nonhuman primate, the total abundance of 12 transporters for which absolute data were available, was 2.1-fold higher ($p = 0.025$) relative to human but the % distribution of these transporters was identical in both the species (Supplemental Figure 3). The abundances of OAT1, OCTN2, SGLT2, MRP1, Na^+K^+ATPase, and MRP3 were highest in rat and lowest in human with fold-range of 2.9 to 7.4 (p value <0.05). MRP4 was highest in nonhuman primate and lowest in mouse. The abundance of OAT2 was ~ 5-fold (p value <0.0001) higher in mouse as compared to human and nonhuman primate and it was undetectable in rat. MATE1, MRP2, OAT3, OAT4 and OATP4C1 were 1.5-2.3-fold (p value <0.05) higher in nonhuman primate than human. OCT1 and BCRP were only detected in rodents. SGLT2 and Na^+K^+ATPase were 2- to 6- fold higher in all four preclinical species than human (p value <0.05). A significant correlation was observed between transporters (Figure 2C).

Sex differences in kidney cortical transporter abundances

Transporter abundance in human and nonhuman primate kidney did not show sex-dependent pattern. Significant sex differences in transporter abundance were observed in mouse followed by rat > dog (Table 1). OAT1 was higher by 3.2 and 1.3-fold (p value <0.05) in male mouse and rat, respectively. OAT2 was 1.6-fold higher in male mouse than the female. OCT2 was higher in male rat and mouse. MDR1 was 2- and 1.4-fold higher (p value <0.05) in female in mouse and dog, respectively. Whereas MDR1 abundance was higher in male rat by 1.6-fold. MRP4 is predominantly expressed in male mouse (2.4-fold, p value <0.05) kidney. OCTN2 abundance was 1.3 and 1.4 fold higher in female mouse and male rats, respectively. SGLT2 was only 1.2 fold higher (p value <0.05) in female rats.

Comparison of LC-MS/MS proteomics and Western blot data of a representative transporter, OAT1

Antibodies are not available for all the studied transporters and when available, these do not work for all the species. Nevertheless, we performed OAT1 quantification by Western blotting using an anti-mouse OAT1 antibody to validate the LC-MS/MS data (method discussed in supplementary information). The sex-difference in OAT1 abundance in mouse is qualitatively confirmed by the Western blot data (Supplemental Figure 4). The higher signal of OAT1 in rat sample was consistent with the proteomics data; however, the antibody did not work for human OAT1 (data not shown).

DISCUSSION

Here we report comparison of nineteen clinically relevant kidney drug transporters between human and four common preclinical animal models used in drug development. The presented data are the total membrane expression because it is technically difficult to reproducibly extract purified plasma membrane from frozen tissues (Kumar et al., 2015). While the plasma membrane isolation involves multiple steps, we have previously demonstrated that the total membrane extraction from different parts of kidney is highly reproducible using the total membrane extraction kit (Prasad et al., 2016).

The quantitative information on cross species transporter abundance is useful in interpreting kidney safety data in humans. Particularly, drug-induced modest (20-30%) rises in sCr in preclinical models could be due to direct tubular damage (i.e., drug-induced kidney injury [DIKI]) or indirect inhibition of kidney transporters. For example, an investigational Janus Kinase inhibitor, INCB039110, resulted in rises in sCr in a healthy volunteer's study (Zhang et al., 2015). A follow-up dedicated kidney investigative clinical study revealed no changes in FDA/EMA qualified nonclinical kidney toxicity biomarkers (e.g., Cystatin C) or glomerular filtration rate (GFR), but rather the rises in sCr were attributed to interference with kidney uptake transporters such as OCT2, OCT3 and OAT2 (Zhang et al., 2015) and efflux transporters such as MATE1 and MATE2K (Lepist et al., 2014; Zhang et al., 2015). Thus, understanding which transporters are potentially involved in uptake or efflux of the therapeutic candidate and knowing the relative abundance of the transporter across nonclinical species and humans will allow better interpretation of clinical data.

This dataset for species and sex specific abundances for kidney transporters is also useful to the biomedical community and drug developers in predicting the drug clearance and safety of new molecule entities. In agreement with our data, OCT1 and BCRP are not detected in human and nonhuman primate but were abundant in rodents (Bleasby et al., 2006; Prasad et al., 2016). The relatively low abundance of MDR1 in mouse kidney as compared to human and rat is consistent with the mRNA data (Bleasby et al., 2006). Similarly, both nonhuman primate and rat have been shown to be associated with kidney toxicity with repeated dosing of tenofovir but such toxicity was not observed in the mouse at a similar dose (Ng et al., 2015; Ustianowski and Arends, 2015).

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High correlation between OAT1 versus MRP2 and MRP4 indicates that the latter are important for the apical efflux of organic anions. Similarly, high correlation of cation transporters, OCT2 and MATE1 in human and nonhuman primate perhaps suggests that these transporters are co-regulated. We also observed that OAT2 (a sCr secreting transporter) is a highly abundant anion transporter in mouse, whereas, OAT1 is predominantly expressed in other species. Therefore, results of individual OAT knockout mouse versus rat are expected to be significantly different.

Differential sex abundance in mouse and rats (Table 1) was supported by negative correlations between transporters (Figure 3). Particularly, in mouse, the basolateral uptake transporters such as OATs and OCT2 were higher in male but the apical transporters (primarily efflux) were higher in females. In rats, sex-dependent regulation of the basolateral uptake transporters was consistent with mouse, however, OCTN2, BCRP and P-gp were also higher in males. Although, mechanisms of sex-dependent expression of these proteins is unknown, these transporters are important in secretion of conjugated sex-hormones (Bush et al., 2017) and therefore can be regulated by the latter. Consistent with our data, abundance or activity of some OATs and OCTs have been shown to be sex-dependent (Cerrutti et al., 2002; Groves et al., 2006; Breljak et al., 2010; da Silva Faria et al., 2015).

Good correlation of OAT1 and OAT3 abundances is supported by the fact that these are transcriptionally co-regulated (Prasad et al., 2016). Because most of the co-regulated proteins work in tandem (OCT vs. MATE and OAT vs. MRPs) in vectorial transport process, a substrate mediated regulation could be tested as potential mechanisms of correlations.

Regarding limitations of this study, it does not allow comparison of all transporters because of the lack of MS-quantifiable or conserved peptides across species (e.g., dog). Nevertheless, our novel

LC-MS/MS proteomics method allowed quantification of a majority of kidney transporters across species, which is not currently feasible using conventional immunoquantification. We also demonstrated that the quality of LC-MS/MS proteomics data is superior to Western blot data using OAT1 as an example (Supplementary Figure 4). In addition, the protein abundance data (in the absence of in vitro functional data) are limited for the prediction of species differences in drug induced kidney injury or PK. However, the transporter abundance data are critical piece of information (i.e., a prerequisite), which constitute key physiological parameters for PBPK modeling (Harwood et al., 2013). Particularly, these data can be integrated with transport kinetics data (e.g., Km, Equation 1) of a drug or new chemical entity to predict in vivo transporter activity in animal models versus human (Equation 2). Further, sex-dependent quantitative differences in the transporter abundance can be directly integrated into a PBPK model to extrapolate drug PK or drug toxicity (Equations 1 and 3); where K_m can be assumed similar between male versus female in a single species.

$$CL = \frac{Vmax}{Km+[S]} = \frac{[Abundance\ x\ Kcat]}{Km+[S]} \qquad \text{(Equation 1)}$$

Thus,

$$CL_{human}\ \alpha\ CL_{animal}\ x\ \frac{[Abundance_{human}]}{[Abundance_{animal}]} \qquad \text{(Equation 2)}$$

$$CL_{female} = CL_{male}\ x\ \frac{[Abundance_{human}]}{[Abundance_{animal}]} \qquad \text{(Equation 3)}$$

Where, CL is intrinsic clearance (pmol/min/mg protein or pmol/min/gm of kidney), V_{max} is the maximum transport activity, K_m is substrate affinity to a transporter, K_{cat} is the turnover number and [S] is the substrate concentration. V_{max} depends on the transporter abundance, whereas K_m and K_{cat} are independent of protein levels (Bhatt and Prasad, 2018). It is also noteworthy that the data obtained from the absolute peptide approach should not be considered as absolute molar protein abundance data because complete trypsin digestion may not be confirmed. However, these data (absolute peptide levels) can be used in allometric scaling of animal to human transporter mediated clearances using Equations 1-3. Because the scaling factor is derived by dividing the peptide abundance values, it does not matter whether absolute protein or absolute peptide values were used in the scaling as long as the trypsin digestion and the sample extraction are reproducible and consistent across species/genders. We have previously described this scaling approach (Bhatt and Prasad, 2018).

In summary, our data on cross-species kidney transporter abundances, particularly in human, monkey and rodents, provide useful quantitative information, which can be leveraged by: 1) allowing for preclinical to clinical translation of transporter mediated secretary clearance using PBPK modeling, 2) distinguishing direct drug-induced kidney toxicity from indirect impact on kidney transporters (e.g., transporter mediated creatinine clearance), and 3) utilizing cross-species transporter levels in conjunction with kidney injury biomarkers to better understand kidney safety signals and human safety risk assessment.

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Author contribution

Participated in research design: A.B., Z.R., V.S.V., and B.P.

Conducted experiments: A.B. and M.K.

Performed data analysis: A.B. and B.P.

Wrote or contributed to the writing of the manuscript: A.B., Z.R., V.S.V., and B.P.

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Footnotes

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Figure legends

Figure 1: Localization of drug transporters in proximal tubule. Kidney proximal tubule contains OAT1-3, OATP4C1, OCT1-3, and MRP1/3 at the basolateral membrane and MATE1, MATE2k, MDR1, MRP2/4, SGLT2, OAT4, and OCTN1/2 at the apical membrane. Both uptake and efflux drug transporters are involved in nephrotoxicity of certain drugs shown in the figure.

Figure 2: **A.** Kidney cortical transporters cross-species comparison of protein abundance (pmol/gram tissue) expression. Each dot represents the individual sample and data are represented as mean ± SD. MATE-2K was not detectable in any species because of poor sensitivity of the surrogate peptide. Only six transporters were detected in dog kidney because of the lack of conserved peptides. **B.** Heat map of relative quantitative abundance of kidney transporters across species. Value in the cell represents the relative abundance of transporters across different species as compared to transporter abundance in human kidney. Yellow to red color indicates increasing abundance of a particular transporter across species.

Figure 3: **A.** Correlation plot for kidney transporter proteins across all five species. Values in the cell represents the coefficient of correlation (r^2) between two transporter proteins. **B.** Positive correlation between the transporter proteins with $r^2>0.70$. Number of samples from individual species used in this study (with few exceptions) was: human (n=34), monkey (n=11), dog (n=12), rat (n=20), and mouse (n=18). NC (not conserved) indicates that a conserved peptide was not found for that particular species. BLQ means below limit of quantification but the peptide was conserved. BLQ values were derived by extrapolation of signal-to-noise ratio to 3. NS indicates that no significant correlation ($r^2 < 0.70$) was found between transporter proteins.

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Table 1: Sex differences in protein abundance of transporters across species.

Proteins	Human	Monkey	Dog	Rat	Mouse
SLC22A6 / Organic anion transporter 1 (OAT1)	↔	↔	↔	M > F *1.3	M > F *3.2
SLC22A7 / Organic anion transporter 2 (OAT2)	↔	↔	NC	BLQ	M > F *1.6
SLC22A8 / Organic anion transporter 3 (OAT3)	↔	↔	NC	NC	NC
SLC22A11 / Organic anion transporter 4 (OAT4)	↔	↔	NC	NC	NC
SLCO4C1 / Organic anion transporter polypeptide 4C1 (OATP4C1)	ND	↔	NC	NC	NC
SLC22A1 / Organic cation transporter 1 (OCT1)	BLQ	BLQ	NC	↔	↔
SLC22A2 / Organic cation transporter 2 (OCT2)	↔	↔	NC	M > F *1.4	M > F *1.6
SLC22A4 / Organic cation/carnitine transporter 1 (OCTN1)	↔	↔	NC	NC	NC
SLC22A5 / Organic cation/carnitine transporter 2 (OCTN2)	↔	↔	NC	M > F *1.4	F > M *1.3
ABCB1 / Multidrug resistance protein 1 (MDR1)	↔	↔	F > M *1.4	M > F *1.6	F > M *2.0
SLC47A1 / Multidrug and toxin extrusion protein 1 (MATE1)	↔	↔	NC	NC	NC
ABCG2 / Breast cancer resistance protein (BCRP)	BLQ	BLQ	NC	M > F *1.6	↔
ABCC1 / Multidrug resistance-associated protein 1 (MRP1)	ND	↔	↔	↔	F > M *1.3
ABCC2 / Multidrug resistance-associated protein 2 (MRP2)	↔	↔	NC	NC	NC
ABCC3 / Multidrug resistance-associated protein 3 (MRP3)	ND	↔	NC	F > M *1.1	F > M *3.1
ABCC4 / Multidrug resistance-associated protein 4 (MRP4)	↔	↔	NC	↔	F > M *2.4
SLC5A2 / Sodium/glucose cotransporter 2 (SGLT2)	↔	↔	↔	F > M *1.2	↔
ATP1A1 / Sodium/potassium-transporting ATPase (Na+K+ATPase)	ND	↔	F > M *1.3	↔	↔

SLC = Solute Carrier; SLCO = solute carrier organic anion transporter; ABCB = ATP-binding cassette sub-family B; ABCG = ATP-binding cassette super-family G; ABCC = ATP-binding cassette sub-family C; ↔=No significant difference, **NC**=Peptide not conserved, *****=Fold difference, BLQ= Below limit of quantification, ND = sex difference was not determined, data was obtained with pooled QC

Figure 1

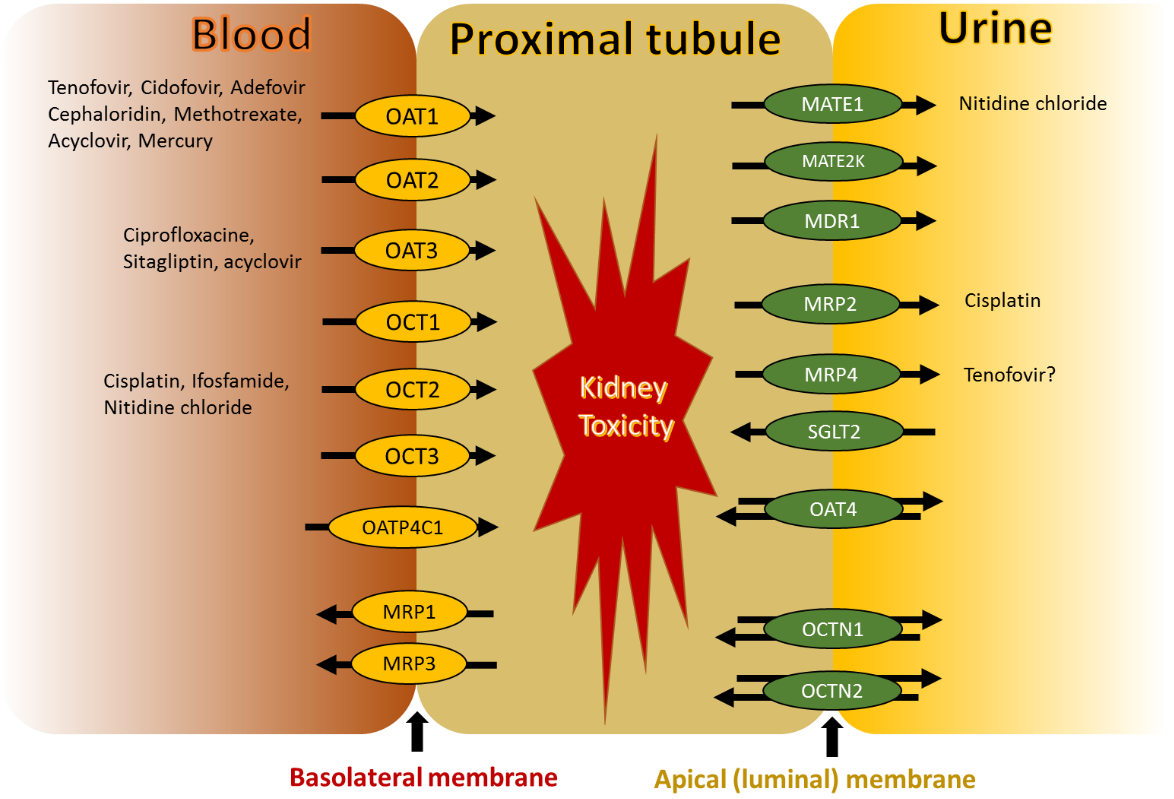
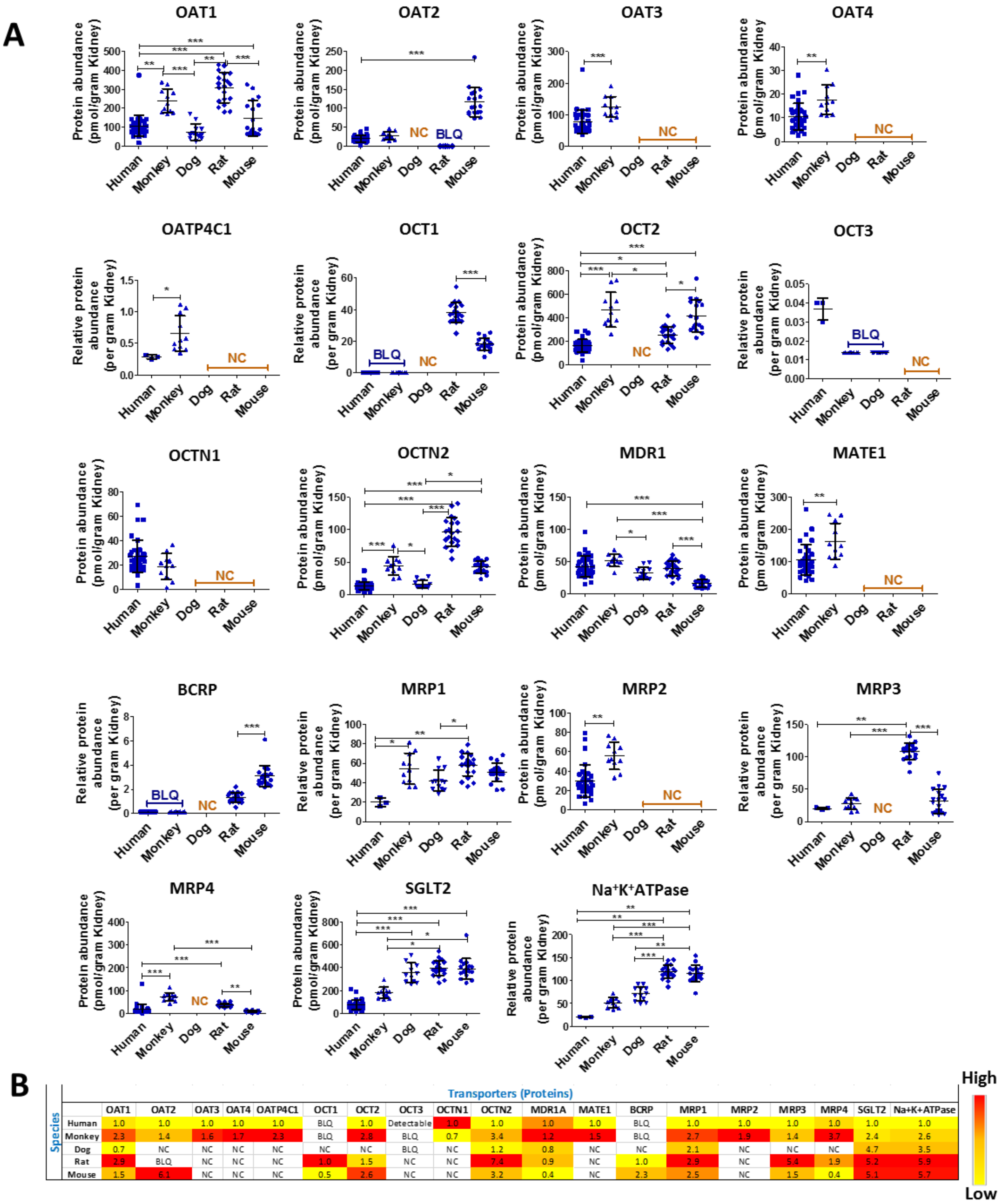

Blood
Proximal tubule
Urine
Tenofovir, Cidofovir, Adefovir
Cephaloridin, Methotrexate,
Acyclovir, Mercury
OAT1
OAT2
Ciprofloxacine,
Sitagliptin, acyclovir
OAT3
OCT1
Cisplatin, Ifosfamide,
Nitidine chloride
OCT2
OCT3
OATP4C1
MRP1
MRP3
Kidney
Toxicity
MATE1
Nitidine chloride
MATE2K
MDR1
MRP2
Cisplatin
MRP4
Tenofovir?
SGLT2
OAT4
OCTN1
OCTN2
Basolateral membrane
Apical (luminal) membrane

Figure 2



Species	OAT1	OAT2	OAT3	OAT4	OATP4C1	OCT1	OCT2	OCT3	OCTN1	OCTN2	MDR1A	MATE1	BCRP	MRP1	MRP2	MRP3	MRP4	SGLT2	Na+K+ATPase
Human	1.0	1.0	1.0	1.0	1.0	BLQ	1.0	Detectable	1.0	1.0	1.0	1.0	BLQ	1.0	1.0	1.0	1.0	1.0	1.0
Monkey	2.3	1.4	1.6	1.7	2.3	BLQ	2.8	BLQ	0.7	3.4	1.2	1.5	BLQ	2.7	1.9	1.4	3.7	2.4	2.6
Dog	0.7	NC	NC	NC	NC	NC	NC	BLQ	NC	1.2	0.8	NC	NC	2.1	NC	NC	NC	4.7	3.5
Rat	2.9	BLQ	NC	NC	NC	1.0	1.5	NC	NC	7.4	0.9	NC	1.0	2.9	NC	5.4	1.9	5.2	5.9
Mouse	1.5	6.1	NC	NC	NC	0.5	2.6	NC	NC	3.2	0.4	NC	2.3	2.5	NC	1.5	0.4	5.1	5.7

Transporters (Proteins)

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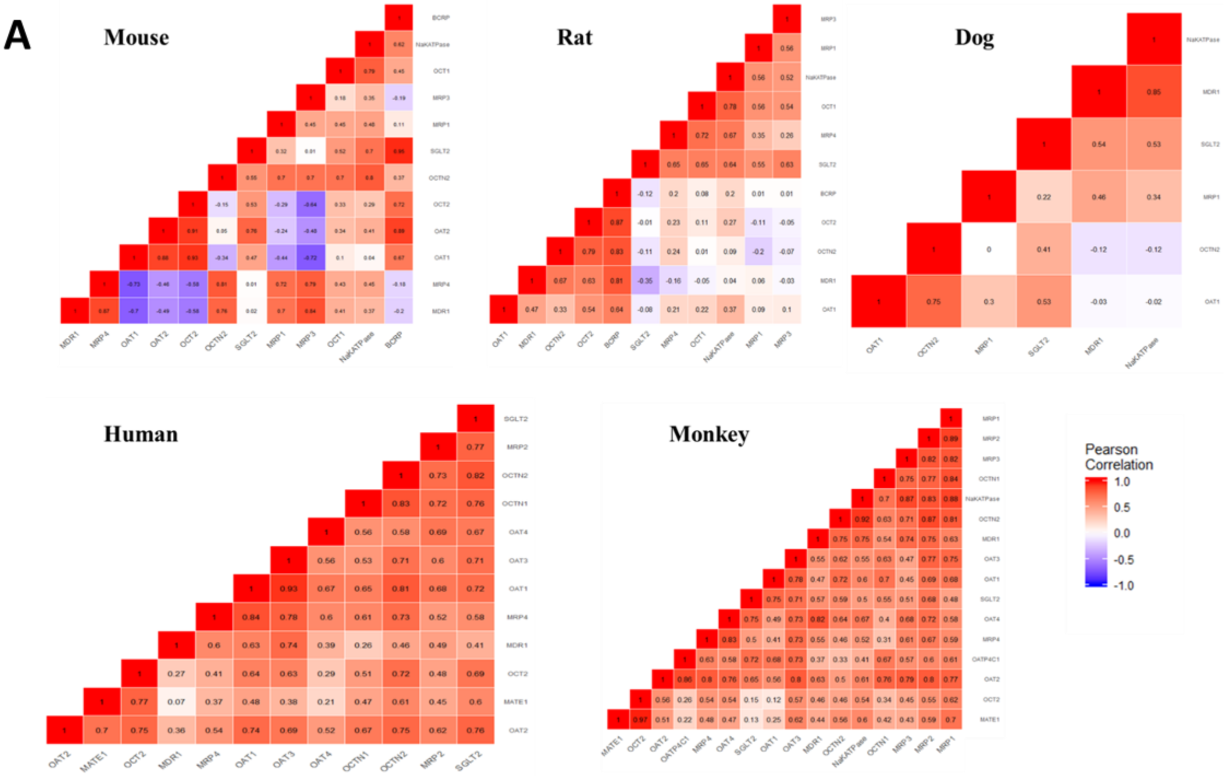
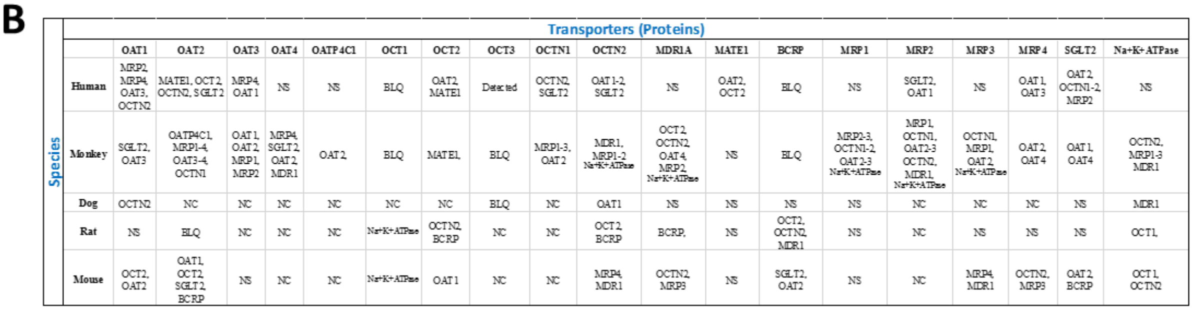
Figure 3:





Species		Transporters (Proteins)																		
		OAT1	OAT2	OAT3	OAT4	OATP4C1	OCT1	OCT2	OCT3	OCTN1	OCTN2	MDR1A	MATE1	BCRP	MRP1	MRP2	MRP3	MRP4	SGLT2	Na+K+ATPase
	Human	MRP2, MRP4, OAT3, OCTN2	MATE1, OCT2, OCTN2, SGLT2	MRP4, OAT1	NS	NS	BLQ	OAT2, MATE1	Detected	OCTN2, SGLT2	OAT1-2, SGLT2	NS	OAT2, OCT2	BLQ	NS	SGLT2, OAT1	NS	OAT1, OAT3	OAT2, OCTN1-2, MRP2	NS
	Monkey	SGLT2, OAT3	OATP4C1, MRP1-4, OAT3-4, OCTN1	OAT1, OAT2, MRP1, MRP2	MRP4, SGLT2, OAT2, MDR1	OAT2,	BLQ	MATE1,	BLQ	MRP1-3, OAT2	MDR1, MRP1-2 Na+K+ATPase	OCT2, OCTN2, OAT4, MRP2, Na+K+ATPase	NS	BLQ	MRP2-3, OCTN1-2, OAT2-3 Na+K+ATPase	MRP1, OCTN1, OAT2-3 OCTN2, MDR1, Na+K+ATPase	OCTN1, MRP1, OAT2, Na+K+ATPase	OAT2, OAT4	OAT1, OAT4	OCTN2, MRP1-3 MDR1
	Dog	OCTN2	NC	NC	NC	NC	NC	NC	BLQ	NC	OAT1	NS	NS	NS	NS	NC	NC	NC	NS	MDR1
	Rat	NS	BLQ	NC	NC	NC	Na+K+ATPase	OCTN2, BCRP	NC	NC	OCT2, BCRP	BCRP,	NS	OCT2, OCTN2, MDR1	NS	NC	NS	NS	NS	OCT1,
	Mouse	OCT2, OAT2	OAT1, OCT2, SGLT2, BCRP	NS	NC	NC	Na+K+ATPase	OAT1	NC	NC	MRP4, MDR1	OCTN2, MRP3	NS	SGLT2, OAT2	NS	NC	MRP4, MDR1	OCTN2, MRP3	OAT2, BCRP	OCT1, OCTN2

SUPPLEMENTARY INFORMATION

Kidney cortical transporter expression across species using quantitative proteomics

Abdul Basit[1], Zaher Radi[2], Vishal Vaidhya[2], Mathew Karasu[1], Bhagwat Prasad[1]

[1] Department of Pharmaceutics, University of Washington, Seattle, P.O. Box 357610, WA 98195 USA

[2] Pfizer Worldwide Research Development, Drug Safety Research Development, One Portland Street, Cambridge, MA 02139 USA

METHODS

LC-MS/MS Data Analysis

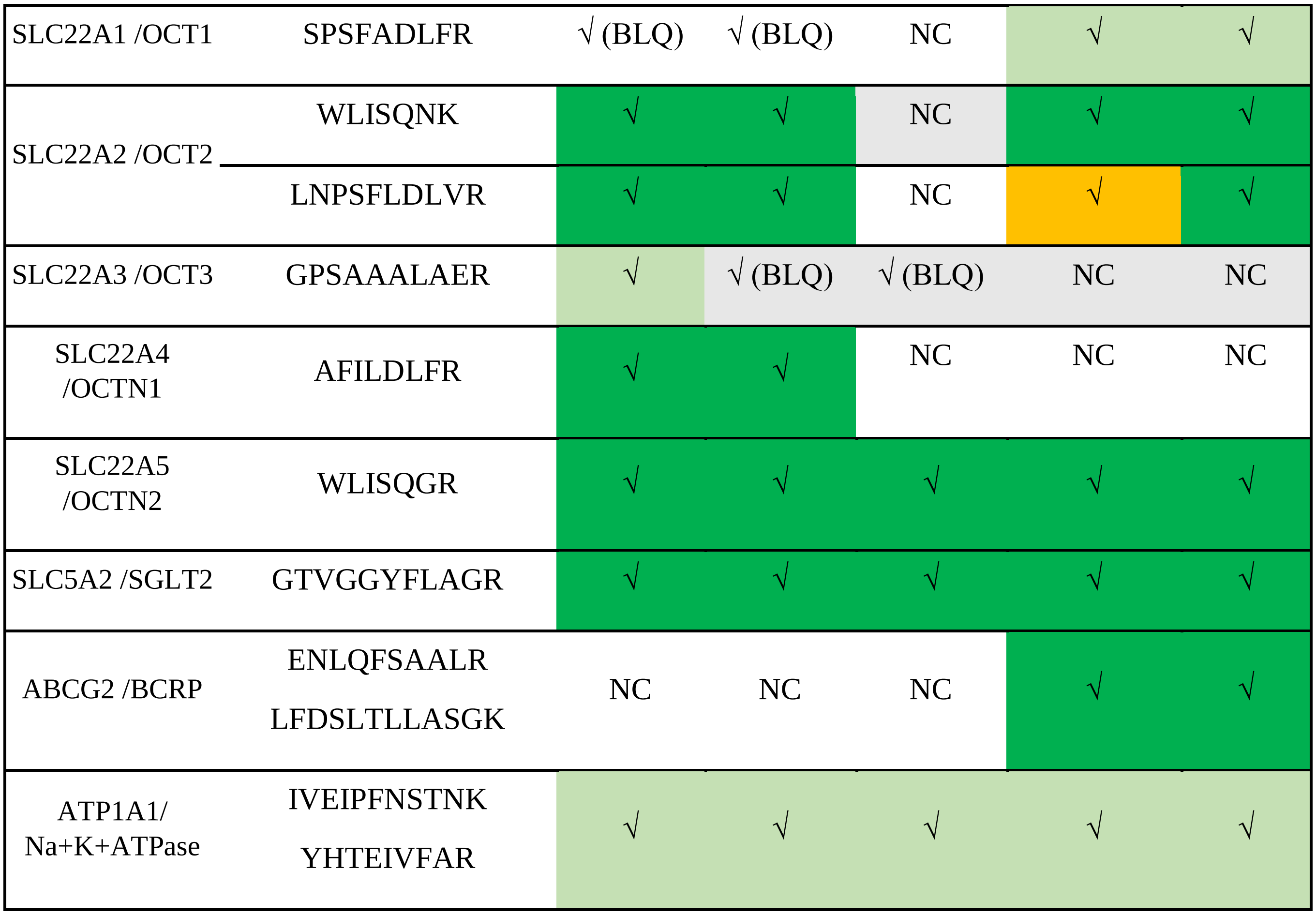
The LC-MS/MS data were analyzed using Skyline 4.1, where the peptide peaks were identified by matching the retention time with heavy internal standards and alignment of selected precursor ion to daughter ion fragments. A previously optimized robust data analysis approach[S4] which considers internal standard protein (BSA), heavy internal standard peptide, and pooled quality control samples was used. In addition, matrix approach was used to estimate transporter protein abundance if a surrogate peptide was not conserved in a particular species (Supplementary Figure 3). The peptide data were initially analyzed as pmol/mg total membrane protein and when more than one peptides were used, the abundance data were presented as the average of peptides. Total membrane protein (mg) per gram kidney tissue (TM-PPGK), was calculated and used for scaling these data to pmol/gram of tissue. The TM-PPGK values for the human ranges from 15.76 to 34.00 mg/g with mean value of 20.1, whereas it was 28.3 ± 5.1, 30.7 ± 7.7, 34.5 ± 4.7, and 28.6 ± 2.0 mg/g for monkey, dog, rat and mouse, respectively (Supplementary Figure 4). Human transporter abundance data were generated previously, which were confirmed using pooled human quality control samples (n=21 previously studied human kidney tissues) in this study. Absolute or relative protein abundance (pmol/g) across species (human, monkey, dog, rat and mouse) was then compared by the Kruskal-Wallis followed by Dunn`s multiple comparison test and Mann-Whitney test. The protein abundance data are presented as mean±SD.

Western Blotting Procedure for OAT1 Quantification

To validate performance of LC-MS/MS data of a representative transporter, OAT1, between male and female mice and across species, Western blotting method, where gel electrophoresis of membrane fractions (2 mg/mL) of mouse, human and rat kidney cortex was performed using 4-15% SDS gel (Mini-PROTEAN TGX, BIO-RAD). Standard Western blotting protocol was followed (Neradugomma et al., 2017). Briefly, 60 µg of protein from each sample was loaded onto the gel electrophoresis. After protein separation on the gel, proteins were transferred onto immune P-nitrocellulose membrane in transfer buffer (25 mM Tris base, 192 mM Glycin in 20% methanol). The blot was then blocked with 5% dry milk in PBS-T buffer (PBS with 0.1% tween 20) incubating for 1 hr at room temperature (RT). The blot was washed three times with PBS-T and incubated with rabbit polyclonal anti-SLC22A antibody (ab135924, Abcam, Cambridge, MA) at 1:1000 dilution as primary antibody for 1 hr at RT. The blot was washed three times with PBS-T buffer and incubated with anti-mouse IgG, HRP-linked secondary antibody (Cell signaling technologies) at 1:10000 dilution for 1 hr at RT. After washing three times with PBS-T buffer, the blot was developed using a ECL Western Development reagent (GE #RPN2132).

Supplemental Table 1: List of surrogate peptides for renal transporters across species. Transporter peptide detected and quantified (green); detected but not quantified (light green); conserved (√); quantified using matrix approach (orange); below limit of quantification (BLQ); and non-conserved peptide (NC).

	Peptide	Human	Monkey	Dog	Rat	Mouse
ABCB1 /MDR1	AGAVAEEVLAAIR LANDAAQVK	√	√	√	√	√
SLC47A1 /MATE1	GGPEATLEVR	√	√	NC	NC	NC
ABCC1 /MRP1	ITIIPQDPVLFSGSLR	√	√	√	√	√
ABCC2 /MRP2	QLLNNILR YFAWEPSFR	√	√	NC	NC	NC
ABCC3 /MRP3	SQLTIIPQDPILFSGTLR	√	√	NC	√	√
	LLEAWQK	🟧	🟧	NC	√	√
ABCC4 /MRP4	SSLISALFR	√	√	NC	√	√
SLC22A6 /OAT1	LVGFLVINSLGR	√	√	√	🟧	🟧
	GQASAMELLR	√	√	🟧	√	√
SLC22A7 /OAT2	ACQQAQVHANLK	√	√	NC	√ (BLQ)	√
	NVALLALPR	√	√	NC	NC	🟧
SLC22A8 /OAT3	TVLAVFGK	√	√	NC	NC	NC
SLC22A11 /OAT4	GKPDQALQELR	√	√	NC	NC	NC



SLC22A1 /OCT1	SPSFADLFR	√ (BLQ)	√ (BLQ)	NC	√	√
SLC22A2 /OCT2	WLISQNK	√	√	NC	√	√
	LNPSFLDLVR	√	√	NC	√	√
SLC22A3 /OCT3	GPSAAALAER	√	√ (BLQ)	√ (BLQ)	NC	NC
SLC22A4 /OCTN1	AFILDLFR	√	√	NC	NC	NC
SLC22A5 /OCTN2	WLISQGR	√	√	√	√	√
SLC5A2 /SGLT2	GTVGGYFLAGR	√	√	√	√	√
ABCG2 /BCRP	ENLQFSAALR LFDSLTLLASGK	NC	NC	NC	√	√
ATP1A1/ Na+K+ATPase	IVEIPFNSTNK YHTEIVFAR	√	√	√	√	√

Supplemental Table 2: Chromatographic conditions for separation of surrogate peptides of kidney transporters.

LC gradient program			
ACQUITY UPLC® HSS T3 C18 column (2.1 × 100 mm, 1.8 µm)			
Time (min)	Flow Rate	A (Water with 0.1% formic acid, %)	B (Acetonitrile with 0.1% formic acid, %)
0	0.3	97.0	3.0
4	0.3	97.0	3.0
8	0.3	87.0	13.0
18	0.3	70.0	30.0
20.5	0.3	65.0	35.0
21.1	0.3	40.0	60.0
23.1	0.3	20.0	80.0
23.2	0.3	97.0	3.0
27	0.3	97.0	3.0

Supplemental Table 3: Multiple reaction monitoring (MRM) parameters for analysis of surrogate peptides of kidney transporters. K and R (shown in bold) were labeled as ^{13}C and ^{15}N.

Protein	Peptide	Peptide labeling	Parent Ion	Daughter Ion	DP	CE
ABCB1 /MDR1	AGAVAEEVLAAI**R**	Light	635.4	971.6	77.4	31.7
			635.4	900.5	77.4	31.7
		Heavy	640.4	981.6	77.4	31.7
			640.4	910.5	77.4	31.7
	LANDAAQV**K**	Light	465.3	745.4	65	25.6
			465.3	631.3	65	25.6
		Heavy	469.3	753.4	65	25.6
			469.3	639.4	65	25.6
SLC47A1 /MATE1	HVGVILQ**R**	Light	461.3	685.4	64.7	25.5
			461.3	529.3	64.7	25.5
			461.3	747.5	64.7	25.5
		Heavy	466.3	695.4	64.7	25.5
			466.3	539.4	64.7	25.5
			466.3	747.5	64.7	25.5
	GGPEATLEV**R**	Light	514.8	688.4	68.6	27.4
			514.8	617.4	68.6	27.4
		Heavy	519.8	698.4	68.6	27.4
			519.8	627.4	68.6	27.4
ABCC1 /MRP1	ITIIPQDPVLFSGSL**R**	Light	878.5	1315.7	95.2	40.5
			878.5	658.4	95.2	40.5
			878.5	441.3	95.2	40.5
		Heavy	883.5	1325.7	95.2	40.5
			883.5	663.4	95.2	40.5
			883.5	441.3	95.2	40.5
ABCC2 /MRP2	QLLNNIL**R**	Light	492.3	742.5	67	26.6
			492.3	629.4	67	26.6
		Heavy	497.3	752.5	67	26.6
			497.3	639.4	67	26.6
	YFAWEPSF**R**	Light	601.8	892.4	75	30.5
			601.8	506.3	75	30.5
		Heavy	606.8	902.4	75	30.5
			606.8	516.3	75	30.5
ABCC3 /MRP3	SQLTIIPQDPILFSGTL**R**	Light	1000.1	1343.7	104	44.9
			1000.1	1003.6	104	44.9
			1000.1	656.4	104	44.9
		Heavy	1005.1	1353.7	104	44.9

			1005.1	1013.6	104	44.9
			1005.1	656.4	104	44.9
	LLEAWQ**K**	Light	444.3	661.3	63.5	24.9
			444.3	532.3	63.5	24.9
			444.3	741.4	63.5	24.9
		Heavy	448.3	669.3	63.5	24.9
			448.3	540.3	63.5	24.9
			448.3	741.4	63.5	24.9
ABCC4 /MRP4	SSLISALF**R**	Light	497.3	706.4	67.4	26.8
			497.3	593.3	67.4	26.8
		Heavy	502.3	716.4	67.4	26.8
			502.3	603.3	67.4	26.8
SLC22A6 /OAT1	GQASAMELL**R**	Light	538.3	819.4	70.4	28.2
			538.3	732.4	70.4	28.2
			538.3	661.4	70.4	28.2
		Heavy	543.3	829.4	70.4	28.2
			543.3	742.4	70.4	28.2
			543.3	671.4	70.4	28.2
	LVGFLVINSLG**R**	Light	644.4	758.5	78.1	32.1
			644.4	659.4	78.1	32.1
		Heavy	649.4	768.5	78.1	32.1
			649.4	669.4	78.1	32.1
SLC22A7 /OAT2	AC[CAM]QQAQVHANL**K**	Light	456.6	568.8	64.4	22.4
			456.6	504.8	64.4	22.4
		Heavy	459.2	572.8	64.4	22.4
			459.2	508.8	64.4	22.4
	NVALLALP**R**	Light	483.8	753.5	66.4	26.3
			483.8	569.4	66.4	26.3
		Heavy	488.8	763.5	66.4	26.3
			488.8	579.4	66.4	26.3
SLC22A8 /OAT3	TVLAVFG**K**	Light	417.8	634.4	61.6	23.9
			417.8	521.3	61.6	23.9
		Heavy	421.8	642.4	61.6	23.9
			421.8	529.3	61.6	23.9
SLC22A11 /OAT4	GKPDQALQEL**R**	Light	418.9	658.4	61.7	20.4
			418.9	545.3	61.7	20.4
		Heavy	422.2	668.4	61.7	20.4
			422.2	555.3	61.7	20.4
SLC22A1 /OCT1	SPSFADLF**R**	Light	520.3	768.4	69	27.6
			520.3	621.3	69	27.6

			520.3	476.7	69	27.6
		Heavy	525.3	778.4	69	27.6
			525.3	631.3	69	27.6
			525.3	481.8	69	27.6
SLC22A2 /OCT2	WLISQN**K**	Light	444.8	702.4	63.5	24.9
			444.8	589.3	63.5	24.9
		Heavy	448.8	710.4	63.5	24.9
			448.8	597.3	63.5	24.9
	LNPSFLDLV**R**	Light	587.3	946.5	73.9	30
			587.3	473.8	73.9	30
		Heavy	592.3	956.5	73.9	30
			592.3	478.8	73.9	30
SLC22A3 /OCT3	GPSAAALAE**R**	Light	471.8	630.4	65.5	25.8
			471.8	559.3	65.5	25.8
		Heavy	476.8	640.4	65.5	25.8
			476.8	569.3	65.5	25.8
SLC22A4 /OCTN1	AFILDLF**R**	Light	497.8	663.4	67.4	26.8
			497.8	550.3	67.4	26.8
		Heavy	502.8	673.4	67.4	26.8
			502.8	560.3	67.4	26.8
SLC22A5 /OCTN2	LGSILSPYFVYLGAYD**R**	Light	967.5	1363.7	101.6	43.7
			967.5	571.3	101.6	43.7
		Heavy	972.5	1373.7	101.6	43.7
			972.5	571.3	101.6	43.7
	WLISQG**R**	Light	430.2	673.4	62.5	24.3
			430.2	560.3	62.5	24.3
		Heavy	435.2	683.4	62.5	24.3
			435.2	570.3	62.5	24.3
SLC5A2 /SGLT2	GTVGGYFLAG**R**	Light	549.3	840.4	71.2	28.6
			549.3	563.3	71.2	28.6
		Heavy	554.3	850.4	71.2	28.6
			554.3	573.3	71.2	28.6
ABCG2 / BCRP	ENLQFSAAL**R**	Light	574.8	792.4	73	29.6
			574.8	664.4	73	29.6
		Heavy	579.8	802.4	73	29.6
			579.8	674.4	73	29.6
	LFDSLTLLASG**K**	Light	632.9	1004.6	77.3	31.6
			632.9	689.4	77.3	31.6
		Heavy	636.9	1012.6	77.3	31.6
			636.9	697.4	77.3	31.6

SLCO4C1 / OATP4C1	HLPGTAEIQAG**K**	Light	407.9	716.4	60.9	19.8
			407.9	645.4	60.9	19.8
			407.9	577.3	60.9	19.8
		Heavy	410.6	724.4	60.9	19.8
			410.6	653.4	60.9	19.8
			410.6	577.3	60.9	19.8
ATP1A1 /Na+K+ATPase	IVEIPFNSTN**K**	Light	631.3	1049.5	77.1	31.6
			631.3	807.4	77.1	31.6
		Heavy	635.4	1057.5	77.1	31.6
			635.4	815.4	77.1	31.6
	YHTEIVFA**R**	Light	568.3	835.5	72.5	29.3
			568.3	734.4	72.5	29.3
			568.3	605.4	72.5	29.3
		Heavy	573.3	845.5	72.5	29.3
			573.3	744.4	72.5	29.3
			573.3	615.4	72.5	29.3

Supplemental Table 4: Abundance of kidney transporters across species.

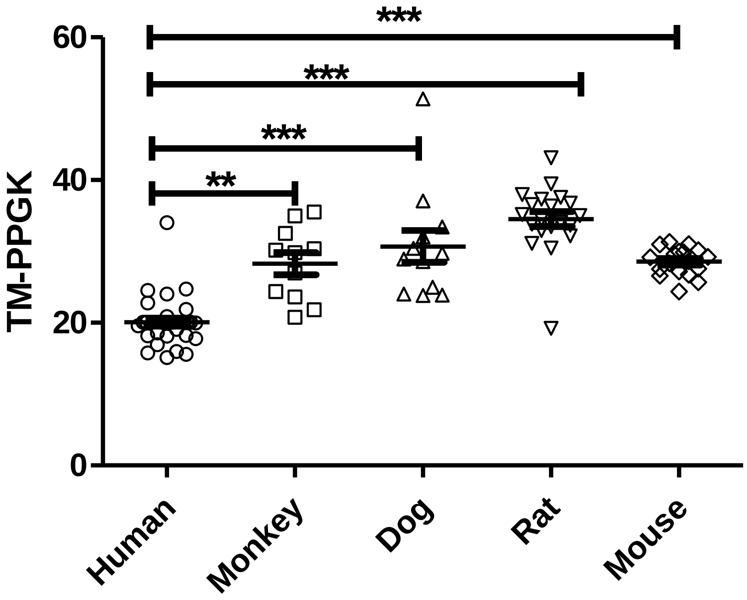
Transporters	Protein abundance (pmol/gram kidney)				
	Human	Monkey	DOG	Rat	Mouse
SLC22A6 /OAT1	107.7 ± 56.83	242.5 ± 62.69	75.4 ± 43.07	308.8 ± 79.24	156.2 ± 92.06
SLC22A7 /OAT2	19.8 ± 8.36	27.3 ± 10.2	NC	BLQ	120.3 ± 39.3
SLC22A8 /OAT3	78.5 ± 37.38	124.7 ± 32.57	NC	NC	NC
SLC22A11 /OAT4	10.6 ± 5.64	17.5 ± 6.28	NC	NC	NC
SLCO4C1 /OATP4C1*	0.3 ± 0.03	0.7 ± 0.28	NC	NC	NC
SLC22A1 /OCT1*	BLQ	BLQ	NC	38.3 ± 6.45	18 ± 3.96
SLC22A2 /OCT2	164.2 ± 53.27	464.8 ± 147.18	NC	253.5 ± 70.92	429.1 ± 134.67
SLC22A3 /OCT3*	0.03 ± 0.01	BLQ	BLQ	NC	NC
SLC22A4 /OCTN1	27.2 ± 13.44	19 ± 10.55	NC	NC	NC
SLC22A5 /OCTN2	13.1 ± 5.8	45 ± 14.35	16 ± 5.84	96.6 ± 22.51	41.4 ± 9.29
ABCB1 /MDR1	42.3 ± 16.16	52 ± 9.44	32.1 ± 9.34	39.3 ± 11.76	15.5 ± 5.99
SLC47A1 /MATE1	105.6 ± 47.52	161.2 ± 56.23	NC	NC	NC
ABCG2 /BCRP*	NC	NC	NC	1.3 ± 0.4	3.1 ± 0.88
ABCC1 /MRP1*	20.1 ± 4.54	54.7 ± 15.7	42.2 ± 10.95	58.3 ± 11.41	50 ± 9.68
ABCC2 /MRP2	30.1 ± 16.52	56 ± 13.99	NC	NC	NC
ABCC3 /MRP3*	20.1 ± 1.78	27.7 ± 9.01	NC	108.7 ± 12.66	29.4 ± 18.42
ABCC4 /MRP4	19.5 ± 20.58	71.3 ± 18.73	NC	37.5 ± 7.51	8.6 ± 3.94
SLC5A2 /SGLT2/	76.4 ± 41.25	182 ± 49.56	356.7 ± 84.85	395.1 ± 67.87	391.7 ± 89.62
ATP1A1 /Na+K+ATPase*	20.1 ± 1.17	51.7 ± 10.91	70.7 ± 15.19	119 ± 14.63	114.9 ± 17.87

*data is presented in relative protein abundance (arbitrary unit)/gram kidney. NC = not conserved peptide, BLQ = Below limit of quantitation

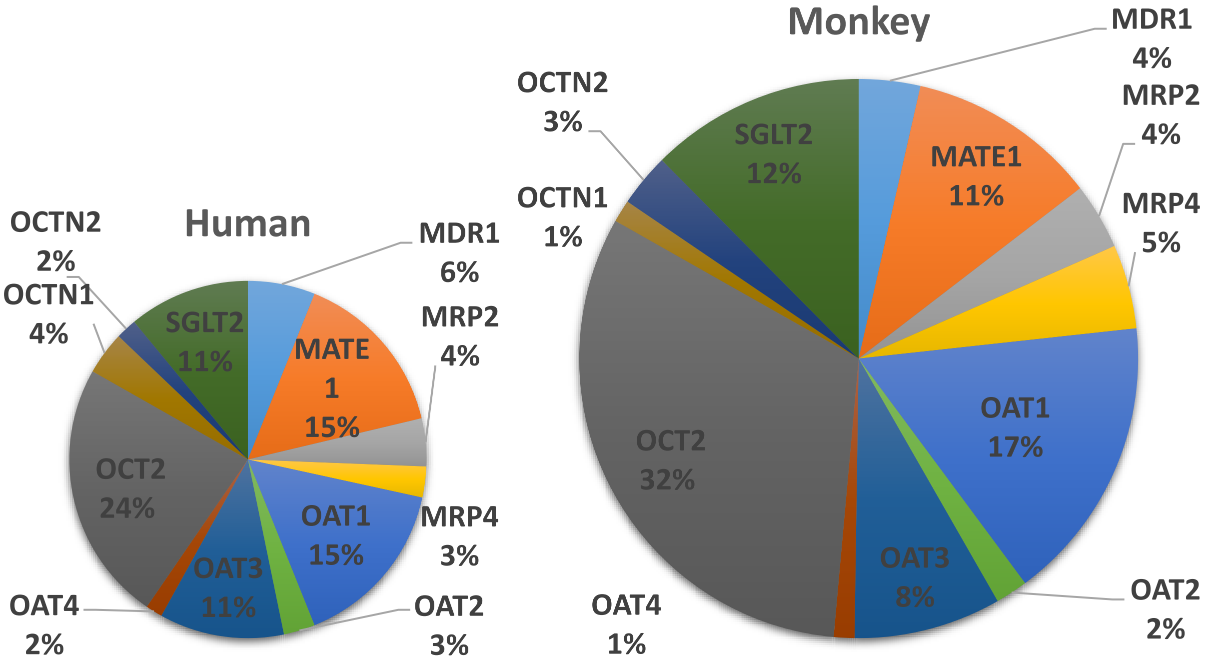
Matrix approach

	Species				
	S1	S2	S3	S4	S5
Peptides	P1	P1	P1		
			P2		P2
			P3	P3	

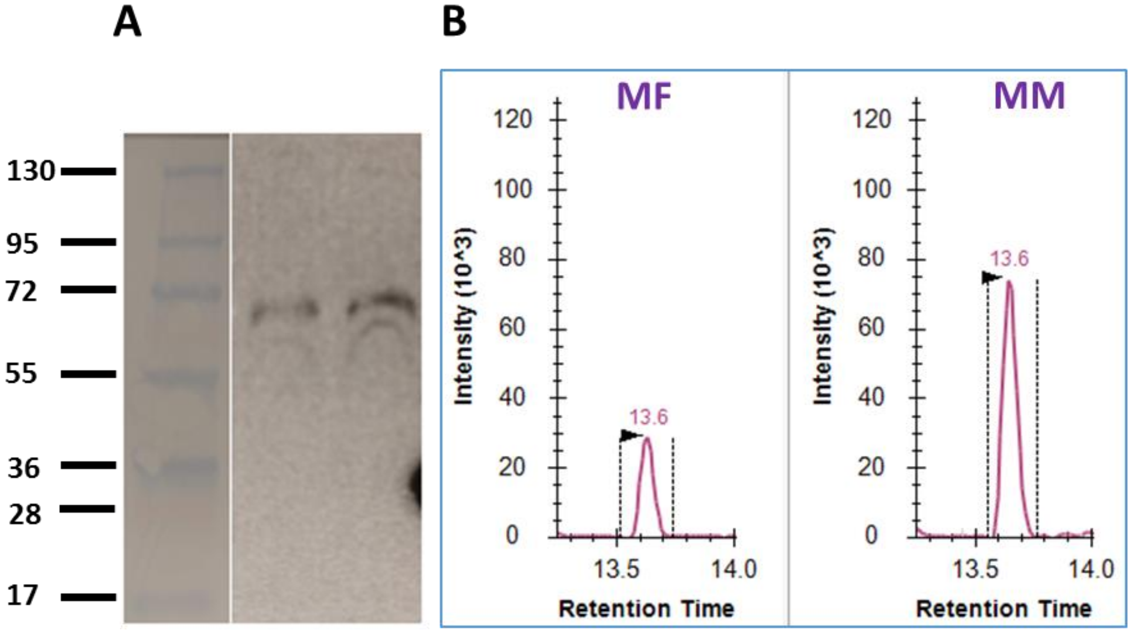
Supplemental Figure 1: A schematic representation of matrix approach for estimating the relative abundance of non-conserved peptides. In this example, S1-S5 are the species and P1 –P3 are the peptides. No peptide is conserved across all five species. P1 is conserved in three species (S1 – S3) whereas P2 and P3 are only conserved in two species. The relative abundance (RA) of P1 is estimated using matrix approach by following formula ($RA_{P1S4} = RA_{P3S4} * RA_{P1S3} / RA_{P3S3}$) Where, RA_{P1S4}, is the estimated relative abundance of P1 in S4, P3. RA_{P3S4}, RA_{P1S3}, and RA_{P3S3} are the measured abundances of P3 in S4, P1 in S3, and P3 in S3, respectively.



Supplemental Figure 2: Milligram of total membrane protein per gram of kidney (TM-PPGK) across species. Dots indicate individual samples and horizontal line and error bars indicate mean and SD, respectively. The mean TM-PPGK content ± SD was 20.1 ± 3.4 for human (n=34), 28.3 ± 5.1 for monkey (n=11), 30.7 ± 7.7 for dog (n=12), 34.5 ± 4.7 for rat (n=20), and 28.6 ± 2.0 for mouse (n=18).



Supplemental Figure 3: Kidney transporters across human and monkey.



Supplemental Figure 4: Comparison of OAT1 abundance data in representative samples [MF= Mouse (Female); MM = Mouse (Male)] using Western blotting (A) and LC-MS/MS proteomics (B). The band (A) and peak (B) intensities represent relative levels of OAT1 in different samples. In general, the quality of proteomics data is significantly better than the Western blotting data. Western blotting data were generated using a rabbit anti-mouse OAT1 antibody. The sex-difference in OAT1 abundance in mouse is qualitatively confirmed by these results. Although the antibody did not work for human OAT1 (likely because of non-specific binding), the higher rat signal is consistent with the proteomics data (data not shown). The peaks in Figure B represent LC-MS/MS responses of a conserved OAT1 surrogate peptide, GQASAMELLR [m/z 538.28 (++) → 819.43(+)]. Panel A (left) represents the molecular weight markers from nitrocellulose membrane while panel A (right) represents the OAT1 bands from developed X-ray film.

References

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