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Title page

MULTISPECIFIC DRUG TRANSPORTER *Slc22a8* (*Oat3*) REGULATES MULTIPLE METABOLIC PATHWAYS

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Running Title Page

Running title: *Oat3 handling of endogenous metabolites and flavonoids*

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Abstract

Multispecific drug transporters of the SLC and ABC families are highly conserved through evolution, but their true physiological role remains unclear. Analyses of the *organic anion transporter 3* (*Slc22a8/Oat3*, originally *Roct*) knockout mouse have confirmed its critical role in the renal handling of common drugs (e.g., antibiotics, antivirals, diuretics) and toxins. Previous targeted metabolomics of the knockout of the closely related *Oat1* have demonstrated a central metabolic role, but the same approach with *Oat3* failed to reveal a set of endogenous substrates. Nevertheless, the *Oat3* knockout is the only Oat described so far with a physiologically significant phenotype suggesting the disturbance of metabolic and/or signaling pathways. Here we analyzed global gene expression in *Oat3* knockout tissue, which implicated *Oat3* in Phase I and Phase II metabolism as well as signaling pathways. Metabolic reconstruction with the recently developed "mouse Recon1" supported the involvement of *Oat3* in the aforementioned pathways. Untargeted metabolomics were used to determine whether the predicted metabolic alterations could be confirmed. Many significant changes were observed; several metabolites were tested for direct interaction with mOAT3, whereas others were supported by published data. *Oat3* thus appears critical for the handling of Phase I (hydroxylation) and Phase II (glucuronidation) metabolites. *Oat3* also plays a role in bioenergetic pathways (e.g., TCA cycle) as well as those involving vitamins (e.g., folate), steroids, prostaglandins, cyclic nucleotides, amino acids, glycans and possibly hyaluronic acid. The data also suggests that *Oat3* is essential for the handling of dietary flavonoids and antioxidants.

Introduction

Oat3 (*Slc22a8*), originally identified as *Roct* (Brady et al., 1999) is a multispecific organic anion drug transporter expressed in renal proximal tubule cells, endothelial cells of the brain and cells of the choroid plexus, where it is believed to comprise part of the "classic" organic anion handling mechanism responsible for the rate-limiting steps in the movement of solutes across the blood-urine, blood-cerebrospinal fluid and blood-brain barriers. While *Oat3* shares several characteristics with related transporters, such as *Oat1* (*Slc22a6*) (Lopez-Nieto et al., 1996; Lopez-Nieto et al., 1997), including the presence of twelve membrane-spanning helices, with several consensus extracellular glycolsylation and intracellular PKC sites (Eraly et al., 2004; You, 2004; Klaassen and Aleksunes, 2010; VanWert et al., 2010; Wu et al., 2011), it is phylogenetically and structurally unrelated to other transport proteins, such as the *Oatps* and drug transporting *ABCs* (Hagenbuch and Meier, 2003). Its nearest non-Oat *SLC22* relations are the organic cation- and carnitine-transporting *Octs* (Burckhardt and Wolff, 2000; Sweet et al., 2001) and *Octns*, as well as the *Flipts* (fly-like putative transporters) (Eraly and Nigam, 2002; Enomoto et al., 2003). As with these related transporters, OAT3 is a multispecific transporter (Kusuhara et al., 1999; Sweet et al., 2003), coupling organic anion influx to dicarboxylate efflux (Sweet et al., 1997; Sweet et al., 2003; Zhou and You, 2007; Anzai et al., 2012), manifesting the physiological properties expected for a transporter underlying "classic" organic anion uptake.

Oat3 is a predominant component of renal organic anion transport apparatus believed to account for the transport of one-third to one-half of the most commonly prescribed drugs (i.e., penicillins, NSAIDs, cephalosporins, ACE inhibitors, diuretics, smallpox and HIV antivirals, methotrexate, statins as well as many toxins (Eraly et al., 2003; Eraly et al., 2004)). In addition, we have analyzed the knockout of *Oat3*, which are born at expected male/female ratio and are fertile (Sweet et al., 2002) and the resulting mutant mice, while free of obvious morphological abnormalities, manifested a distinct physiological phenotype characterized by a loss of organic

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anion transport in the kidney and choroid plexus (Sweet et al., 2002). In addition, basal glomerular filtration rate (determined by ^{3}H-inulin clearance) as well as PAH clearance were not different between WT control and *Oat3* deficient mice, suggesting that *Oat3* is not absolutely required for renal PAH secretion (Sweet et al., 2002). *Oat3*-deficient mice also display systolic blood pressure 10 to 15% lower than wild-type mice, raising the possibility that OAT3 mediates the specific transport of an endogenous compound(s) involved in the regulation of blood pressure (Vallon et al., 2008a). Nevertheless, despite its potential considerable pharmaceutical and pharmacological worth, representing a high priority target of interest (Giacomini et al., 2010), the endogenous physiological function(s) of this highly conserved transporter remains to be fully elucidated. In contrast to the *Oat1* knockout (Eraly et al., 2006) targeted metabolomics analysis of the *Oat3* knockout have revealed surprisingly few changes (Eraly et al., 2008; Vallon et al., 2008a; Vallon et al., 2008b; Vallon et al., 2012).

A role for *Oat3* and other SLC and ABC multispecific drug transporters in remote sensing and signaling during inter-organ communication has been proposed (Ahn and Nigam, 2009; Wu et al., 2011); implicit in this hypothesis is a central role for “drug” transporters like OAT3 in regulating key metabolites and signaling molecules. By using a systems-biology approach combining computational and wet-lab “omics” data obtained from the *Oat3* deficient mouse, we observed altered molecular gene networks and concentrations of a battery of cellular metabolites involved in energy production and cell growth, as well as plasma accumulation of a large number of secondary metabolites (Phase II) of plant ingredients of dietary origin, particularly those of flavonoid antioxidants. Metabolic reconstruction using transcriptomic data from the knockout and wild type was then performed, followed by testing of predictions by two different untargeted metabolomics methods (Figure 1). In general, the data are consistent with a central role for *Oat3* in regulating the cellular metabolism and remote communication.

Materials and Methods

Materials- Chemicals used in in vitro tests were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were analytical grade pure.

Animal- Oat3/Slc22a8 deficient mice were born with expected sex ratio. Male mice between 12 and 20 weeks of age were used in these experiments. For microarray analysis, *Oat3/Slc22a8* deficient mice were backcrossed to C57BL/6J for five generations. For metabolomic analysis, *Oat3* deficient mice were backcrossed to C57BL/6J for a total of ten generations and both control and *Oat3* deficient mice were fed the same standard diet. Blood and urine samples were collected and plasma was isolated and stored at −80 °C until analysis was carried out.

Microarray analysis- Total RNA was prepared and purified from wild-type (n=3) and knockout kidneys (n=3) and microarray analyses were performed as previously described (Wikoff et al., 2011). The amplified RNA was labeled by incorporation of biotinylated nucleotides during in vitro transcription and then hybridized to Affymetrix microarrays, washed, and scanned per the standard Affymetrix protocol. Hybridization and scanning were carried out at the UCSD/Department of Veterans Affairs Medical Center (VAMC) GeneChip core laboratory (http://www.vmrf.org/research-websites/gcf), and microarray data analysis was performed as described (Tsigelny et al., 2008).

Recon 1 analysis. A variation of Recon 1 (Duarte et al., 2007), a global human metabolic network reconstruction, was used to analyze the differential transcriptomic data from the wild-type and *Oat3* deficient animals. As described previously (Ahn et al., 2011), the NCBI Homologene Database (www.ncbi.nlm.nih.gov/homologene) was used to map human Entrez Gene IDs to their mouse homologs. Specific WT and *Oat3* deficient models were created using the transcription profiles (binary classification using Affymetrix MAS5.0 presence/absence calls) as data with the GIMME algorithm (Becker et al., 2007) and the COBRA toolbox

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(Schellenberger et al., 2011). The mouse biomass pseudo-reaction was used as the objective function. Identical generalized uptake conditions were used for both models and flux spans were calculated following Flux Variability Analysis (FVA) (Mahadevan and Schilling, 2003; Ahn et al., 2011).

Predicted changes in the metabolic capabilities of the two models were classified into two categories; the active reactions that were shared between the models and the reactions that were not shared between KO and WT. The most substantive flux changes for the set of shared reactions were identified by rank ordering the reactions and identifying reactions with flux spans that either increased by 2-fold or decreased by 50%. Of note, changes in the flux spans will not necessarily be reflected as increases or decreases in flux, but rather increases or decreases in the range of possible flux for a particular reaction.

Untargeted Metabolomics- 950 MHz NMR: Blood and spontaneous urine samples (at the time of blood collection) from adult male wild-type control and *Oat3* deficient mice (n=3) were obtained and individual, unpooled samples were subjected to 950 MHz NMR analysis at University of North Carolina metabolomics laboratory (Research Triangle Park, NC 20779).

An untargeted, mass-spectrometry-based LC/MS approach for metabolomics was carried out by the core facility at Scripps Center for Metabolomics and Mass-spectrometry. Plasma samples were obtained from adult male *Oat3* deficient and control wild-type mice (n=3) and prepared in a manner similar to that previously described for mOAT1 metabolomics (Wikoff et al., 2011). Mass-spectrometry data were evaluated using XCMS (https://xcmsonline.scripps.edu/), with nonlinear data alignment together with intensity integration. METLIN database (http://metlin.scripps.edu/) was then used for compound identification. KEGG was used for pathway analysis. Plasma samples from *Oat3* deficient mice were compared with plasma samples from wild-types. The samples for metabolomics were then run together and analyzed as a single group. The concentration ratios of revealed metabolites

indicate those molecules with altered plasma distribution in the knockout versus wild type samples.

Cellular uptake assay- As previously described, confluent monolayers of Chinese hamster ovary cells permanently expressing mouse OAT3 (mOAT3-CHO) growing in 96-well tissue culture plates were employed in uptake assays using 5-carboxyl fluorescein as a tracer molecule (Ahn et al., 2011).

Statistical Analysis- The statistical analysis was performed using unpaired t-test. The values are expressed as the means ± standard deviation.

Ethics Statement- All animals were handled in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines (http://iacuc.ucsd.edu/index.aspx).

Results

Detailed transcriptomic analyses revealed altered expression of genes involved in key metabolic pathways in the Oat3 deficient kidney. *Oat3* is predominantly expressed in the kidney where it mediates the uptake of organic anionic solutes in proximal tubular cells (VanWert et al., 2010). Despite the importance of *Oat3* in the uptake and elimination of a wide variety of drugs and toxins (Table 1), *Oat3* deficient animals are healthy and viable. In order to determine if a molecular compensatory mechanism exists in the kidney of *Oat3* deficient mice, a microarray analysis was carried out comparing renal gene expression in the knockout to that of wild-type controls. Among the profiled genes, significant expression changes (≥2-fold change in expression either up or down) were observed for more than 100 genes, including *Oat3/Slc22a8* (absent in in the *Oat3* deficient mice). Detailed annotation of 67 of those gene transcripts that were most significantly elevated (>2.5X) in the *Oat3* deficient kidney revealed that at least 23 of them encode gene products related to metabolism suggesting elevated enzymatic activities (Table 2). For example, expression of the gene related to the human cytochrome P450 2b10 isoform 1, a Phase I drug metabolizing enzyme, was significantly elevated (4.2 fold) in the *Oat3* deficient kidney (Table 2). In addition, the expression of Ugt2b5 homolog, a Phase II drug metabolizing enzyme which catalyzes glucuronide conjugation, was also elevated (over 300 fold), while elevated expression was also observed for 3-oxo-5-alpha-steroid 4-dehydrogenase 2 (an enzyme involved in steroid metabolism) (3.7 fold). Taken together, these expression changes indicate that *Oat3*-deficiency results in alterations in the expression of Phase I and Phase II drug metabolizing enzymes, as well as Phase III drug transporters and appears to link Oat3 to the normal physiological functions of metabolic enzymes and transporters.

Utilizing available bioinformatic tools including GeneSpring, GOBY and Ingenuity IPA, the genes were further analyzed in order to investigate the possibility that networks of genes involved in drug metabolism and transport were altered in the knockout animals. These

analyses revealed that TCA cycle and energy metabolism, as well as nucleotide and amino acid metabolism, were among some of the most significantly impacted pathways (Table 3). In addition to the core cellular functions implicated, altered functionality was also observed in vitamin and mineral metabolism. Thus, our analyses not only link *Oat3* to the normal physiological functions of metabolic enzymes and transporters, but they also suggest the involvement of *Oat3* in the handling of nutrients and vitamins.

Metabolomic Reconstruction of potential physiological roles of Oat3 by Recon 1. To further investigate this possibility as well as decipher the cellular response to *Oat3* deficiency, global transcriptomic clustering was followed by pathway analysis using mouse Recon 1, a global metabolic network reconstruction largely based on human Recon 1 which is comprised of known biochemical and physiologic data (Sigurdsson et al., 2010; Bordbar et al., 2012). These computational analyses revealed alterations in a number of metabolic pathways related to transcriptional regulation, solute handling, and endogenous enzymatic activities (also see Table 2). Among the 285 reactions linked to transcriptomic alteration in *Oat3* deficient kidneys by mouse Recon 1, 220 were related to the cell mass growth functionality that includes energy (ATP) production and metabolism of nucleic acids, amino acids and fatty acids, raising the possibility that compensatory molecular responses in cell growth and bio-mass occur in the kidneys of *Oat3* deficient mice. This genome-wide reconstruction of mouse metabolism based on transcriptomic data also identified 19 additional processes/reactions, including bile acid synthesis, glucuronidation, sulfation, prostaglandin synthesis, hyaluronan metabolism and steroid metabolism, as well as vitamin metabolism, including folate (vitamin B9 derived from dietary sources) (Table 4). As an example, the affected folate metabolism pathway is depicted here and three reactions involved in the metabolism of folate (DHFR, MTHFD and MTHFC) which, based on mouse Recon1 reconstruction, should be enhanced in *Oat3* deficient kidney are highlighted (Figure 2). An overview of the predicted changes in metabolic pathways reactions can be found in Supplemental Figure 1 and Supplemental Tables 1-4 respectively.

Thus, comparison of the WT and *Oat3* deficient metabolic networks using transcriptomic data highlighted alterations in multiple areas of metabolism, including amino acid, fatty acid, cholesterol, and nucleotide pathways. Moreover, hydroxylation as well as glucuronidation and conjugation reactions (which are usually involved in Phase I and Phase II drug metabolism) were among the largest differences noted between the WT and *Oat3*-deficient mouse.

Metabolomic analysis revealed altered urinary excretion of cellular metabolites in Oat3 deficient mice. In contrast to the *Oat1* deficient animal, targeted metabolomics analysis of the *Oat3* deficient animal revealed minimal alterations in a set of about 30 of the most abundant organic anionic endogenous metabolites (Vallon et al., 2008a). Taken together with subsequent analyses which revealed modest alterations in the concentration of urate, thymidine and flavin mononucleotide in the *Oat3* deficient animals (Eraly et al., 2008; Vallon et al., 2008a; Vallon et al., 2008b), it seems likely that the metabolic pathways disturbed in the *Oat3* deficient mouse are different from those altered in the *Oat1* deficient mouse. In order to confirm and validate the systems biology predictions from the analyses described above (Table 3), global untargeted metabolomics analyses were performed on serum and urine samples derived from wild type and *Oat3* deficient mice. After normalization of the concentrations of the detected metabolite to creatinine, 950 MHz NMR-based untargeted metabolomics of urine revealed significant concentration changes in metabolites related to cellular energy metabolism, (e.g., α-ketogluterate, citrate, 4-hydroxyphenylacetate), and growth related metabolites such as amino acids (e.g. valine) (Figure 3), providing support for the Recon 1 predictions in Table 3. However, 950 MHz NMR-based untargeted metabolomics of serum samples did not yield broad concentration changes in the *Oat3* deficient animal (data not shown), although non-significant reductions in the concentrations of several amino acids, including valine, were detected in the *Oat3* deficient animal.

Global untargeted mass-spectrometric metabolomic profiling in Oat3 deficient mice revealed significant plasma accumulation of metabolites conjugated by Phase I and Phase II

reactions, including derivatives of plant flavonoids. A global untargeted, mass-spectrometry-based analysis of blood samples derived from adult *Oat3* deficient mice verses wild-type controls was also performed. Plasma samples from adult C57/BL6 control mice and comparable *Oat3* deficient mice were obtained. These samples were subject to LC/MS untargeted metabolomics analysis at Scripps Centers for Metabolomics and Mass Spectrometry and the METLIN metabolomics database was used for compound identification. The plasma concentrations of 1538 features were found to be significantly different between the *Oat3* deficient and wild type control mice, 982 of which were altered 1.5 fold or higher (either up or down). Of these 982 features, 220 of them were associated with known molecular fragments, of which 30 were clearly identified as known metabolites (Figure 4 and supplemental table 5).

Consistent with metabolic reconstructions, behenic acid (a cholesterol-raising saturated fatty acid in humans), as well as a propionoate (a metabolite linked to carboxylic acid metabolism) and modified serotonin was significantly reduced (11 fold and 5 fold decrease relative to Wt, respectively) in the plasma of *Oat3* deficient mice (Figure 4a). In addition, a glucuronidated molecule (2-Amino-3-hydroxy-5-nitrobenzophenone glucuronide) was also among the highest accumulated in the *Oat3* deficient mice, consistent with the Recon 1 analysis (Figure 2, Table 4). Furthermore, methylguanosine, a uremic toxin, was also found to be significantly accumulated in the plasma of *Oat3* deficient mice. The two molecules which were found to be most highly accumulated in *Oat3* deficient plasma were pongamoside A and 9-amino-nonanoic acid, both of which are derived from plants (Figure 4a). Pongamoside A is a plant derivative flavonoid antioxidant, while 9-amino-nonanoic acid is a modified nonanoic acid derived from soybeans.

Known mass-spect features associated with a group of metabolites that are dietary phenolic derivatives. The potential molecules associated with the mass-spectrometry fragment features that were most highly accumulated in the Oat3 deficient mice were also examined. Of the top ten features with a minimum of a 5-fold increase in their concentrations in the *Oat3*

deficient mice (Top 4 are listed in Table 5), at least 7 of them were found to be associated with metabolites of plant origin. For example, feature M457T_1, elevated 13.8 fold *Oat3* deficient plasma, is associated with a group of metabolites of dietary phenolic derivatives including Epicatechin 3-O-(3-O-methylgallate), also known as an internal metabolite of the ester of epigallocatechin and gallic acid (epigallocatechin gallate, EGCG). EGCG is a potent antioxidant found in a number of plants, but it is the most abundant catechin in tea leaves. Interaction of EGCG with OAT3 was confirmed in a cell based assay that employed mOAT3-expressing CHO cells (mOAT3-CHO). In this assay, EGCG, as well as typical OAT3 substrates (ie., estrone sulfate) were tested for their ability to inhibit uptake of a preferential OAT3 tracer 5CF. The uptake of 5CF in mOAT3-CHO cell was inhibitable by epicatechin (Sigma 49045-U), as well as epicatechin gallate (Sigma 49060-U) (Figure 5). Thus, the data support the notion that the broad accumulation of multiple phytophenolic metabolites of dietary origin is most likely the result of a deficiency in OAT3-mediated transport.

Discussion

Utilizing a systems biology approach comparing wild-type and *Oat3* deficient mice, the correlation of transcriptomic, computational pathway and metabolomics analyses (950 MHz NMR plus LC/MS) revealed that the absence of this transporter leads to significant alterations in: 1) a number of cellular metabolic pathways (e.g., TCA cycle, nucleotide and amino acid metabolism) (Table 2-3 and Figure 2 and 3, Supplemental Figure S1); 2) the expression of genes encoding enzymes with critical roles in Phase I and Phase II xenobiotic metabolism, as well as Phase III drug transport (Table 2 and 4); 3) pathways involved in the regulation of secondary metabolites, including endogenous signalling molecules (e.g., prostaglandins and steroids), and dietary plant derivatives (e.g., vitamins) (Table 2-4, and Figures 2 and 4); and 4) pathways involved in the handling of dietary flavonoids (Table 5, Figure 4 and 5). Among the dietary flavonoids found to accumulate in the plasma of the *Oat3*-deficient animal, epicatechin is interesting as this molecule has been suggested to impact blood pressure (and have potential cardiovascular benefits), and the *Oat3* deficient mice have decreased blood pressure (Vallon et al., 2008a). Nevertheless, taken together, the data help to define the role of this transporter in normal physiology by linking its activity to a variety of pathways regulating levels of key metabolites and signalling molecules.

Oat3 is expressed in the proximal tubules of the kidneys, where it plays an important role in the elimination of numerous organic anions of physiological, pharmacological and toxicological relevance (Burckhardt and Burckhardt, 2003; Eraly et al., 2004; Nigam et al., 2007; Ahn and Bhatnagar, 2008; Di Giusto et al., 2008; Ahn and Nigam, 2009; Wu et al., 2009; Wu et al., 2011). There is significant overlap in the expression of *Oat3* and its close homolog, *Oat1*, with many proximal tubular cells expressing both of these transporters (Lopez-Nieto et al., 1997; Hwang et al., 2010). Nevertheless, immunocytochemical localization reveals that OAT1 appears to be expressed preferentially in the S1 and S2 segments while OAT3 is found more in

the S2 and S3 segments (Hwang et al., 2010), a finding consistent with their functional localization in knockout animals (Truong et al., 2008). In addition to its renal expression, *Oat3* is also expressed in the brain (on the endothelial cell as part of blood brain barrier), as well as in choroid plexus where it is believed to be part of the transport apparatus mediating the elimination of solutes from the brain and central nervous system (Sweet et al., 2002; Vanwert et al., 2007; Truong et al., 2008; Vallon et al., 2008a; Vallon et al., 2008b; VanWert and Sweet, 2008; Ose et al., 2009; Nagle et al., 2011; Sweeney et al., 2011; Xue et al., 2011).

While it is believed that OAT3 is involved in mediating the disposition, distribution and elimination of a wide variety of common pharmaceuticals (VanWert et al., 2010; Burckhardt and Burckhardt, 2011), the role of *Oat3* in normal physiology has been less clearly defined. In a recent targeted metabolomics study, of the 30 most abundant plasma small organic anion molecules investigated, none were found to accumulate in the plasma of *Oat3* deficient mice, which is contrary to the finding from *Oat1 deficient* mice (Vallon et al., 2008a). The data from this study indicate that *Oat3*-deficiency leads to changes in the concentration of a variety of small organic metabolites in the blood. Furthermore, transcriptomic and pathway analyses revealed alterations in Phase I and Phase II xenobiotic metabolism such as sulfation and glucuronidation a notion supported by metabolomics analyses which revealed alterations in the levels of sulfated and glucuronidated small molecules, including components of bio-mass cell growth and flavonoid antioxidants of dietary origin (Table 6). Thus, *Oat3* is not only likely key to the handling of endogenous metabolites and signaling molecules with important physiological functions, but it is also important in the handling of metabolites derived from the gut microbiome. The results seem generally compatible with the proposed role of *Oat3* and remote sensing and signaling (Kaler et al., 2006; Ahn and Nigam, 2009; Wu et al., 2011).

Since this set of metabolites was not found to be altered in a untargeted metabolomics analysis of plasma from *Oat1*-deficient animals (Ahn et al., 2011; Wikoff et al., 2011), the data presented above support the notion that *Oat3* plays a critical and perhaps primary role in the

uptake and elimination of these dietary antioxidants. A metabolic reconstruction of the *Oat1* deficient animal using similar transcriptomic and metabolomics data revealed alterations in largely different but somewhat overlapping set of pathways (Ahn et al., 2011). Considering the importance of OAT3 in the uptake and elimination of a wide variety of commonly prescribed drugs (e.g. antibiotics, antivirals used for HIV, diuretics, NSAIDs, methotrexate) (Giacomini et al., 2010; Klaassen and Aleksunes, 2010; VanWert et al., 2010; Wu et al., 2011), together with the fact that the use of dietary supplements, including herbal medicines, botanicals, and probiotics, is increasing worldwide (Bardia et al., 2007; Williamson et al., 2007; Wong et al., 2011a; Wong et al., 2011b), the data raise the possibility of competition between dietary metabolites and drugs for access to OAT3. Moreover, based on the expression of *Oat3* such metabolite-drug interactions are likely to affect multiple tissues (Figure 6).

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Authorship Contributions

Participated in research design: Wu, Jamshidi, Palsson, Eraly, and Nigam.

Conducted experiments: Wu, Jamshidi, Eraly, Bush and Liu.

Contributed new reagents or analytic tools: Palsson, and Nigam.

Performed data analysis: Wu, Jamshidi, Eraly, Bush and Liu.

Wrote or contributed to the writing of the manuscript: Wu, Jamshidi, Bush, Palsson, and Nigam.

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Footnotes

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The abbreviations used are: Oat3, organic anion transporter 3/Slc22a8; Oat1, organic anion transporter 1/Slc22a6; EGCG, Epigallocatechin gallate.

Figure Legends

Figure 1. **Overall strategy of this study.**

Figure 2. **Graphic pathway diagram depicting altered components (enhanced) of folate metabolism in *Oat3* knockout kidney generated by Recon 1 analysis.** The enhanced pathways of DHFR, MTHFC and MTHFD were also listed in table 4. This diagram is a portion of the global overview of predicted global changes in pathways by Recon 1 analysis, which can be found in Supplemental Figure 1.

Figure 3. ***Oat3*-deficiency results in alterations in the urinary concentration of metabolites.** Relative urine concentrations of selected compounds in wild-type and *Oat3* deficient mice. Spontaneous urine samples were obtained from adult mice and subjected to untargeted metabolomic analysis using $^{(1)}$H nuclear magnetic resonance (NMR) spectroscopy and multivariate statistical analyses to generate metabolic fingerprints. Urine concentrations of metabolites were normalized to creatinine. n=3. *, $p< 0.05$.

Figure 4. ***Oat*3-deficiency results in the plasma accumulation of a variety of chemical compounds.** A. List of significantly altered (>2.5X) natural metabolites in the plasma of *Oat3* deficient mice by untargeted LC-MS analysis. Additional significantly altered mass-spect features are listed in Supplemental Table 5. B. Boxplots of relative plasma concentrations for four most highly accumulated compounds in *Oat3* deficient plasma. Pongamoside A (*p*-value $=3.3 \times 10^{-2}$) is a flavonoid metabolite of dietary origin.

Figure 5. **Inhibitory effects of metabolites on uptake of 5CF in Chinese hamster ovary cell permanently expressing mouse OAT3 (mOAT3-CHO).** mOAT3-CHO cells were plated in 96-

well plate overnight and uptake of an OAT3-preferred tracer (5-carboxyl fluorescein, 20 microM) was carried out for 5 min at room temperature. (A) Bar graph illustrating inhibitory effects of epicatechin and epicatechin gallate on OAT3-mediated uptake of 5CF. As expected, probenecid inhibits the uptake in a range that is consistent with previous data. (B-C) Molecular structures and some chemical properties of epicatechin (B) and epicatechin gallate (C). n=3; *p<0.01.

Figure 6. **Diagram depicting potential interaction of commonly prescribed pharmaceuticals and endogenous substrates at the site of organic anion transporter 3 for excretion.**

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Tables

TABLE 1
Known Functional Alterations in the Oat3 Knockout Kidney

Molecule	Class	Reference
Methotrexate	Drug	VanWert and Sweet, 2007
Penicillin G	Drug	VanWert et al., 2007
Furosemide	Drug	Vallon et al., 2008
Bendroflumethiazide	Drug	Vallon et al., 2008
Ciprofloxacin	Drug	VanWert et al., 2008
Urate	Metabolite	Eraly et al., 2008
Ro 64-0802 (active form of Oseltamivir)	Drug	Ose et al., 2009
Aristolochic acid	Toxin	Xue et al., 2011
Zidovudine	Drug	Nagle et al., 2011
Acyclovir	Drug	Nagle et al., 2011
Tenofovir	Drug	Nagle et al., 2011
Lamivudine	Drug	Nagle et al., 2011
Creatinine	Metabolite	Vallon et al., 2012

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TABLE 2
Partial List of Enzyme Transcripts (including some Ests) Upregulated in *Oat3*-KO Kidney

Affy. ID	Gene Name	Description	Fold Change
1423397_at	Ugt2b5	UDP glucuronosyltransferase 2B5	371.9
1440339_at	Enpp1	ectonucleotide pyrophosphatase/phosphodiesterase 1	120.2
1459311_at	Est	cAMP-specific 3',5'-cyclic phosphodiesterase 4D-like	14.6
1449486_at	Ces1	carboxylesterase 1	4.5
1422257_s_at	Cyp2b10	cytochrome P450, family 2b10 isoform 1	4.2
1440463_at	Est	similar to peptide N-glycanase (Ngly1) gene	4.0
1422960_at	Srd5a2	3-oxo-5-alpha-steroid 4-dehydrogenase 2	3.7
1444032_at	Keg1	glycine N-acyltransferase-like protein Keg1	3.7

NOTE. Multiple probes for Phase I (i.e., cytochrome P450, family 2b10 isoform 1) and Phase II (i.e., UDP glucuronosyltransferase 2B5) drug metabolizing enzyme displayed similar levels of expression change. The elevated expression observed for 3-oxo-5-alpha-steroid 4-dehydrogenase 2 suggests enhanced enzymatic metabolism for steroid hormones, many of which are known OAT3 substrates.

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TABLE 3
Subsystem Prediction of Altered Metabolic Pathways in *Oat3* Knockout

Up	Down
Arginine and Proline Metabolism	Galactose metabolism
Blood Group Biosynthesis	Glutathione Metabolism
Chondroitin/heparan sulfate biosynthesis	Glycine, Serine & Threonine Metabolism
Fatty Acid Metabolism	Glycolysis/Gluconeogenesis
Galactose metabolism	IMP Biosynthesis
Glycerophospholipid Metabolism	Pentose Phosphate Pathway
Inositol Phosphate Metabolism	Pyrimidine Biosynthesis
Nucleotides	Pyruvate Metabolism
O-Glycan Biosynthesis	Transport, Extracellular
Sphingolipid Metabolism	Vitamin B12 Metabolism
Steroid Metabolism	

Note. Transcriptomic analysis was carried out using Affymetrix mouse gene expression array. The most significantly altered sybsystems were listed. It appears that Arginine and Proline Metabolism, Blood Group Biosynthesis, Fatty Acid Metabolism and others were predicted to be increased in *Oat3* deficient kidney; While Glutathione Metabolism, Glycine, Serine, and Threonine Metabolism and others were predicted to be reduced in the *Oat3* deficient kidney.

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TABLE 4
Enhanced RECON 1 Enzyme Reactions in the *Oat3* Knockout

Enzyme Reactions	Reaction Name	Subsystem
P4508B11r	Sterol 12-alpha-hydroxylase	Bile Acid Biosynthesis
P4508B13r	Sterol 12-alpha-hydroxylase (nadh)	Bile Acid Biosynthesis
B3GNT51g	UDP-GlcNAc:bGal b-1,3-N-acetylglucosaminyltransferase 5	Glycan Biosynthesis
ST3GAL31g	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	Glycan Biosynthesis
HMGCOASim	Hydroxymethylglutaryl CoA synthase (ir)	Cholesterol Metabolism
GALT2g	UDP-D-galactose:galactosylxylose galactosyltransferase, Golgi	Chondroitin/heparan sulfate biosynthesis
S3T2g	heparin-glucosamine 3-O-sulfotransferase	Chondroitin/heparan sulfate biosynthesis
LTA4H	Leukotriene A-4 hydrolase	Eicosanoid Metabolism
PGISr	Prostaglandin I2 synthase	Eicosanoid Metabolism
DHFR*	dihydrofolate reductase	Folate Metabolism
MTHFD*	methylenetetrahydrofolate dehydrogenase (NADP)	Folate Metabolism
HAS1	hyaluronan synthase	Hyaluronan Metabolism
HAS2	hyaluronan synthase	Hyaluronan Metabolism
S23T3g	beta-galactoside alpha-2,3-sialyltransferase (complex N-glycan)	Keratan sulfate biosynthesis
HSD17B3r	testicular 17-beta-hydroxysteroid dehydrogenase	Steroid Metabolism
PYDXDH	pyridoxal dehydrogenase	Vitamin B6 Metabolism
S23T3g	beta-galactoside alpha-2,3-sialyltransferase (T antigen)	O-Glycan biosynthesis
MTHFC*	methenyltetrahydrofolate cyclohydrolase	Folate Metabolism
34HPLFM	3-(4-hydroxyphenyl-)lactate formation	Ubiquinone Biosynthesis

NOTE: This is a list of significantly enhanced RECON 1 enzyme reactions (without exchange, transport or biomass-growth related reactions) in the *Oat3* knockout.
*Enhanced reactions related to folate metabolism (DHFR, MTHFD and MTHFC) are highlighted in Fig. 2.

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TABLE 5
Mass Spectrometry Features Highly Accumulated in the Plasma of *Oat3* Deficient Mice

Name	Fold Change	p-value	m/z	Retention Time	Putative Associated Molecule
M301T2_5	17.7	0.0045	301.0746	1.59	Sulfaquinoxaline and others
M457T9_1	13.8	0.0125	457.1144	9.37	**Epicatechin 3-O-(3-O-methylgallate) and others**
M573T10	9.9	0.0075	573.1626	10.4	**Licuroside and 3 others**
M441T11_1	8.3	0.0325	441.1205	11.27	**Pongamoside**

NOTE. Flavonoid metabolites are in bold

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TABLE 6
Known OAT3 Metabolites

Metabolite	Assay	Reference
Pomgamoside A	Up in Oat3KO plasma	This study
9-amino-nonanoic acid	Up in Oat3KO plasma	This study
Flavin mononucleotide	Up in Oat3KO plasma	Vallon, 2008
Thymidine	Up in Oat3KO plasma	Vallon, 2008
Urate	Down in Oat3KO urine	Eraly, 2007
Dideoxycytidine	mOat3, X. laevis	Truon, 2008
17b-estradiol-D-17bglucuronide	mOat3, LLC-PK1	Nagata, 2002
Estrone sulfate	hOAT3, X. laevis	Windass, 2007
Homovanillic acid	rOat3, X. laevis	Mori,2003
3-Carboxy-4-methyl-5-propyl-2-furanpropionate	hOAT3, HEK293	Deguchi,2004
Indoxyl sulfate	hOAT3, HEK293	Deguchi, 2004
Prostaglandin E2	hOAT3, S2 segment	Kimura, 2002
Prostaglandin F2a	hOAT3, S2 segment	Kimura, 2002
Dehydroepiandrosterone sulfate	hOAT3, HEK293	Ueo, 2005
Genistein-7-O-glucuronide	hOAT3, HEK293	Wong, 2011
Glycitein-7-O-glucuronide	hOAT3, HEK293	Wong, 2011
Quercetin-3′-O-glucuronide	hOAT3, HEK293	Wong, 2011

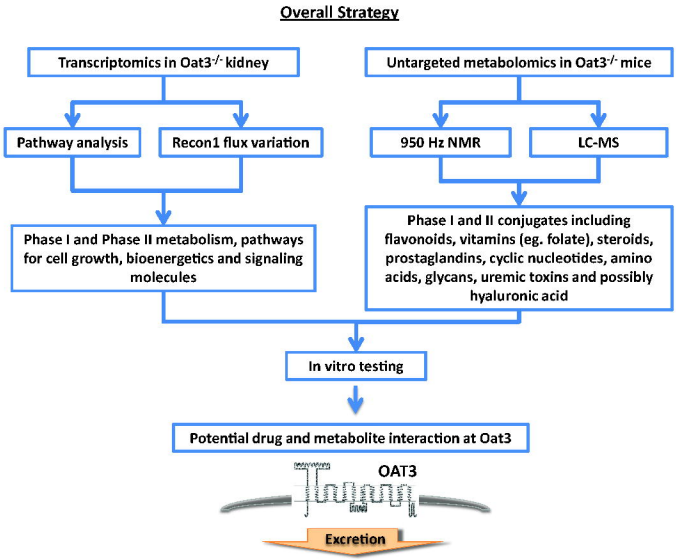
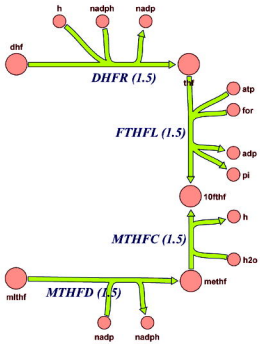

Overall Strategy
Transcriptomics in Oat3-/- kidney
Untargeted metabolomics in Oat3-/- mice
Pathway analysis
Recon1 flux variation
950 Hz NMR
LC-MS
Phase I and Phase II metabolism, pathways for cell growth, bioenergetics and signaling molecules
Phase I and II conjugates including flavonoids, vitamins (eg. folate), steroids, prostaglandins, cyclic nucleotides, amino acids, glycans, uremic toxins and possibly hyaluronic acid
In vitro testing
Potential drug and metabolite interaction at Oat3
OAT3
Excretion

Figure 1.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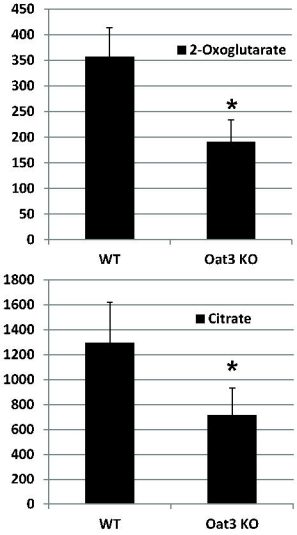
nadp
nadph

Figure 2.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300
250
200
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Oat3 KO
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25
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15
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5
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4-Hydroxyphenylacetate
*
WT
Oat3 KO
1800
1600
1400
1200
1000
800
600
400
200
0
Citrate
*
WT
Oat3 KO
20
18
16
14
12
10
8
6
4
2
0
Valine
*
WT
Oat3 KO

Figure 3.

A. List of significantly altered (>2.5X) natural metabolites in the plasma of Oat3 deficient mice.

Feature	m/z	Fold Change Oat3KO/Wt	Molecule
M441T11_1	441.1205	**8.3**	Pongamoside A
M174T10_2	174.1496	**7.2**	9-amino-nonanoic acid
M310T13_1	310.1137	**6.9**	9-O-Acetylneuraminic acid
M457T11_1	457.0867	**6.5**	2-Amino-3-hydroxy-5-nitrobenzophenone glucuronide
M454T13_1	454.1812	**4.6**	1-Hydroxyfluphenazine
M175T3_2	175.0242	**3.9**	Dehydroascorbic acid
M241T9	241.0885	**3.5**	2-Oxo-9-methylthiononanoic acid
M320T10_1	320.0954	**2.7**	7-Methylguanosine
M415T14_3	415.3282	**0.4**	Palmitoyl Serotonin
M195T14	195.0385	**0.2**	Hydantoin-5-propionic acid
M366T17	366.3406	**0.1**	Behenic acid(d3)

B.

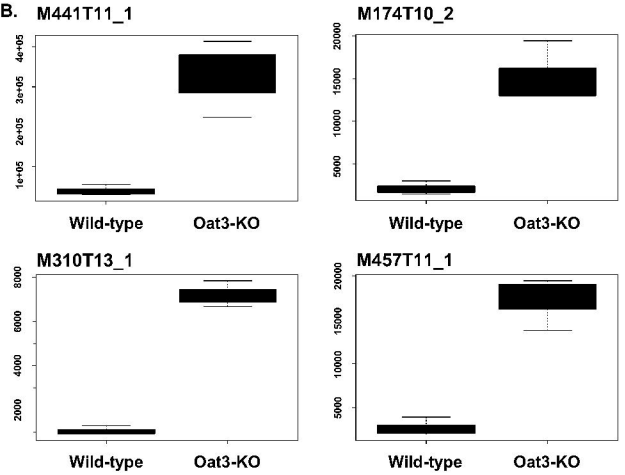
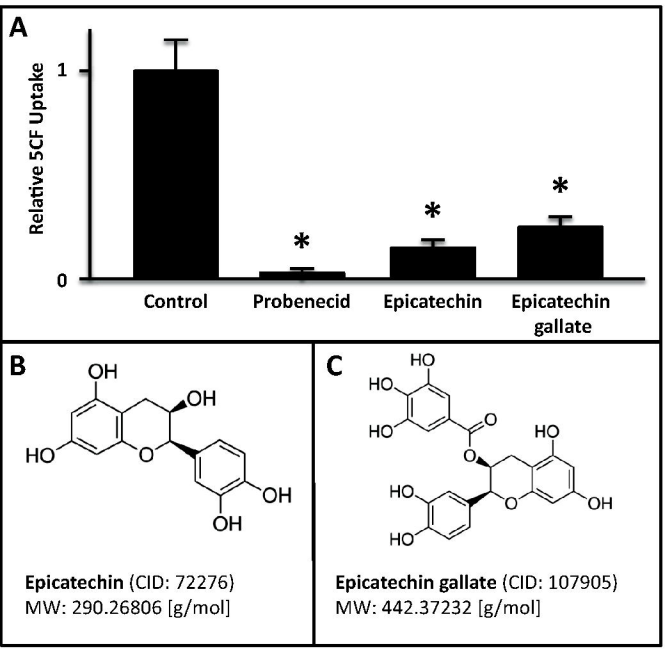


Figure 4.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*
*
*
Control
Probenecid
Epicatechin
Epicatechin gallate
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OH
OH
Epicatechin (CID: 72276)
MW: 290.26806 [g/mol]
C
HO
HO
HO
O
O
HO
HO
O
OH
HO
Epicatechin gallate (CID: 107905)
MW: 442.37232 [g/mol]

Figure 5.

	OAT3 Substrate
Cellular Metabolite	Homovanillic acid
Pharmaceuticals	Zidovudine, Pravastatin
Toxin	Indoxyl sulfate
Prostaglandins	Prostaglandin E2, F2
Steroid/Flavonoid	ES, DHES, Quercetin-3′-O-glucuronide

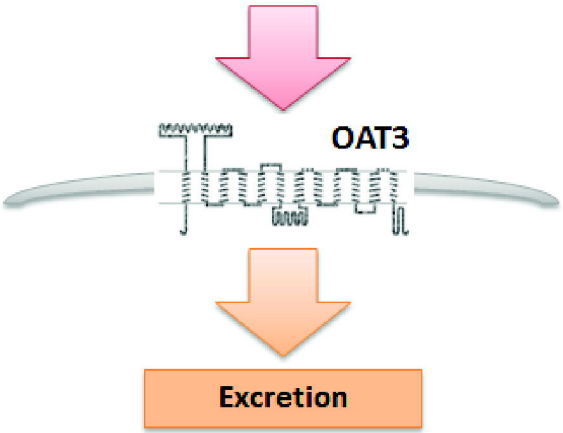

Figure 6.