Linkage of Organic Anion Transporter-1 to Metabolic Pathways through Integrated “Omics”-driven Network and Functional Analysis*

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The main kidney transporter of many commonly prescribed drugs (e.g. penicillins, diuretics, antivirals, methotrexate, and non-steroidal anti-inflammatory drugs) is organic anion transporter-1 (OAT1), originally identified as NKT (Lopez-Nieto, C. E., You, G., Bush, K. T., Barros, E. J., Beier, D. R., and Nigam, S. K. (1997) J. Biol. Chem. 272, 6471–6478). Targeted metabolomics in knockouts have shown that OAT1 mediates the secretion or reabsorption of many important metabolites, including intermediates in carbohydrate, fatty acid, and amino acid metabolism. This observation raises the possibility that OAT1 helps regulate broader metabolic activities. We therefore examined the potential roles of OAT1 in metabolic pathways using Recon 1, a functionally tested genome-scale reconstruction of human metabolism. A computational approach was used to analyze in vivo metabolomic as well as transcriptomic data from wild-type and OAT1 knock-out animals, resulting in the implication of several metabolic pathways, including the citric acid cycle, polyamine, and fatty acid metabolism. Validation by in vitro and ex vivo analysis using Xenopus oocyte, cell culture, and kidney tissue assays demonstrated interactions between OAT1 and key intermediates in these metabolic pathways, including previously unknown substrates, such as polyamines (e.g. spermine and spermidine). A genome-scale metabolic network reconstruction generated some experimentally supported predictions for metabolic pathways linked to OAT1-related transport. The data support the possibility that the SLC22 and other families of transporters, known to be expressed in many tissues and primarily known for drug and toxin clearance, are integral to a number of endogenous pathways and may be involved in a larger remote sensing and signaling system (Ahn, S. Y., and Nigam, S. K. (2009) Mol. Pharmacol. 76, 481–490, and Wu, W., Dnyanmote, A. V., and Nigam, S. K. (2011) Mol. Pharmacol. 79, 795–805). Drugs may alter metabolism by competing for OAT1 binding of metabolites.

The kidney is responsible for the elimination of toxic substances and wastes as well as for the reabsorption of 99% of the glomerular ultrafiltrate each day (~60% of which occurs in the proximal tubule) (1). Among the transporters that drive this reabsorption and secretion process are the organic anion transporters (OATs), which transport an extensive range of endogenous metabolites, such as tricarboxylic acid (TCA) cycle intermediates, cyclic nucleotides, and prostaglandins, and also serve as “drug/xenobiotic transporters” (2). Their abundance in a wide range of tissues other than the kidney, including tissues not generally considered to be sites of xenobiotic elimination, as well as their conservation down to fly and worm (3), indicates an important physiological role for these transporters. The OATs have been shown to mediate the transport of numerous endogenous/exogenous solutes, and a role in blood pressure regulation for OAT3 (4) and remote sensing for OAT6 (5) has been suggested. In addition, the highly active metabolic reactions that occur in the proximal tubule in order to fuel its extensive transport processes as well as the fact that the OATs transport many important metabolic intermediates suggest a vital role in the proximal tubule’s endogenous metabolic pathways and/or whole body metabolism linked to proximal tubule function. Indeed, it has been suggested that OATs and other SLC transporters as well as ABC transporters are involved in a remote sensing and signaling system (2). We therefore attempted to elucidate this role by using an “omics”-driven approach that integrates computational analyses of metabolomic and gene expression profiles followed by experimental verification of predictions on potential metabolic pathways involved.

One of the primary goals of systems biology is to integrate high throughput data generation and computational approaches to systematically analyze biological systems (2, 6–9). Constraint-based analysis of metabolic network reconstructions, in particular, have been shown to be predictive of biological behavior in a number of organisms (10, 11) and have recently been extended to human metabolism (12). With this tool, various methods for analyzing reconstructed networks have been successfully developed and implemented in a wide range of studies for microbial and high order organisms, includ-

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.

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3 The abbreviations used are: OAT, organic anion transporter; mOAT1, mouse OAT1; FBA, flux balance analysis; 6CF, 6-carboxyfluorescein.
ing prediction of gene deletion phenotypes in microbes (13–15), functional characterization of novel and putative genes in *Escherichia coli* (16), and analyzing disease and dietetic effects on cardiac mitochondrial metabolism (17).

We employed an integrative transcriptomic and metabolomic approach using the Recon 1 network to systematically identify metabolites and their associated pathways associated with OAT1 function. OAT1, the prototypical OAT originally identified as NKT (18), is primarily expressed in the kidney and is located on the basolateral side of the proximal tubule, where it plays a principal role in the transport of organic anions, coupling their entry into the tubular cell to the exit of dicarboxylates along their concentration gradient.

Based on metabolomic and gene expression data from OAT1 KO mice, we used Recon 1 to identify complementary metabolic pathway signatures linked to OAT1 function. The pathways identified included the TCA cycle, pentose phosphate, cholate, fatty acid, and the polyamine pathways, among others. Although TCA intermediates and β-hydroxybutyrate have previously been shown to interact with OAT1 (6, 7, 19), the polyamine pathway had not previously been implicated. Spermidine and spermine, intermediates in this pathway, were found to bind mouse OAT1 (mOAT1), and are putative novel endogenous substrates of this transporter. We thus present a computational approach, based on metabolomic and gene expression constraints on a genome-scale metabolic network reconstruction, to generate and test predictions concerning metabolic pathways linked to OAT1. The results indicate that OAT1 is part of a broader metabolic network linking various endogenous pathways.

**EXPERIMENTAL PROCEDURES**

**Microarray Data from OAT1 Knock-out Mice**—The generation and breeding of OAT1 KO mice and the collection of microarray and targeted metabolomic data were performed as described previously (7, 20, 21). Mice heterozygous for the Oat1 null allele (Deltagen Inc., Redwood City, CA) were backcrossed to C57BL/6J mice for 4 generations. Heterozygous mice from the final back-cross were bred to each other to generate knock-out and wild-type mice.

**Overall Computational Strategy**—The human metabolic network reconstruction, Recon 1, was used as the framework for analysis of the experimental data generated from the WT and KO animals. The metabolomic and transcriptomic data were each analyzed independently with Recon 1, and results that were shared by both of them were prioritized for experimental verification as shown by the schematic in Fig. 1. Because Recon 1 contains reactions across many tissues, the network content needs to be culled and made condition-specific because different data types are effective in constraining different parts of the network. The network in these analyses included 2,786 compartment-specific metabolites (1,511 unique metabolites) interacting via 3,985 reactions (transport as well as catalysis). Because flux balance analysis (FBA) was used extensively in both analyses, we describe it briefly here, noting that there is extensive literature on FBA (22), which is, in short, the application of linear programming to metabolic networks by applying physical (e.g. mass conservation) and physiological constraints, as appropriate.

A quasi-steady state assumption is made, and mass conservation is enforced,

\[ S \cdot v = 0 \] (Eq. 1)

for a system with \( m \) metabolites and \( n \) reactions, in which \( S \) is the \( m \times n \) stoichiometric matrix, and \( v \) is a reaction flux vector of length \( n \). The metabolomic and transcriptomic data were used to specify reaction bounds,

\[ \alpha \leq v \leq \beta \] (Eq. 2)

in which \( \alpha \) and \( \beta \) are lower and upper reaction bound vectors, respectively. Irreversible reactions have either \( \alpha \) or \( \beta \) equal to zero. A binary vector, \( c \), specifies the objective function to be optimized (maximized or minimized), which in these studies is a linear combination of reaction fluxes.

**Global Modeling Using Metabolomic Data**—Urine metabolite measurements normalized to creatinine were converted into 24-h urine excretions based on the known rates of creatinine clearance in OAT1 KO and WT mice (7), respectively, and used as global excretion flux constraints on the network. Uptake constraints were not available; however, because absolute fluxes were not being calculated and the simulations were carried out to compare the KO and WT, identical uptake constraints were used for the KO and WT (2 \( \text{mM/h} \) uptake of unmeasured metabolites with free exchange of protons and water). This enables FBA simulations to be carried out in an unbiased manner, with the calculated differences reflecting changes in the urine excretion profiles. Flux variability analysis (23) was then used to determine the “bounding box” for the network. This required carrying out \( 2n \) FBA optimizations (where \( n \) is the number of reactions in the network), during which every reaction in the network was individually minimized and maximized, in order to determine all of the corners of the solution spaces for the OAT1 wild-type and knock-out mice. Concentrations and fluxes are not correlated; however, a change in concentration necessarily implies a change in the production or consumption fluxes for the metabolite in ques-

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**Organic Anion Transporter-1 and Network Analysis**

**FIGURE 1. Overall schema for data analysis and experimental testing of predictions.** The gene expression profiles and urinary metabolomic data were analyzed separately and compared with one another, and based on the results, *in vitro* assays in *Xenopus* oocytes and cell cultures, together with *ex vivo* uptake assays in kidney slices, were carried out.
Organic Anion Transporter-1 and Network Analysis

Network-based Gene Expression Analysis—The NCBI Homologene Database (available on the World Wide Web) was first used to map human Entrez Gene IDs to their mouse homologs, followed by the mapping of normalized expression detection $p$ values to their corresponding reactions based on gene-protein-reaction associations. The detection $p$ values were determined by Affymetrix Microarray Suite Version 5.0. We implemented the GIMME algorithm (24) to analyze mouse kidney gene expression profiles mapped to homologous human genes in the Recon 1 network. Briefly, the GIMME algorithm maps gene expression data as reaction weighting cues in a network model and uses FBA to derive context-specific subnetworks that can achieve predetermined metabolic objectives. The algorithm allows for quantifying the predicted subnetwork’s agreement with gene expression data for an imposed physiological or biochemical criterion (i.e. objective function), through a “consistency” score.

We used this approach to assess all reactions in the metabolic network as an objective function by score-evaluating weighted flux distributions required for optimizing each reaction in the metabolic network. The Recon 1 network was first converted into an irreversible network to restrict flux values to be positive, and the optimal flux value for each reaction objective was calculated through FBA as described earlier,

$$v_{\text{max},i} = \max$$

(Eq. 3)

where $c_{v_i}$ is a binary vector with the $i$th objective function entry equal to 1 and all others equal to 0, such that the vector $v$ contains the maximum flux for metabolic reaction objectives. The lower bound of each reaction objective was set to its calculated optimal flux value, and each objective was minimized to determine the most probable flux distribution (i.e. lowest summation of $p$ value weighted reactions) that enabled its optimal activity,

$$x_i = \min(p \cdot v ; S \cdot v = 0, \alpha \leq v \leq \beta, \alpha_i = v_{\text{max},i})$$

(Eq. 4)

where mapped expression detection $p$ values, $p$, were stored in the weighting vector $c_p$ and each vector component $c_{p,i} = p_i$ mapped to its corresponding $i$th reaction. Raw scores across all data sets were z-standardized for each reaction to enable normalization between reactions within each profile. This was followed by z-transformation of the scores to correct internally within each profile (i.e. compare from a normalized score mean of zero). A z-test was used to determine reactions that were significantly different between each knock-out and wild-type set. Permutation testing of 1,000 randomizations for each set was performed to determine statistical significance of the z-test analysis for the standardized scores. The resulting scores indicate functional reaction activity perturbations in the knockout compared with the wild-type that are statistically significant.

Xenopus Oocyte Transport Assays—Putative OAT substrates (spermidine, spermine, pyruvate, β-hydroxybutyrate, arginine, and α-ketoglutarate), probenecid, and 6-carboxyfluorescein (6CF) were obtained from Sigma-Aldrich.

Xenopus oocyte assays were performed as described previously (7, 19, 25, 26). Briefly, oocytes were defolliculated by treatment with collagenase A and maintained overnight in Barth’s buffer growth medium containing gentamycin (0.05 mg/ml). Stage V and VI oocytes were injected the following day with capped RNA solution (1 µg/µl, 23 nl/oocyte) synthesized from linearized plasmid DNA (for mOAT1, Image clone ID: 4163278) using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Transport assays were performed 3–4 days after microinjection. Experimental groups of 16–20 oocytes were placed in a 24-well plate with 1 ml of Barth’s buffer containing a 30 µM concentration of the fluorescent anion tracer, 6CF. Samples contained different concentrations of unlabeled test compounds. After a 1-h incubation at 25 °C, oocytes were washed five times with 4 °C Barth’s buffer, and each experimental group was divided into four samples of 4–5 oocytes. The oocytes were subsequently lysed and centrifuged, and the supernatant was measured for fluorescence (Wallac Victor 1420 Multilabel counter, PerkinElmer Life Sciences).

Background tracer uptake was measured in uninjected oocytes and subtracted from uptake in OAT1-injected oocytes to isolate the OAT1-mediated component of transport. Fluorescent tracer uptake was determined in the presence of test compounds at various concentrations in 10-fold increments. These values were used to calculate the IC$_{50}$ for the test compounds. Each data point is the average of at least three experiments. Inhibition data were plotted using nonlinear regression analysis in Prism software version 5.0 (GraphPad Inc., San Diego, CA) to calculate the IC$_{50}$ ± S.E. as described previously (7).

Chinese Hamster Ovary Cell Culture Transport Assays—Chinese hamster ovary (CHO) cells constitutively expressing mOAT1 (mOAT1-CHO) (27) were plated out on a 96-well plate at a uniform density and allowed to grow to confluence overnight. The growth medium was removed, and the cells were rinsed with PBS. Fresh PBS containing a 5 µM concentration of the fluorescent tracer, 6CF, without and with inhibitor at various concentrations was added to the wells, and the cells were incubated for 5 min at room temperature. Following the incubation, the cells were washed three times in PBS, and fluorescence was subsequently measured (Wallac Victor 1420 Multilabel counter, PerkinElmer Life Sciences). For the inhibition assays using a radiolabeled tracer, mOAT1-CHO cells were incubated for 10 min in PBS containing 1,000 µM inhibitor and
1 μCi of [3H]methotrexate (specific activity 20 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO). The control group did not contain an inhibitor. The cells were then washed three times with PBS and lysed, and radioactivity was subsequently measured by scintillation counting (Rackbeta, Beckman-Coulter, Fullerton, CA).

Uptake Assay in Adult Renal Slices—The uptake assay in renal slices was done as described previously. Briefly, kidneys from C57BL6 mice were dissected and maintained in 4°C L-15 medium. Coronal slices of 0.2-mm thickness were cut with a Stadie–Riggs microtome (7) and maintained in 4°C PBS. Symmetrical pieces for each condition were cut from single slices and then placed in 24-well plates containing minimal saline with 1 μM 6CF in the presence of either PBS or an inhibitor (probenecid (2.0 mM), spermidine (2.0 mM), or arginine (2.8 mM)). The concentrations of the polyamines corresponded to the IC50 values obtained from *Xenopus* oocyte data. Slices were incubated at 25°C for 1 h, washed three times with PBS, and then placed on a slide with Fluoromount (Southern Biotech, Birmingham, AL) and imaged with the Nikon D-ellipse C1 confocal microscope (A. G. Heinze, Lake Forest, CA). The OAT-mediated component of transport was determined by the difference in uptake of 6CF in the absence and presence of the OAT inhibitor probenecid.

RESULTS

Traditionally, research on OATs has focused on their role in drug and toxin handling. However, our earlier work in knockouts of OAT1 and OAT3 has suggested an important link between metabolism and the transport of organic anions (4, 19, 20). The proximal tubule of the kidney, where expression of these genes is largely or exclusively localized, is responsible for reabsorption of 60% of the glomerular ultrafiltrate and thus is the site of active metabolism. Ultrastructurally, the proximal tubule cells are very rich in mitochondria. These considerations as well as the suggestion that subtle alterations in the physiology of OAT knockouts may be due to changes in renal metabolite excretion led us to consider approaches to better understand the relationship between renal organic anion transport and metabolism. In order to extract information from and gain insight into organ level as well as systems level changes resulting from knock-out of the OAT1 transporter, kidney gene expression profiles were used in conjunction with metabolic profiling to assess the resulting biochemical phenotype changes between the KO and WT. A global biochemical network reconstruction was used as the platform for transcriptomic and metabolomic data integration and analysis.

Network Analysis of Urine Metabolomic Data—We previously described the quantification of 30–60 of the most abundant endogenous organic anions in OAT1 WT and KO mice by GC-MS (7). Changes in urinary metabolite excretion were used to conduct a global, organism level assessment of the changes resulting from knocking out the OAT1 transporter (Fig. 2). Because at steady state the net “ins and outs” of every element and compound must be balanced, the rate of urinary excretion for any compound will be equivalent to its net rate of production (biosynthesis minus degradation and/or elimination via extrarenal routes). Thus, the calculated ratio of the flux spans between KO and WT OAT1 (a ratio of 1 would indicate no change between KO and WT, and values greater than or less than 1 would indicate increased or decreased activity, respectively) enabled evaluation of the changes in global metabolism resulting from loss of transport activity by using Recon 1 (see “Experimental Procedures”). The resulting predictions yielded changes in 396 compartment-specific metabolite exchanges (336 unique metabolites) and 2,153 catalysis and intercompartmental transport reactions (supplemental Table 1). Because this was a global analysis focused on interactions between the kidneys and all other organs, we focused on the metabolites involved in the exchange reactions because they could be transported into/out of organs and tissues. In order to identify the most substantive metabolite changes, the flux span ratios of the metabolite exchanges were ranked, and metabolites resulting in a decrease of at least 50% of the flux span (64 transporter reactions, corresponding to 58 unique metabolite species) were tabulated for comparison with the expression profiling results. Metabolite exchange or transport between extracellular and intracellular compartments altered in the OAT1 knockout included urate, prostaglandins, bile acid intermediates, fatty acids, glyoxylate, and polyamines (spermine and spermidine).

Network Analysis of Transcriptomic Data—The kidney is the main OAT1-expressing organ and the site of its primary physiological function. We used a network-based approach to analyze differences in gene expression profiles due to the deletion of OAT1 in WT and KO kidneys in order to identify metabolic reaction perturbations associated with the deleted OAT1 function. As described under “Experimental Procedures,” the GIMME algorithm was used to score-assess biochemical or physiological function criteria in light of gene expression-modulated reaction usage. This analysis resulted in the identification of 243 intracellular reactions (catalysis and intercompartmental transport) that are significantly perturbed (p < 0.05).
and predicted to be associated with the OAT1 deletion in mice kidneys (supplemental Table 2). Many enzymatic intermediates of intracellular pathways in the following categories were shown to have either increased or decreased functional activities: nucleotide metabolism, fatty acid metabolism, tyrosine metabolism, arginine and proline metabolism, pyruvate metabolism, and pentose phosphate pathway (Fig. 3). Tyrosine transaminase, the first step in the tyrosine catabolic pathway, was among the reactions implicated to be perturbed and is consistent with substrates identified from our previous targeted metabolomics analysis (7). Predominantly decreased activities include pathways involved with arginine-related polyamine biosynthesis and included the spermine synthase function that converts spermidine to spermine. Ninety-three reactions pertaining to the extracellular exchange and/or transport of metabolites were also found to be significantly altered \((p < 0.05)\) that account for the 68 unique metabolites. The statistically significant reaction perturbations included transport pathways involved with fatty acid metabolism (acetone and acetoacetate), steroid metabolism (estrone-3-sulfate and estradiol glucuronide), polyamine metabolism (arginine, spermine), and nucleotide metabolism.

The most significant metabolites predicted to have altered for each approach are tabulated in Table 1. The scope of the metabolomic data analysis reflects more global changes \(\text{(i.e., not only changes in kidney function). Although the transcriptomic analysis is more specific to renal function changes, it is worth emphasis that the kidney is the site of maximal OAT1 expression. The overlapping predictions between the two were of particular interest. The two approaches shared six specific overlapping metabolite predictions (spermine, urate, oxalate, two \(\omega\)-hydroxy fatty acids, and one leukotriene) and additional overlaps in classes/pathways of metabolites, such as multiple bile acid intermediates and amino acid degradation products. These overlapping predictions were subjected to further examination.}

**In Vitro and ex Vivo Analysis of in Silico Predictions**—Metabolic networks altered in the OAT knockouts could be a direct result of altered transport of components of these networks or be due to an indirect effect on a metabolic pathway. To test the possibility that some of the predicted pathway metabolites were due to direct interaction of key substrates with OAT1, we assessed the interaction with OAT1 of selected metabolites from pathways predicted to be altered in the knockouts.

For various compounds, we tested the concentration-dependent inhibition of fluorescent substrate uptake in *Xenopus* oocytes and CHO cells expressing OAT1. Several metabolites that were identified as significantly different in functionality in the knockouts were found in these assays to interact with OAT1. Specifically, we found that \(\beta\)-hydroxybutyrate interacted with OAT1 with an IC\(_{50}\) of 8.7 \(\pm\) 2.4 mM, whereas pyruvate and \(\alpha\)-ketoglutarate interacted with an IC\(_{50}\) of 4.3 \(\pm\) 0.6 and 0.039 \(\pm\) 0.003 mM, respectively (Fig. 4), consistent with prior observations (7). Moreover, an interaction between intermediates of the TCA cycle, such as fumarate, and intermediates of fatty acid oxidation and steroid metabolism with OAT1 has been shown (supplemental Table 3) (19). The data supporting interaction with OAT1 suggest that the constraint-based mod-

**FIGURE 3.** Number of increased and decreased intracellular reaction activities \((p < 0.05)\) in OAT1-associated metabolic pathways based on kidney gene expression data. Consistency scores for each metabolic reaction determined based on the GIMME algorithm were compared between OAT1 knock-out versus wild-type mice. Intracellular pathways mediating nucleotide, fatty acid, arginine, tyrosine, and pyruvate metabolism were implicated to be broadly perturbed in the kidney when the OAT1 function was absent. Only metabolic pathway subsystems containing more than one perturbed reaction are shown in this figure.
eling approach can direct further investigation of metabolites in pathways highlighted in the computational analysis but not previously implicated in OAT biology. These include intermediates in polyamine metabolism.

We were able to confirm the prediction of a novel interaction between spermidine and spermine (intermediates in the polyamine pathway) and OAT1, with an IC50 of 2.0 ± 0.7 and 1.6 ± 0.6 mM, respectively, when 6CF is used as a tracer. Arginine also interacted with OAT1 at an IC50 of 1.6 ± 0.3 mM in transfected cells. It is now clear that OAT1 can transport cations as well as anions, and a number of accepted OAT1 substrates, such as methotrexate and cimetidine, have \( K_r \) values in the millimolar range (25). We have observed almost no specific tracer uptake in untransfected CHO cells. Moreover, in the OAT1-transfected cells, probenecid almost completely blocks tracer uptake (data not shown). Thus, this appears to be a specific phenomenon.

We further examined the interaction of the polyamines with OATs at the level of the tissue, using adult kidney slices as a model of OAT-dependent transport in its more native location within the renal tubule. Spermidine and arginine inhibited uptake of the OAT substrate, 6CF, by 46 and 68%, respectively (Fig. 5), consistent with interaction with OATs. These results are similar to our data from the *Xenopus* oocyte and CHO cell culture assays. We also studied the interaction of the polyamines with known OAT1 drug substrates and found that sper-

| TABLE 1 |

Top metabolites linked to OAT1 in metabolomic and transcriptomic analyses

| Unique metabolites, urinary metabolome | Unique metabolites, renal transcriptome |
|----------------------------------------|----------------------------------------|
| (Z,Z,Z)-8,11,14-Eicosatrienoate         | 2-Hydroxybutyrate                       |
| 2,6-Dimethylheptanoyl carnitine        | 4-Aminobutyrate                        |
| (2AR,25)-Dihydroxyvitamin D3           | Acetoacetate                           |
| 2-Hydroxyphenylacetate                 | Acetone                                |
| 3,4-Dihydroxy-1-phenylalanine exchange | Alanine                                |
| 3,4-Dihydroxymandelate                 | Ammonia                                |
| 3,4-Dihydroxyphenyletheneglycol exchange | Bisconitate                        |
| 3α,7α,12α-Trihydroxy-5β-cholanoate     | Bilirubin monoglucuronide              |
| 4-Hydroxyretinoic acid                 | Chloride                               |
| 4,17-Dihydroxyestradiol                | Cholate                                |
| 4-Methylpentanal                       | Choline                                |
| 4-Oxorenoic acid                       | 2-Alanine                              |
| 4-Pyridoxate                          | Dehydroascorbate                       |
| 6β-Hydroxytestosterone                 | Dehydroepiandrosterone sulfate         |
| 7α-(24S)-Dihydroxycholesterol         | Deoxyribonucleic acid                  |
| 7α-25-Dihydroxycholesterol             | Fructose                               |
| 7α-27-Dihydroxycholesterol             | Glucose                                |
| Adrenaline                             | Dihydroxyacetone phosphate             |
| Aldosterone                            | L-Lactate                              |
| Arachidonic acid                      | L-Mannose                              |
| Carbon monoxide                       | Dopamine 3-O-sulfate                   |
| Cervonic acid                         | Estradiol glucuronide                  |
| Chenodeoxyglycineolate                | Estrone 3-sulfate                      |
| Clupanodic acid                       | Galactose                              |
| Corticosterone                        | Glyceraldehyde 3-phosphate             |
| Cortisol                              | Glycine                                |
| Docosa-4,7,10,13,16-pentaenoic acid (n-6) | Glycocholate                       |
| Glutathione (oxidized)                | Glutathione (oxidized)                 |
| Glycochenodeoxycholate                | Histidine                              |
| Glyoxylate                            | Isoleucine                             |
| Hydrogen peroxide                     | L-Leucine                              |
| Hypotaurine                           | Methionine                             |
| L-Carnitine                           | Phenylalanine                          |
| Leukotriene A4                        | Proline                                |
| Leukotriene B4                        | Serotonin                              |
| Linoleic acid                         | Spermine                               |
| Norepinephrine                        | Taurocholate                           |
| \( α \)-Hydroxydodecanoate \( n-C12:0 \) | Thromboxane A2                        |
| \( α \)-Hydroxyhexadecanoate \( n-C16:0 \) | T3                                     |
| \( α \)-Hydroxytetradecanoate \( n-C14:0 \) | Taurine                                |
| Orotate                               | Vaccenic acid                          |
midine inhibited the uptake of [3H]methotrexate (Table 2 and Fig. 6). Taken together, these findings are particularly interesting because of the known importance of polyamines in kidney proximal tubule (the primary site of OAT1 expression) metabolism (28) and because of recent data indicating that OATs can bind a variety of weakly cationic organic molecules as well as anions (25).

DISCUSSION

The kidney proximal tubule is the site of highly active metabolic functions that supply the considerable energy and substrate demand posed by its extensive transport processes. The OATs, among the most abundantly expressed proximal tubule genes, mediate an important component of these transport processes and are thus intimately linked to the metabolic reactions occurring within this long, convoluted nephron segment (2, 29, 30). However, the interactions between OATs and renal metabolic pathways are not well understood.

We previously described potential endogenous OAT1 substrates identified by targeted GC-MS analysis of plasma and urine from wild-type and OAT1 knockouts (7). These substrates included 4-hydroxyphenyllactate and 4-hydroxyphenylacetate (intermediates of tyrosine catabolism), 3-hydroxyisobutyrate (an intermediate in valine catabolism), and 3-hydroxybutyrate (an intermediate in ketone metabolism), suggesting involvement of OAT1 in amino and fatty acid metabolism. This analysis, however, was simply limited to identifying statistically significant differences of individual substrate concentrations in plasma and/or urine of wild type and knockouts. The present analysis takes an integrated "omics"-driven network approach that 1) enabled a multilevel analysis of different data types (i.e. transcriptomic and metabolomic); 2) highlights complementary metabolic pathways that are potentially perturbed for the given physiological condition (i.e. OAT1-deleted transport); and 3) experimentally verified novel metabolites of interest that were linked to the OAT1 function.

First, we assessed the reaction pathways interconnected between these observed metabolite changes within the context of a genome-scale network reconstruction. We further analyzed gene expression data from OAT1 wild type and knockout to identify complementary pathway signatures.

FIGURE 4. Inhibition of OAT1-mediated tracer uptake by endogenous metabolites. Concentration-dependent inhibition of fluorescent tracer (6CF) uptake by mOAT1 was noted for β-hydroxybutyrate, spermidine, spermine, arginine, pyruvate, and α-ketoglutarate. β-Hydroxybutyrate, spermidine, spermine, and α-ketoglutarate were tested in Xenopus oocyte transport assays, whereas arginine and pyruvate were tested in CHO cell culture assays.
that indicated significant functional activity changes between wild-type and OAT1 knock-out mice. The identified metabolic pathways were consistent with in vitro, ex vivo, and previously reported in vivo data, suggesting that computational analyses employing Recon 1 can be useful tools in systematically elucidating metabolic pathways that are linked to transporter functions.

The flux balance gene expression analysis implicated OAT1 in the pentose phosphate shunt. The pentose phosphate shunt, by oxidizing glucose, provides NADPH, which is required for the biosynthesis of steroids, nucleotides, and fatty acids. The microarray analysis showed significant differences in the enzymatic intermediates of the pentose phosphate shunt, including ribulose-5-phosphate 3-epimerase and phosphopentomutase. This pathway has been reported in renal tissue slices and nephron segments; moreover, the highest catalytic activity of glucose-6-phosphate dehydrogenase, the first enzyme in this pathway, has been found to be in the proximal and distal tubule (1, 31).

There were also significant increases in metabolic functions involving intermediates of the TCA cycle and energy production in the OAT1 KO compared with WT mice. The proximal tubule depends on the TCA cycle to provide the ATP necessary for the energy requirements of this tissue.

The polyamine pathway was also implicated by the metabolomic and microarray FBA analyses. The proximal tubule contains ornithine decarboxylase, which is the key enzyme in the polyamine pathway and converts l-ornithine into putrescine (33). Ornithine aminotransferase, also found in proximal tubules (34), produces l-glutamate and glutamate-γ-semialdehyde from l-ornithine and α-ketoglutarate. l-Ornithine serves as a source of energy for the proximal tubular cell by being completely oxidized in the TCA cycle. Polyamines serve as important regulators of cellular proliferation and play a key role in tissue repair after kidney injury (35, 36). We were able to

![Figure 5](image)

**FIGURE 5.** Inhibition of 6CF uptake by polyamines in adult kidney slices. **A–D**, fluorescent photomicrographs of 6CF (1.0 μM) uptake (white) in adult kidney slices in the presence of either vehicular control (A), 2.0 mM probenecid (B), 2.0 mM spermidine (C), or 2.8 mM arginine (D). The bar graph (E) shows quantitative analysis of the fluorescent signal from 6CF absorption in adult kidney slices (*, p < 0.05). Images are representative of quadruplicate slices from the same experiment. Scale bar, 0.2 mm. Error bars, S.E.

**TABLE 2**

Interaction between metabolites identified from the computational analysis and OAT1 drug substrates and tracers

| Endogenous metabolite (reference) | OAT1 substrates |
|----------------------------------|----------------|
| Spermidine (this work)           | Methotrexate   |
| Uric acid (46)                   | Olmesartan     |
| Cholate (19)                     | PAH*           |
| Taurocholate (47)                | PAH            |
| Corticosterone (48)              | PAH            |
| Estrone 3-sulfate (19)           | PAH            |
| Pyruvate (19)                    | PAH            |
| Dehydroepiandrosterone sulfate (49) | PAH          |

* PAH, p-aminobenzoic acid.
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demonstrate a novel interaction between several intermediates in the polyamine pathway (arginine, spermidine, and spermine) and OAT1.

Among the overlapping metabolite predictions from the metabolomic and microarray constraint-based analyses was uric acid. Uric acid is the major breakdown product of purine metabolism and is mainly excreted by the kidneys in humans. Elevated levels of uric acid are associated with many important clinical conditions, including kidney stones, gout, cardiovascular disease, and the metabolic syndrome (37). Due to its primary elimination by the kidneys, the transporters involved in the reabsorption and secretion of uric acid have been extensively studied, including OAT1. The involvement of OAT1 in uric acid transport has been supported by studies demonstrating decreased secretion of uric acid in OAT1 null mice (38) and in vitro studies showing transport of urate by human OAT1 (39).

In general, the focus on OATs has been primarily on their role in drug and toxin handling (40). Given that the proximal tubule of the kidney is highly active in transport and metabolism, a key question is the extent to which these processes are linked. If so, OATs, particularly OAT1, are likely to be the main transporters involved because it is known, from isolated experiments, that key metabolites can be transported by OAT1 (2, 7, 19, 41).

Our results show that the mathematically structured global metabolic network model is a powerful tool that can integrate and analyze data from different levels (i.e. transcriptomic and metabolomic) to identify and link these metabolites and endogenous pathways that involve OAT1 and can, therefore, more broadly identify other aspects of kidney function that have remained unknown until now. It can also be used to predict severe adverse metabolic disturbances that arise from accumulation of endogenous metabolites resulting from conditions that perturb the functional activity of the OATs, such as chronic kidney disease, renal ischemia, drug interaction, or drug-induced nephrotoxicity. For example, some OAT1 metabolites have been found to accumulate in the setting of chronic kidney disease and mediate uremic toxicity (42). During renal failure, indoxyl sulfate, a product of dietary protein metabolism that interacts with OAT1, OAT3, and OAT4, accumulates within the proximal tubule cell and triggers free radical production and nephrotoxicity (43). Accumulated indoxyl sulfate may also perturb metabolism in other organs, such as the bone, where it is taken up by OAT3 expressed in bone osteoblasts and may generate free radicals and lead to reduced level of parathyroid hormone receptor expression (44). The global metabolic network model can be used to predict these systemic metabolic changes, which seem consistent with the remote sensing and signaling hypothesis for multispecific drug transporters (2).

Other potential applications of such a model are numerous, including the large scale analysis of high throughput data of substrate interactions with the OATs, which could lead to further discoveries of novel metabolic pathways that involve the OATs and a better understanding of the binding and transport properties of the OATs. A possible future area of study would be to utilize the global metabolic network model to analyze OAT1-mediated changes in biological systems treated with known OAT1 drug substrates (i.e. comparing the metabolome in OAT1 WT and KO mice treated with known OAT1 substrates) to assess the role of OAT1 in excretion and reabsorption of specific drugs and the corresponding physiological effects. Competition between drugs, such as HIV antiretrovirals and diuretics (which have been shown to be handled by OAT1 (21, 26, 45)) and endogenous metabolites for transport and handling via OAT1 (Table 2), could lead to alterations in metabolite concentration and contribute to drug-mediated metabolic syndromes, an important clinical problem. For example, we showed an inhibitory effect on methotrexate uptake by spermidine in vitro, suggesting that spermidine metabolism may potentially be altered in patients receiving methotrexate as treatment. Angiotensin II receptor blockers, such as olmesartan, were also shown to inhibit uptake of uric acid by OAT1 in a concentration-dependent manner (46) (Table 2). In summary, combining a global metabolic network analysis model with experimental validation is a previously unattempted approach that has numerous potential capabilities that can broaden our understanding of the diverse functions of OAT1 in substrate transport and metabolic regulation as well as its hypothesized role in “remote sensing and signaling” (2, 50).

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