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Key structural features for substrate binding to organic anion transporter 1 (Oat1; slc22a6) identified by global untargeted metabolomics of Oat1null plasma

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Abstract

Untargeted metabolomics on the plasma and urine from wild-type and organic anion transporter-1 (Oat1/Slc22a6) knockout mice identified a number of physiologically important metabolites, including several not previously linked to Oat1-mediated transport. Several, such as indoxyl sulfate, derive from Phase II metabolism of enteric gut precursors and accumulate in chronic kidney disease (CKD). Other compounds included vitamins (pantothenic acid, 4-pyridoxic acid), urate, and metabolites in the tryptophan and nucleoside pathways. Three metabolites, indoxyl sulfate, kynurenine and xanthurenic acid, were elevated in the plasma and interacted strongly and directly with Oat1 *in vitro* with IC₅₀ of 18μM, 12μM and 50μM, respectively. A pharmacophore model based on several identified Oat1 substrates was used to screen the NCI database and interaction candidate compounds with Oat1 was validated in an *in vitro* assay. Together, the data suggest a complex, previously unidentified remote communication between the gut microbiome, Phase II metabolism in the liver, and elimination via Oats of the kidney, as well as indicating the importance of Oat1 in the handling of endogenous toxins associated with renal failure and uremia. The possibility that some of the compounds identified may be part of a larger remote sensing and signaling pathway is also discussed.

INTRODUCTION

The human kidney is a complex organ responsible for the clearance of endogenous metabolites, toxins, and xenobiotics from the body while maintaining the correct balance of fluid, ions, and many small molecules. The kidney, together with other epithelial organs, maintains this homeostatic balance through polarized distribution of numerous channels and

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transporters. The organic anion transporters (OATs) are members of the SLC22 family of solute carriers and, with other SLC transporter families (eg. OATPs, SLC21), as well as members of the ATP-binding cassette (ABC) transporter family, are critical for the transport of drugs and toxins in the kidney and a variety of epithelial tissues, including liver, choroid plexus, placenta, olfactory mucosa, intestine.¹ Some of these same transporter proteins also operate in other contexts, such as transport of drugs and metabolites across the blood-brain barrier.

Organic anion transporters (Oats) expressed on the brush border and basolateral membrane of proximal tubular cells of the kidney and belonging to the SLC22A family of drug transporters have now been well-characterized functionally by the targeted *in vitro* testing of substrates one-by-one.² Of these, Oat1 (SLC22A6) plays a major role, along with Oat3 (SLC22A8), in the rate-limiting step of excretion of toxins and metabolites from the body into urine. Thus the Oats, which have been widely studied using transfected cells, renal slices, *Xenopus* oocytes, and kidneys in live organ cultures³ are known to play a significant role in eliminating xenobiotics, environmental toxins, and endogenous metabolites.^{4, 5}

Oat1 is a member of a new subfamily of solute carriers functioning as drug transporters capable of handling both organic anionic and cationic substrates.^{6, 7} The SLC22 family also includes the organic cation transporters (OCTs), organic carnitine transporters (Octns) fly-like-putative transporters (flips) and unknown substrate transporters (USTs).^{8, 9} Many Oats and Octs are multispecific transporters and, in some cases, such as Oat1, the range of substrates (mostly drugs) includes over a hundred small molecules.⁵ Although recent pharmacophore modeling has begun to define the molecular determinants of substrate interactions,^{7, 10, 11} most data on substrate binding and transport by Oat1 and other SLC transporters has been obtained by targeted transport assays in microinjected *Xenopus* oocytes or transfected cells. Since the choice of substrates to test has often been dictated by pharmaceutical relevance, the impression is that these proteins are primarily transporters of drugs, a view that has recently been questioned.^{12, 13} These transporters have been evolutionarily quite conserved as a family, and their diverse expression patterns, both in adult and embryonic tissue suggest other roles.² Moreover, injury to one organ, such as the liver, often alters expression of family members in another organ, such as the kidney.¹⁴ Since it is clear that these transporters have a role in the handling of endogenous substrates, it has been further hypothesized, based on a large body of circumstantial evidence, that these “multispecific drug transporters” (SLC and ABC families) play an important role in remote sensing and signaling between organs and, possibly, between organisms.^{12, 13} Nevertheless, the identification of key physiological metabolites transported by any single transporter has not been systematic and this has not been the primary focus of standard *Xenopus* oocyte and transfected cell assays which have usually focused on individual pharmaceuticals (e.g., antibiotics, antivirals and diuretics), as well as toxins.⁵

Metabolomics is the systems-wide analysis of small molecule compounds, including endogenous metabolites, xenobiotic compounds, drug metabolites and others. Mass spectrometry in combination with liquid chromatography (LC/MS) has proved a sensitive and effective approach to profiling hundreds to thousands of molecules in a particular tissue or biofluid. In addition to investigations directly in humans¹⁵, rodent models of kidney injury and disease have been investigated by untargeted metabolomics methods, including both NMR and mass spectrometry approaches^{16–19}

In this study untargeted metabolomic analysis of plasma and urine from Oat1 KO animals was applied in an attempt to identify, *en masse*, endogenous substrates without prior knowledge of transporter selectivity. This was followed by confirmation of the substrate interaction with Oat1 using *in vitro* assays. Using this global approach, metabolites were

identified whose excretion into the urine or retention in the plasma was altered by the absence of Oat1-mediated transport. Several of these are derived from Phase II metabolism of precursors produced by the enteric gut bacteria and were not previously considered Oat1 substrates. The ability of several of these metabolites to bind Oat1 was confirmed in *in vitro* *Xenopus* oocyte transport assays. In addition to identifying novel endogenous substrates of Oat1 using untargeted metabolomics, we designed pharmacophore models based on these metabolites and used them to virtually screen the Open NCI Database. (<http://cactus.nci.nih.gov/download/nci/>). Significant binding to Oat1 for some of the compounds obtained from the screen was confirmed in *in vitro* assays. Taken together, the data suggest secondary or indirect connections between the kidney, via Oat1-mediated handling, and enterobiome metabolites. Some of these metabolites have been very recently hypothesized, and clinically identified by metabolomic profiling, to mediate uremic toxicity in patients with end stage renal disease (chronic kidney disease (CKD)).²⁰ Thus, the data further suggest that Oat1 potentially plays a major role in the rate limiting step in the uptake and excretion of these putative toxic metabolites.

EXPERIMENTAL METHODS

Untargeted Metabolomics

An untargeted, mass-spectrometry-based approach to metabolomics was used and included reverse phase chromatography and electrospray ionization with accurate mass determination. Data were analyzed using XCMS, with non-linear data alignment and intensity integration.²¹ This approach can locate very small differences in very large data sets. Molecules were identified using accurate mass, database searching, and LC/MS/MS (QTOF) (see Supplemental Data Table S1). Plasma samples from Oat1 knockouts were compared with plasma samples from wild type, similarly, urine samples from both knockout and wild type were compared. The concentration ratios of metabolites revealed those molecules with altered plasma/urine distribution. Because Oat1 is predominantly an anionic transporter, the ratios of negatively charged, highly polar molecules were expected to be affected. Methods were developed to measure polar, negatively charged compounds since many of these are not captured with routine metabolomics methods.

All animals were handled in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Six mice were used from each group of knockout and wild type (n=6). Oat1 KO mice were backcrossed to C57BL6 for 10 generations (N10). Age matched Oat1 KO mice and wild type control (in separate cages) were used. Simultaneous blood and urine samples were collected and plasma and urine were stored at -80°C until metabolite analysis was done. The metabolomics experiments were then run together and analyzed as a single group.

Metabolites were extracted from plasma and urine with methanol. Four volumes of ice cold methanol were added to 50 µl of either plasma or urine, vortexed, and incubated at -20°C for one hour. Samples were centrifuged 10 min. at 14,000 g, the supernatant was collected, and the centrifugation was repeated. The supernatant was dried in a SpeedVac, resuspended in 50 µl 95:5 water:acetonitrile, and clarified for 5 min at 14,000 g. Urine samples were normalized based upon measured creatinine concentrations.

4 µl of extracted plasma maintained at 4°C in an autosampler was applied to a Waters T3 HPLC column (2.1 mm ID × 150 mm length) via an LC stack consisting of a binary pump, degasser, and thermostatted autosampler (Agilent). The flow rate was 200 µl/min, with solvent A composed of water containing 0.1% formic acid, and solvent B composed of acetonitrile containing 0.1% formic acid. A gradient from 0% (hold for 2 min) to 95% B over 20 min, followed by a hold for 1 min at 95% B, a gradient to 10% B over 1 min, at

which point the flow rate was increased to 300 μ l/min, and a gradient back to 95% B over 5 min, hold for 0.5 min, and a gradient back to 0% B over 1 min, and 5 min for re-equilibration. The method incorporated a wash, and the data collection ended at 30 min, with a total injection-to-injection time of 36.5 min. Data were collected separately in positive and negative ion electrospray mode on a TOF 6210 (Agilent). The MS parameters were capillary 3500V, nebulizer 30 psig, drying gas 10 L/min, gas temp 325°C and fragmentor 120V. The mass range was scanned from m/z 50 – 1000, at ~1 scan/sec. Auto tuning, including calibration, was performed immediately before the run.

To reduce systematic error associated with instrument drift, samples were run in random order. Data in instrument specific format were converted to cdf or mzXML format files. The program XCMS was used for non-linear alignment of the data in the time domain and automatic integration and extraction of the peak intensities.²¹ These integrated peak intensities form the basis of the relative metabolite quantitation.

A combination of approaches was used for metabolite identification using mass spectrometry. Firstly, accurate mass information from the TOF (within 5ppm) was available from the profiling data. This accurate mass information was used to search metabolite databases for matches based on mass, and these hits were used to provide a list of “informed guesses” as to the identity of the metabolites of interest. This was followed by LC/MS using a QTOF, reproducing the LC conditions so that the retention time of each metabolite would be the same as from the profiling experiment and then each metabolite (consisting of an accurate m/z, retention time pair) was targeted for MS/MS using the QTOF.

The experiment used an Agilent 6510 QTOF, with source parameters set identically to those on the TOF. Both the TOF and the quadrupole were tuned, including calibration, immediately before use. After an initial run with collision energy of 15V, the fragmentation patterns were evaluated and the collision energy adjusted when necessary, and the run repeated. Metabolite standards for comparison were run under identical conditions. Three metabolomics databases were used for compound identification: METLIN²², LIPIDMAPS²³, and HMDB²⁴. In addition, PUBCHEM and SciFinder Scholar were used for chemical searches. KEGG²⁵ was used for pathway analysis.

Microinjected *Xenopus* oocyte Binding Assay

Some of the compounds identified by the untargeted metabolomics were obtained from Sigma-Aldrich (St. Louis, MO) and included indoxyl sulfate, xanthurenic acid and kynurenine,. Additionally, compounds identified from the Open NCI Database (<http://cactus.nci.nih.gov/download/nci/>) on basis of the Oat1 pharmacophore model were obtained from the NCI Open Repository Of Synthetic And Pure Natural Products (http://dtp.nci.nih.gov/branches/dscb/repo_open.html) including Chemical Abstract Service (CAS) 5435-73-4. *Xenopus* oocyte assays were performed as previously described in detail.^{7, 10, 11} Briefly, mouse Oat1 (Image clone ID 4163278) was linearized using NotI restriction enzyme and capped RNA was synthesized by in vitro transcription using the mMessage Machine kit from Applied Biosciences/Ambion (Austin, TX). *Xenopus* oocytes were freshly isolated, defolliculated and used for microinjection of 23 nl/oocyte of the capped mOat1 RNA. The inhibition of compounds in presence of 30 μ M of 6-carboxyfluorescein (6CF)¹¹ as a tracer, was tested by incubating the injected oocytes with mOat1 for one hour, followed by washing with ice cold PBS (containing Ca and Mg). Inhibition of 6CF uptake was measured using a fluorometer as previously described.¹¹

Calculations and Statistics

The compounds (inhibitors) were tested at 3–4 different concentrations (from the highest concentration of 10mM to the lowest of 10 μ M) and the averages of four measurements (n=16 oocytes) were used as single data points. Using the Prism software 5.0 (GraphPad Inc., San Diego, CA) the inhibition data was curve-fitted by nonlinear regression to calculate $IC_{50} \pm$ S.E. as described in detail previously.⁷

Computational Modeling

The pharmacophore models were developed using plasma and urine metabolites identified by untargeted mass spectrometry,²⁶ including nine compounds increased in plasma: 4-pyridoxic acid, indole-3-lactic acid, indoxyl sulfate—indican, kynurenine, L-methionine, *p*-hydroxyphenyllactic acid, pantothenic acid, phenylacetylglycine, and phenyl sulfate; and six decreased in urine: 5-methylcytidine, acetylglycine, *N*2-dimethylguanosine, thymidine, urate—trioxopurine, and xanthurenic acid. Development of pharmacophores included several stages: (1) clustering (grouping) the metabolites; (2) designing a common pharmacophore hypothesis for each cluster; (3) searching compound databases using designed pharmacophores.⁷

Creating three-dimensional (3D) structures for each metabolite

Two-dimensional (2D) structures were data-mined from public databases and peer-reviewed articles. Three-dimensional structures were created from the 2D structures using the GlycoBioChem PRODRG2 Server (<http://davapc1.bioch.dundee.ac.uk/prodrg/>)²⁷ with JME Molecular Editor.^{28, 29} The algorithm for the conversion of 2D molecular structures into 3D structures utilizes the GROMINGEN Machine for Chemical Simulations (GROMACS) library of three-atom combination geometries employing a combination of short molecular dynamics simulations and energy minimizations.³⁰ The 3D structures were converted from 2D structures and calculated either as neutral molecules or anions, depending of their molecular formula.

Superimposition and clustering of metabolites

The 3D molecular structures of the compounds were superimposed on each other and conformationally aligned using Molecular Operational Environment (MOE, Chemical Computing Group, Montreal) software and five clusters of compounds based on the geometrical structural fit and chemical features were identified for untargeted blood plasma data and two for untargeted urine data.

Designing consensus pharmacophore models for each aligned cluster

Since most metabolites have the following features: hydrogen bond donor, hydrogen bond acceptor, hydrophobic groups, aromatic ring, and double oxygen functions, consensus pharmacophore models of metabolite clusters were designed from flexible alignment of compounds in each cluster using MOE software with the above mentioned pharmacophore features. According to the complexity of selected clusters, the hypotheses for different metabolite clusters contain different number of features, varying from five to twelve. Finally, a pharmacophore-directed search of the NCI database of compounds was performed. To avoid exclusion of possible high scoring candidates, a less restrictive criteria than “all features search” for the pharmacophore-directed search was used, for example: four out of five features, five of six, six of seven, etc.

RESULTS

Metabolite identification by untargeted metabolomics

A combination of an Oat1 gene deletion in mice and mass spectrometry-based untargeted metabolomics was used to characterize the molecular transport specificity of Oat1 and to identify new endogenous substrates. By deleting the transporter and determining the changes in small molecule concentration in plasma and urine (representing the upstream and downstream sides of kidney transport), a wide range of endogenous metabolites was surveyed. Molecules affected by the knockouts were identified.

The metabolomics workflow is shown in Figure 1. Because the Oat1 transporter is known to primarily transport charged and highly polar compounds, methods were added to the routine profiling approach that were optimized to capture polar compounds and to include negatively charged molecules (anions). These are among the most challenging molecules to profile using LC/MS and which may have been missed in the original report of the targeted analysis in the Oat1 knockouts, that utilized a GC/MS approach with derivatization, targeting small organic anions known to be important in human metabolic disease.³¹ Compounds such as amino acids and organic anions were used as control substances to determine the baseline conditions under which such molecules would chromatograph and ionize. A number of columns were compared, including standard reverse phase C18, hydrophilic interaction (HILIC), and polar reverse phase. A polar end-capped column which can run in 100% aqueous phase had optimal overall properties, and was ultimately used for the experiment.

Figure 2 shows the plots that represent the average intensity in WT and KO for the 100 most significant ions, based upon a t-test calculation. The deletion of Oat1 results in a systematic increase of plasma metabolites (Fig. 2a), a trend that is reversed in urine (Fig. 2b) (i.e., specific molecules accumulate in the plasma whereas their concentration is reduced in urine due to the absence of the transporter). More than a dozen of the affected molecules were identified, including phenylacetyl glycine, p-hydroxyphenyl lactic acid, orotic acid, indole-3-lactic acid (cinnamoyl glycine), indoxyl sulfate, and phenyl sulfate (Tables 1–2). As described in a separate study, these compounds, absent from the plasma of germ-free mice, are produced both directly or indirectly by the gut microbiome or “enterobiome.”²⁶ Indoxyl sulfate and phenyl sulfate have also been shown to be uremic toxins increased in the plasma of patients with chronic kidney disease.³² Additionally, a recent clinical study identified a subset of Oat1 KO metabolites in patients with end stage renal disease by metabolomic profiling.²⁰

Other affected molecules included pantothenic acid, 4-pyridoxic acid (two vitamins not previously associated with Oat1 transport), urate and xanthurenic acid (Tables 1–2). All of the identified compounds share some structural similarities and identification of the core structural motifs may allow for the prediction of the transport of new drugs. The affected pathways include those involved in the metabolism of tryptophan, vitamins, and nucleosides. Molecules that are part of the microbiome cycle, including those which represent phase II biotransformations of gut microbiome products (i.e., indoxyl sulfate, phenyl sulfate, p-aminocresol sulfate and phenylacetyl glycine) were particularly affected²⁶ (Tables 1–2). Boxplots of the relative plasma concentrations for four compounds in Oat1 wild type and KO are also shown in Figure 3.

In vitro validation of identified metabolites in *Xenopus* oocyte binding assay

Validation of significantly altered uremic toxins identified by *in vivo* untargeted metabolomics was performed using the *in vitro* *Xenopus* oocyte assay. The identified compounds that were tested included: indoxyl sulfate, xanthurenic acid, and kynurenine.

IC₅₀ (affinity) values were determined by measuring the inhibition of 6-carboxyfluorescein uptake.^{7, 11} Based on the IC₅₀ values of the indoxyl sulfate (18μM) (a known uremic toxin), kynurenine (12μM) and xanthurenic acid (50μM) (both from the tryptophan pathway), these compounds showed a strong affinity for mOat1 transporter (Fig. 4).

Pharmacophore model and in vitro validation

Using flexible alignment in the MOE software, we clustered plasma metabolites identified with the untargeted metabolomic approach into five groups based on geometrical and chemical properties. One of the clusters consisted of a single molecule, 4-pyridoxic acid, a vitamin metabolite.

A pharmacophore hypothesis takes into account functionally important atoms and their geometrical locations. After the model is developed, experimental validation can be obtained by the screening of 3D structure databases; hits are those with significant superimposition scores with the hypothesis. Using the pharmacophore, one can search known compound databases to find a set of molecules that might be employed as templates for drug design.

For pharmacophore modeling, a ligand-based approach was used, which presumes that the conformation of the ligand used for pharmacophore-hypothesis design interacts in the environment of the transporter to which it binds. The structure of one of the plasma metabolites, 4-pyridoxic acid (Fig. 5A) and the 3D representation of the ligand-based pharmacophore model (Fig. 5B), as well as the compound identified in the screen (CAS 5435-73-4; C₁₂H₆N₄O₇) (Fig. 5C) are shown. Color spheres represent the seven pharmacophore features: green—two hydrophobic (Hyd), orange—one aromatic (Aro), red—one hydrogen-bond acceptor (Acc) and one double oxygen (O2), and cyan—two hydrogen-bond donors and/or acceptors (Don&Acc). The pyridine aromatic ring has both aromatic and hydrophobic features. The hydrophobic feature has a greater size because it includes the pyridine aromatic ring and a methyl group. Another methyl group is identified by a separate hydrophobic feature. There are two OH groups that could be either hydrogen-bond donors or hydrogen-bond acceptors. The remaining group contains two oxygens and is a stronger feature than a single oxygen. It is defined either by two “anionic or acceptor” features, or both atoms are defined with the feature “double oxygen,” which we used in further analysis.

We conducted the pharmacophore search using the National Cancer Institute (NCI) Open database, containing chemical information for over 250,000 compounds with 3D coordinates. The screen was initiated using six of the seven features of the developed pharmacophore model which made it possible to test various combinations of features. Since *a priori* prediction of critical features in the screen is difficult, reducing the number of features enabled more flexibility. However, each screen had to be optimized because selecting too few features generates too many hits. For example, the search of the NCI database using the consensus pharmacophore from a single cluster generated 2991 hits and, in the majority of cases, the selected compounds represented fragments of larger compounds. To further reduce the number of hits, the list was also filtered by molecular weight and solvent accessible surface (SAS). Applying a molecular weight cutoff of 400 and a SAS of 500Å², the list was reduced to 556 hits. Further application of a root mean square deviation (RMSD) filter (RMSD <1) narrowed the list to 221 compounds (see Supplemental Data Table S2). From this list, one compound, C₁₂H₆N₄O₇ (CAS 5435-73-4), which was calculated to have the lowest total self-consistent field (SCF) energy of compound conformers (-1890026.395 kJ/mol)—corresponding to the greatest probability of existence of this conformer since there is a clear dependence between SCF energy and relative Boltzman probability of specified conformer³³—was selected and tested in vitro for its ability to interact with Oat1 (Fig. 5C).

In vitro testing of best hit compound

The compound predicted by the pharmacophore was tested for a functional effect on organic anion transport by Oat1 which was curve fitted using a nonlinear regression with Prism software 5.0 (Fig. 5D). The compound, CAS 5435-73-4, showed higher affinity for Oat1 compared to other tested substrates for the same transporter in the *in vitro* *Xenopus* oocyte assay (Fig. 5C). Inhibition of uptake of the Oat1 specific tracer, 6-carboxyfluorescein, was achieved with concentrations of CAS 5435-73-4 as low as 100 μ M and the IC₅₀ was calculated as 22 μ M (Fig. 5D). Thus the untargeted metabolomic analysis of the plasma and urine of the Oat1^{-/-} mice identified enterobiome-derived metabolites, putative uremic toxins, and other metabolites which can be used to find potentially novel substrates (some with a higher affinity for Oat1) by virtual screening of chemical libraries.

DISCUSSION

Previous targeted work, which focused on metabolic disease-related organic anions and used targeted metabolomics in Oat1 knockouts, demonstrated transport defects in the absence of this transporter.³¹ Similar targeted metabolomics analyses aimed at identifying endogenous substrates of the efflux transporter multidrug resistance protein 3 (MRP3) demonstrated a key role for MRP3 in the disposition of dietary phytoestrogens.³⁴ In this study, however, we have performed *en masse* untargeted metabolomics on urine and plasma samples collected from Oat1 knockout and wild type mice and have identified endogenous metabolites belonging to several different pathways. Most of the metabolites are clearly organic anions whereas others, such as 4-pyridoxic acid, are likely to be so at physiological pH.³⁵ Several of these metabolites have been shown to directly interact with the Oat1 transporter by *in vivo* and *in vitro* assays. The identified known and novel compounds, from the untargeted metabolomic analysis have allowed us to design Oat1 metabolite-specific pharmacophores using computational modeling methods and predicted further compounds with the pharmacophore-directed search of the NCI database. *In vitro* testing of the compound, CAS 5435-73-4 (Fig. 5C–D), identified in the pharmacophore search, showed a very strong and direct interaction with Oat1. It is worth noting here that Oat1 transport can be inhibited by both organic anions and cations.⁷

The findings also point to the potentially important connection between Oat1-mediated uptake of endogenous metabolites and the gut enterobiome. The importance of mammalian-microbiome interactions in conditions including cancer, diabetes, obesity, IBD (inflammatory bowel disease) and autoimmune diseases is becoming increasingly clear.^{36–42} In fact, a clinical study describing the metabolomic profiling of plasma from patients with end stage renal disease identified a subset of the same metabolites identified in the plasma of Oat1 KOs, a number of which are known to function as uremic toxins.²⁰ Major toxic compounds of the microbiome altered in the Oat1 knockouts included indoxyl sulfate,^{43, 44} a retention solute (not excreted in uremic syndrome)⁴⁵ as well as phenyl sulfate and indole-3 lactic acid.²⁶ Also found to accumulate in the Oat1 knockouts were enterobiome metabolites that have been modified by Phase II metabolism in the liver. Previous studies in transfected cell lines have shown that Oat1 plays a significant role in handling of tryptophan pathway metabolites in mammalian cell systems.⁴⁶ Here we report that some of these (kynurenine and xanthurenic acid) actually accumulated *in vivo* in the Oat1 knockouts and validated direct transporter interactions using the *Xenopus* oocyte assay.

Taken together, these data indicate a novel direct role of Oat1 in the handling and transport of enterobiome and uremic toxins and suggest that the complex connections between the enteric bacterial flora, liver metabolism, and kidney transport, are mediated, at least in part, by Oat1 (as shown in Figure 6). Whether this connection has additional physiological significance beyond the elimination of potential endogenous toxins remains to be

determined, but it has been suggested that some Oat1 metabolites play a role in remote sensing and signaling between organs and organisms.^{12, 13} For example, earlier targeted metabolomics in the Oat1 knockouts revealed that many odorant molecules, which act on olfactory odorant G-protein-coupled receptors and are substrates for the olfactory-specific transporter Oat6 enter the urine via Oat1.³¹ Odorant receptors have recently been identified in the kidney and elsewhere,^{47–49} raising other possibilities for remote communication regulated by SLC and ABC transporters, which themselves are regulated by injury to other organs through the secretion of growth factors, cytokines and other molecules. Thus, the larger pathway, in which Oats, other SLC genes, and ABC transporters participate may be quite complex. The type of approach we have employed here is an essential step toward understanding the broader role of multispecific drug transporters (SLC and ABC) in both normal and pathological states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Zair ZM, Eloranta JJ, Stieger B, Kullak-Ublick GA. Pharmacogenetics of OATP (SLC21/SLCO), OAT and OCT (SLC22) and PEPT (SLC15) transporters in the intestine, liver and kidney. *Pharmacogenomics*. 2008; 9(5):597–624. [PubMed: 18466105]
2. Nigam SK, Bush KT, Bhatnagar V. Drug and toxicant handling by the OAT organic anion transporters in the kidney and other tissues. *Nat Clin Pract Nephrol*. 2007; 3(8):443–8. [PubMed: 17653123]
3. Sweet DH, Eraly SA, Vaughn DA, Bush KT, Nigam SK. Organic anion and cation transporter expression and function during embryonic kidney development and in organ culture models. *Kidney Int*. 2006; 69(5):837–45. [PubMed: 16518343]
4. Dresser MJ, Leabman MK, Giacomini KM. Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters. *J Pharm Sci*. 2001; 90(4):397–421. [PubMed: 11170032]
5. VanWert AL, Gionfriddo MR, Sweet DH. Organic anion transporters: discovery, pharmacology, regulation and roles in pathophysiology. *Biopharm Drug Dispos*. 2010; 31(1):1–71. [PubMed: 19953504]
6. Lopez-Nieto CE, You G, Bush KT, Barros EJ, Beier DR, Nigam SK. Molecular cloning and characterization of NKT, a gene product related to the organic cation transporter family that is almost exclusively expressed in the kidney. *J Biol Chem*. 1997; 272(10):6471–8. [PubMed: 9045672]
7. Ahn SY, Eraly SA, Tsigelny I, Nigam SK. Interaction of organic cations with organic anion transporters. *J Biol Chem*. 2009; 284(45):31422–30. [PubMed: 19737926]
8. Eraly SA, Monte JC, Nigam SK. Novel slc22 transporter homologs in fly, worm, and human clarify the phylogeny of organic anion and cation transporters. *Physiol Genomics*. 2004; 18(1):12–24. [PubMed: 15054140]
9. Wu W, Baker ME, Eraly SA, Bush KT, Nigam SK. Analysis of a large cluster of SLC22 transporter genes, including novel USTs, reveals species-specific amplification of subsets of family members. *Physiol Genomics*. 2009; 38(2):116–24. [PubMed: 19417012]
10. Kaler G, Truong DM, Khandelwal A, Nagle M, Eraly SA, Swaan PW, Nigam SK. Structural variation governs substrate specificity for organic anion transporter (OAT) homologs. Potential

remote sensing by OAT family members. *J Biol Chem*. 2007; 282(33):23841–53. [PubMed: 17553798]

11. Truong DM, Kaler G, Khandelwal A, Swaan PW, Nigam SK. Multi-level analysis of organic anion transporters 1, 3, and 6 reveals major differences in structural determinants of antiviral discrimination. *J Biol Chem*. 2008; 283(13):8654–63. [PubMed: 18174163]
12. Ahn SY, Nigam SK. Toward a systems level understanding of organic anion and other multispecific drug transporters: a remote sensing and signaling hypothesis. *Mol Pharmacol*. 2009; 76(3):481–90. [PubMed: 19515966]
13. Wu W, Dnyanmote AV, Nigam SK. Remote Communication through Slc and Abc Drug Transporter Pathways: An Update on the Remote Sensing and Signaling Hypothesis. *Mol Pharmacol*. 2011
14. Brandoni A, Anzai N, Kanai Y, Endou H, Torres AM. Renal elimination of p-aminohippurate (PAH) in response to three days of biliary obstruction in the rat. The role of OAT1 and OAT3. *Biochim Biophys Acta*. 2006; 1762(7):673–82. [PubMed: 16844357]
15. Beger RD, Holland RD, Sun J, Schnackenberg LK, Moore PC, Dent CL, Devarajan P, Portilla D. Metabonomics of acute kidney injury in children after cardiac surgery. *Pediatr Nephrol*. 2008; 23(6):977–84. [PubMed: 18320237]
16. Hwang GS, Yang JY, Ryu do H, Kwon TH. Metabolic profiling of kidney and urine in rats with lithium-induced nephrogenic diabetes insipidus by (1)H-NMR-based metabonomics. *Am J Physiol Renal Physiol*. 2010; 298(2):F461–70. [PubMed: 19923409]
17. Lenz EM, Bright J, Knight R, Westwood FR, Davies D, Major H, Wilson ID. Metabonomics with 1H-NMR spectroscopy and liquid chromatography-mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat. *Biomarkers*. 2005; 10(2–3):173–87. [PubMed: 16076731]
18. Taylor SL, Ganti S, Bukanov NO, Chapman A, Fiehn O, Osier M, Kim K, Weiss RH. A metabolomics approach using juvenile cystic mice to identify urinary biomarkers and altered pathways in polycystic kidney disease. *Am J Physiol Renal Physiol*. 2010; 298(4):F909–22. [PubMed: 20130118]
19. Williams RE, Major H, Lock EA, Lenz EM, Wilson ID. D-Serine-induced nephrotoxicity: a HPLC-TOF/MS-based metabonomics approach. *Toxicology*. 2005; 207(2):179–90. [PubMed: 15596249]
20. Rhee EP, Souza A, Farrell L, Pollak MR, Lewis GD, Steele DJ, Thadhani R, Clish CB, Greka A, Gerszten RE. Metabolite profiling identifies markers of uremia. *J Am Soc Nephrol*. 2010; 21(6):1041–1051. [PubMed: 20378825]
21. Benton HP, Wong DM, Trauger SA, Siuzdak G. XCMS2: processing tandem mass spectrometry data for metabolite identification and structural characterization. *Anal Chem*. 2008; 80(16):6382–9. [PubMed: 18627180]
22. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. *Ther Drug Monit*. 2005; 27(6):747–51. [PubMed: 16404815]
23. Fahy E, Sud M, Cotter D, Subramaniam S. LIPID MAPS online tools for lipid research. *Nucleic Acids Res*. 2007; 35(Web Server issue):W606–12. [PubMed: 17584797]
24. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel HJ, Querengesser L. HMDB: the Human Metabolome Database. *Nucleic Acids Res*. 2007; 35(Database issue):D521–6. [PubMed: 17202168]
25. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*. 1999; 27(1):29–34. [PubMed: 9847135]
26. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci U S A*. 2009; 106(10):3698–703. [PubMed: 19234110]

27. Schüttelkopf AW, van Aalten DM. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr D Biol Crystallogr*. 2004; 60(Pt 8):1355–63. [PubMed: 15272157]
28. Ertl P. Molecular structure input on the web. *J Cheminform*. 2010; 2(1):1. [PubMed: 20298528]
29. Tetko IV, Tanchuk VY, Kasheva TN, Villa AE. Internet software for the calculation of the lipophilicity and aqueous solubility of chemical compounds. *J Chem Inf Comput Sci*. 2001; 41(2): 246–52. [PubMed: 11277705]
30. van Aalten DM, Bywater R, Findlay JB, Hendlich M, Hooft RW, Vriend G. PRODRG, a program for generating molecular topologies and unique molecular descriptors from coordinates of small molecules. *J Comput Aided Mol Des*. 1996; 10(3):255–62. [PubMed: 8808741]
31. Eraly SA, Vallon V, Vaughn DA, Gangoi JA, Richter K, Nagle M, Monte JC, Rieg T, Truong DM, Long JM, Barshop BA, Kaler G, Nigam SK. Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. *J Biol Chem*. 2006; 281(8):5072–83. [PubMed: 16354673]
32. Kikuchi K, Itoh Y, Tateoka R, Ezawa A, Murakami K, Niwa T. Metabolomic analysis of uremic toxins by liquid chromatography/electrospray ionization-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2010; 878(20):1662–8.
33. Weltman JK, Skowron G, Lorient GB. The HF-SCF energy of HIV-1 MNgp120 V3 hairpin loop conformers. *J Mol Model*. 2004; 10(5–6):367–72. [PubMed: 15597205]
34. van de Wetering K, Feddema W, Helms JB, Brouwers JF, Borst P. Targeted metabolomics identifies glucuronides of dietary phytoestrogens as a major class of MRP3 substrates in vivo. *Gastroenterology*. 2009; 137(5):1725–35. [PubMed: 19577570]
35. Bueno C, Guerrero J, Encinas MV. Spectroscopic Properties of 4-Pyridoxic Acid as a Function of pH and Solvent. *Helvetica Chimica Acta*. 2004; 87(4):940–948.
36. Lampe JW. The Human Microbiome Project: getting to the guts of the matter in cancer epidemiology. *Cancer Epidemiol Biomarkers Prev*. 2008; 17(10):2523–4. [PubMed: 18842991]
37. Mai V, Draganov PV. Recent advances and remaining gaps in our knowledge of associations between gut microbiota and human health. *World J Gastroenterol*. 2009; 15(1):81–5. [PubMed: 19115471]
38. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C, Baker CC, Di Francesco V, Howcroft TK, Karp RW, Lunsford RD, Wellington CR, Belachew T, Wright M, Giblin C, David H, Mills M, Salomon R, Mullins C, Akolkar B, Begg L, Davis C, Grandison L, Humble M, Khalsa J, Little AR, Peavy H, Pontzer C, Portnoy M, Sayre MH, Starke-Reed P, Zakhari S, Read J, Watson B, Guyer M. The NIH Human Microbiome Project. *Genome Res*. 2009; 19(12):2317–23. [PubMed: 19819907]
39. Petrosino JF, Highlander S, Luna RA, Gibbs RA, Versalovic J. Metagenomic pyrosequencing and microbial identification. *Clin Chem*. 2009; 55(5):856–66. [PubMed: 19264858]
40. Proal AD, Albert PJ, Marshall T. Autoimmune disease in the era of the metagenome. *Autoimmun Rev*. 2009; 8(8):677–81. [PubMed: 19393196]
41. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. *J Physiol*. 2009; 587(Pt 17):4153–8. [PubMed: 19491241]
42. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature*. 2007; 449(7164):804–10. [PubMed: 17943116]
43. Deguchi T, Kouno Y, Terasaki T, Takadate A, Otagiri M. Differential contributions of rOat1 (Slc22a6) and rOat3 (Slc22a8) to the in vivo renal uptake of uremic toxins in rats. *Pharm Res*. 2005; 22(4):619–27. [PubMed: 15846470]
44. Motojima M, Hosokawa A, Yamato H, Muraki T, Yoshioka T. Uraemic toxins induce proximal tubular injury via organic anion transporter 1-mediated uptake. *Br J Pharmacol*. 2002; 135(2):555–63. [PubMed: 11815391]
45. Dou L, Bertrand E, Cerini C, Faure V, Sampol J, Vanholder R, Berland Y, Brunet P. The uremic solutes p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair. *Kidney Int*. 2004; 65(2):442–51. [PubMed: 14717914]
46. Bahn A, Ljubojevic M, Lorenz H, Schultz C, Ghebremedhin E, Ugele B, Sabolic I, Burckhardt G, Hagos Y. Murine renal organic anion transporters mOAT1 and mOAT3 facilitate the transport of

- neuroactive tryptophan metabolites. *Am J Physiol Cell Physiol*. 2005; 289(5):C1075–84. [PubMed: 15944205]
47. Feldmesser E, Olender T, Khen M, Yanai I, Ophir R, Lancet D. Widespread ectopic expression of olfactory receptor genes. *BMC Genomics*. 2006; 7:121. [PubMed: 16716209]
48. Zhang X, Firestein S. Genomics of olfactory receptors. *Results Probl Cell Differ*. 2009; 47:25–36. [PubMed: 19132320]
49. Zhang X, Rogers M, Tian H, Zou DJ, Liu J, Ma M, Shepherd GM, Firestein SJ. High-throughput microarray detection of olfactory receptor gene expression in the mouse. *Proc Natl Acad Sci U S A*. 2004; 101(39):14168–73. [PubMed: 15377787]

Figure 1. Metabolomic profiling of Oat1 knockout plasma and urine samples

Workflow diagram of the strategy used for untargeted metabolomic profiling of potential Oat1 substrates. a) Plasma and urine samples were collected from wild type and Oat1^{-/-} mice and compounds either increased in the plasma and/or decreased in the urine of Oat1^{-/-} mice were confirmed by LC/MS/MS by using a QTOF (model 6510, Agilent). The screening platform included reverse phase liquid chromatography and electrospray mass spectrometry with accurate mass determination. Data were analyzed using XCMS, with non-linear data alignment and intensity integration.²¹ b) Accurate masses of the identified molecules displaying significant differences in either the urine or plasma were searched against the METLIN, KEGG, HMDB, and LIPIDMAPS databases.

Figure 2. Overview of metabolic changes produced in OAT1 knockouts

Scatter plots of the average intensity of the 100 most significantly altered ions in the plasma (a) and urine (b) of WT and Oat1-KO (based upon p-value from a t-test calculation). In plasma, most of the changes are due to an increase in concentration in the knockouts compared to wild type, whereas in the urine this trend is reversed.

Figure 3.

Boxplots indicate the relative plasma concentrations for four compounds in Oat1 wild type and KO; two of these metabolites, phenyl sulfate (p-value = 1.3×10^{-3}) and indoxyl sulfate (p-value = 8.1×10^{-3}) represent phase II biotransformation of gut microbiome products. Structure images are from ChemSpider.

Figure 4. Interactions of metabolomic metabolites with mOat1

A number of metabolites were identified by non-targeted metabolomics and several (i.e., indoxyl sulfate, xanthurenic acid and kynurenine) were tested for Oat1-mediated handling in vitro utilizing microinjected *Xenopus* oocytes (3–4 different concentrations ranging from 10 μ m to 10mm were employed). Curves were plotted using non-linear regression with Prism software 5.0 and IC₅₀ values were calculated (GraphPad Inc., San Diego, CA). Each data point represents the mean $\pm$ S.E values of four groups of 4 oocytes each. All the tested compounds showed strong inhibition of uptake of fluorescent tracer (6-carboxyfluorescein) and interacted strongly with mOat1 transporter.

Figure 5. Oat1 Pharmacophore model designed from untargeted metabolites elevated in Oat1 knockout plasma

A) 3D structure of 4-pyridoxic acid: red, oxygen; blue, nitrogen; gray, carbon; white, hydrogen. B) Oat1 pharmacophore model based on 3D chemical structure of 4-pyridoxic acid. Colored spheres represent the structural features of the pharmacophore: green—hydrophobic (F5 & F7:Hyd), orange—aromatic (F4:Aro), red—hydrogen-bond acceptor (F6:Acc) and one double oxygen (F1:O2), and cyan—two hydrogen-bond donors and/or acceptors (F2 & F3:Don&Acc). C) The top hit compound derived from the pharmacophore model shown in panel B (CAS #5435-73-4). D) *In vitro* validation of the pharmacophore model using the *Xenopus* oocyte binding assay. CAS 5435-73-4, identified by the pharmacophore model in the NCI database, was tested at 5 different concentrations; the IC₅₀ was determined to be 22 μM.

Figure 6.

An illustration indicating the physiological connections and putative crosstalk between different Oats found in multiple tissues/organs of body utilizing endogenous metabolites as a remote sensing mechanism. Oat1 remains the major transporter across all these tissues.

TABLE 1

Comparison of Plasma

compound	formula	functional groups	biochemical pathways	Micr.	XlogP	Hbond don/acc	fold	p-value	up in
indoxyl sulfate *	C ₈ H ₇ NO ₄ S	indole, sulfate, hydroxyl	tryptophan, phase II metabolism	X	1.3	2/4	9.4	8.1 × 10 ⁻³	KO
indole lactic acid	C ₁₁ H ₁₁ NO ₃	indole, carboxylic acid	tryptophan metabolism	X	1.5	3/3	3.4	1.7 × 10 ⁻²	KO
phenyl sulfate	C ₆ H ₅ O ₄ S	phenyl, sulfate	phase II metabolism	X	0.7	0/4	2.9	1.3 × 10 ⁻³	KO
hippuric acid	C ₉ H ₉ NO ₃	phenyl, carboxylic acid	phenylalanine metabolism	R ^P	0.3	2/3	N/A	N/A	N
phenylacetyl- glycine	C ₁₀ H ₁₁ NO ₃	amide, phenyl, carboxylic acid	phenylalanine metabolism	R ^P	0.7	2/3	2.5	1.1 × 10 ⁻²	KO
pantothenic acid *	C ₉ H ₁₇ NO ₅	amide, carboxylic acid, hydroxyl	vitamin B5 & CoA biosynthesis; beta-alanine metabolism		-1.1	4/5	5.1	1.7 × 10 ⁻⁴	KO
p-hydroxy- phenyllactic acid	C ₉ H ₁₀ O ₄	phenyl, carboxylic acid, hydroxyl	tyrosine metabolism; ubiquinone synthesis		0.3	3/4	2.5	6.4 × 10 ⁻⁴	KO
4-pyridoxic acid	C ₈ H ₉ NO ₄	carboxylic acid, hydroxyl	vitamin B6 metabolism		0.1	3/5	3.8	1.5 × 10 ⁻⁴	KO
kynurenine	C ₁₀ H ₁₂ N ₂ O ₃	carboxylic acid, amine	tryptophan degradation; NAD biosynthesis		-2.2	3/5	2.1	3.7 × 10 ⁻²	KO
methionine *	C ₅ H ₁₁ NO ₂ S	carboxylic acid, amine	cysteine and methionine metabolism		-1.9	2/3	2.1	1.5 × 10 ⁻²	KO

Micr.: X indicates that the compound is found exclusively in wild type, not in germ free; R^P indicates regulation of the metabolite as found previously. ²⁶Compound:

* indicates a known Oat1 substrate.

Comparison of Urine

TABLE 2

compound	formula	functional groups	biochemical pathways	Micr.	XlogP	Hbond don/acc	fold	p-value	up in
Urate [*]	C ₅ H ₄ N ₄ O ₃	xanthine	purine metabolism	R ^P	-1.9	4/3	2.2	6.74 × 10 ⁻⁵	WT
thymidine	C ₁₀ H ₁₄ N ₂ O ₅	hydroxyl	pyrimidine metabolism		-1.2	3/5	2.7	5.10 × 10 ⁻⁴	WT
5-methyl- cytidine	C ₁₀ H ₁₅ N ₃ O ₅	hydroxyl			-2.0	4/6	1.5	8.08 × 10 ⁻⁴	WT
N-methyl- adenosine	C ₁₁ H ₁₅ N ₅ O ₄				-0.4	4/8	1.3	3.13 × 10 ⁻²	WT
N2-N2-dimethyl- guanosine	C ₁₂ H ₁₇ N ₅ O ₅		tRNA degradation		-2.1	4/8	2.1	5.58 × 10 ⁻⁶	WT
xanthurenic acid	C ₁₀ H ₇ NO ₄	carboxylic acid, hydroxyl groups	tryptophan metabolism		0.9	3/5	1.9	9.75 × 10 ⁻⁵	WT
N-acetyl glycine	C ₄ H ₇ NO ₃	carboxylic acid, amide			-1.2	2/3	2.3	6.11 × 10 ⁻³	WT
orotic acid	C ₅ H ₄ N ₂ O ₄	carboxylic acid, uracil	pyrimidine biosynthesis, microbiome	X	-1.4	3/4	1.7	3 × 10 ⁻³	WT
amino-cresol sulfate ^T	C ₇ H ₉ NO ₄ S	amine, phenyl, sulfate	microbiome, phase II	P	0.7	2/3	9.5	1.54 × 10 ⁻⁴	WT

Micr.: X indicates that the compound is found exclusively in wild type, not in germ free; R^P indicates regulation of the metabolite as found previously²⁶; P indicates probable microbiome product. Compound: T indicates a tentative ID.

^{*} indicates a known Oat1 substrate.