THE CHARACTERIZATION OF rbOAT1 AND rbOAT3

by

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DEDICATION

This work is dedicated to those that have given me their unconditional love and support. To my family, I would like to thank you for all your guidance and help during my years as a student. To my best friend Eric, thank you for helping me find the strength within myself to persevere and achieve my goals. I love you all.

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ABSTRACT

Understanding renal transport mechanisms is essential to predicting molecular interactions and targeting drugs to specific transporters. This study focuses on characterizing rbOAT1 and rbOAT3 with respect to interactions with compounds that varied in charge, length and hydrophobicity. Straight chain dicarboxylates of five carbons or more inhibited both transporters well. Maximum inhibition occurred with glutarate (5C). Monocarboxylates interacted poorly with both transporters, yet exhibited greater inhibition as the chain length increased. Aromatic dicarboxylates inhibited both transporters optimally at a charge separation similar to that of glutarate. Both OAT1 and OAT3 were inhibited by reduced and oxidized 2,3-dimercapto-1-propane sulfonate (DMPS). To test the chemical properties of DMPS that facilitated its interaction with rbOAT1 and rbOAT3, I used 3-mercapto-1-propane sulfonate and found that the SH groups are essential in stabilizing DMPS to the binding sites. These results indicate that the size, hydrophobicity, charge, and H-bonding capabilities of a molecule work together to stabilize it to the transporter binding site.

INTRODUCTION

The organic anion (OA) transport pathway is essential in the secretion of endogenous and exogenous compounds such as anionic drugs and their metabolites. It is also one of the first lines of defense against toxic molecules such as the zwitterionic nephrotoxic cysteine S-conjugates S-dichlorovinylcysteine (DCVC) and S-chlorotrifluoroethylcysteine (CTFC) [12,13]. Until recently, the classical OA transport pathway had been most thoroughly described in rat tubule perfusion studies which focused on the effect of structurally different compounds on OA secretion [26,31,32,33]. With an increasing knowledge of how OAs are transported at the molecular level, there is a growing need to identify the secretory pathway of anionic molecules in detail. Comprehending the mechanisms that handle OA transport will help predict drug interactions and target OA molecules to specific transport pathways. Therefore, my research extends our present knowledge to include the molecular characteristics of transport of two such processes, the OA exchangers OAT1 and OAT3.

The gene for the novel kidney transporter (NKT) also known as the organic anion transporter 1 (OAT1) was first isolated from the mouse kidney [21]. Its sequence has 546 amino acids and was predicted to have 11 to 12 transmembrane domains (TMD). The gene also has a large extracellular loop with glycosylation sites between TMD1 and TMD2 and an intracellular loop with PKC sites between TMD6 and TMD7 [21]. The NKT/OAT1 gene shares the topology and homology of several members of the "Major Facilitator Superfamily" (MFS), a superfamily of eukaryotic and prokaryotic nutrient

transporters [30]. Within the MFS the NKT/OAT1 gene has high homology to the organic cation transporter (OCT1) gene. In fact OCTs and OATs not only share sequence similarities but also selected transport characteristics and common substrates [34,35].

The study of the molecular basis of OA transport has increased in parallel with the cloning of multiple orthologs. OAT1 has been cloned from the rat [25,28], human [14,22], flounder [36] and rabbit [2]. In addition, several homologous transporters have been identified within the OA Transporter family: OAT2 [11,24], OAT3 [3,20,23] and OAT4 [4]. While the role of OAT2 in the kidney appears to be relatively minor [24], both OAT1 and OAT3 have been located to the basolateral membrane of proximal tubule [19] and been shown to mediate the exchange of mono and divalent OAs [29]. OAT4 has been localized to the luminal membrane [1], and is expected to be involved in the second step of epithelial membrane transport.

It is appropriate to describe how these discrete transport processes fit into the current physiological model of renal OA secretion. OA secretion is a two-step process that occurs throughout the renal proximal tubule epithelium and involves transport of substrate across two membranes: basolateral and luminal. In the basolateral membrane, the organic anion exchangers OAT1 and OAT3 exchange intracellular α-ketoglutarate for extracellular organic anions, a process driven by a combination of primary and secondary energy-requiring transporters. Sodium-dicarboxylate cotransporters account for 60% of the outwardly directed α-ketoglutarate gradient that drives the tertiary active transporters OAT1 and OAT3 [6]. The remaining 40% of the gradient is maintained by metabolic processes. The electrochemical gradient that drives the secondary active process of

Na^+dicarboxylate cotransport is established by the Na^+/K^+ATPase. The second step of secretion, which is the transport of OA's from the inside-negative intracellular compartment through the luminal membrane is not well understood, although it has been shown to be electrochemically downhill [6].

An immunolocalization study using rat nephrons located both rOAT1 and rOAT3 to the S_1, S_2 and S_3 segments of the proximal tubule [19]. Observations from single rabbit tubule studies show the majority of PAH transport (an OAT1-specific substrate in the rabbit) occurring in the S_2 segment of the proximal tubule [27], while the majority of the transport of estrone sulfate (an OAT3-specific substrate) occurs throughout the S_1 and S_2 segments and minimally in the S_3 segment of the proximal convoluted tubule of the rabbit kidney [Anusorn Lungkaphin, personal communication]. This is suggestive of the distribution of OAT1 and OAT3 transport in the renal proximal tubule.

As noted previously, the classical process of renal OA secretion results in excretion from the body of a wide array of anionic xenobiotic compounds. Of particular interest has been the role of this process in the clearance of heavy metals (e.g., Hg, As, Fe), through the clinical application of anionic metal chelators, including DMPS. Chelation of metals involves interaction of the metal with one or more of the thiol groups contained in this class of compound. DMPS has been used both therapeutically and diagnostically [7,15]. A number of studies have demonstrated the utility of DMPS at clearing metals from renal tissue [8,15,17,38,39,40,41].

Because of the negative charge and low molecular weight of DMPS (refer to Table 1), it has been assumed that DMPS entry into renal cells involves interaction with

the classical OA transport pathway, and substantial evidence supports that assumption. In isolated perfused rat kidneys DMPS was not only found to be an effective chelator of $HgCl_2$ but also found to have a net tubular secretion that was saturable and blockable by probenecid and p-aminohippurate [17]. In a study where rabbit renal tissue was exposed to $HgCl_2$ and subsequently to a chelating agent it was found that DMPS was the most effective agent in removing Hg from both in vivo and in vitro tissue, and this effect was blocked by application of probenecid [15]. Studies done on S2 segments of rabbit proximal tubules have indicated that DMPS protects tissue from toxicity induced by heavy metals, and is moved from the bathing compartment into the luminal compartment [38]. Moreover, it has been found that DMPS is likely to be transported into proximal tubule cells via basolateral membrane transporters [2,16,18]. In light of the previously noted observation that PAH is an OAT1-specific substrate in the rabbit [2], these observations implicate OAT1 as a pathway for DMPS entry into renal cells. In fact, both reduced and oxidized DMPS have recently been shown to inhibit PAH transport in Xenopous oocytes transfected with hOAT1, with K_is of 22.4 ± 8.4 μM and 66 ± 13.6 μM respectively [16]. DMPS was also shown to inhibit fluorescein transport in Cos-7 cells transiently transfected with rbOAT1 (K_i of 102 μM [2]). However, the extent to which OAT3 interacts with the various forms of DMPS is not known. Thus I have included in this study experiments that assess the molecular properties of DMPS that aid in a favorable interaction with both transporters. Specifically, my experiments examined the influence of charge, hydrophobicity, and structure on binding of substrates to OAT1 and OAT3. I have focused on the selectivity of the rabbit orthologs of OAT1 and OAT3

because of the particular advantages this species has for studies of renal OA secretion. The availability of rabbit kidney tissue, our ability to obtain data from intact isolated proximal tubules, and an 89% and 85% sequence homology to the human OAT1 and OAT3 respectively [2, Zhang X., personal communication] are all favorable points that supported the use of rabbit OA transporters for this study.

From a broader perspective, the significance of addressing the substrate specificity of members of the OAT family lies in mapping out the basis of possible drug interactions, designing structurally similar drugs targeted for this pathway, and understanding the mechanism of transport for similar compounds. My hypothesis was that both transporters interact with compounds that possess similar chemical properties, yet these substrates will differ in affinity for the transporters.

MATERIALS AND METHODS

I. Materials

The transport substrates included [^{3}H]PAH (3.97Ci/mmol; Life Science Products, Inc.) and 6-carboxyfluorescein (Molecular Probes). Additional unlabeled substrates were purchased from Sigma-Aldrich. Other chemicals were obtained from routine sources and were typically the highest quality available.

II. Cell Culture

a. *Creation of the Chinese Hamster Ovary (CHO) cell line that stably expresses rbOAT3 and rbOAT1.* The Flp-In System (Invitrogen Life Technologies, Inc.) was used to place the genes of interest into Flp-In CHO cells. Flp-In CHO cells were electroporated in a 4mm gap cuvette containing 10 μg of pcDNA5/FRT vector with either the rbOAT3 or rbOAT1 insert and 10 μg of salmon sperm (Invitrogen Life Technologies, Inc.), using the following conditions: 260 V, 160 μF with zero resistance. Subsequently, dilution cloning was performed in order to select a single clone that stably expressed the gene of interest. Cells that expressed transport activity were identified by exposing them for 10 min to 5 μM 6-carboxyfluorescein and then visualizing accumulated fluorescent substrate using an epifluorescence-equipped microscope. The stable cells were maintained in Ham's F-12 Kaighn's modification media (Sigma-Aldrich) in the presence of 10% non-heat inactivated Fetal Bovine Serum (Hyclone, Logan, UT) and 200 μg/ml of Hygromycin B (Invitrogen Life Technologies, Inc.).

b. *Uptake Experiments.* The stable cells were seeded at a density of $3.5x10^5$ in twelve well cell culture plates (Cellstar) for next-day experiments. All experiments were

performed at room temperature. Uptakes of fluorescent substrates were performed with overhead fluorescent lights turned off (to reduce photobleaching of the dye). Before an experiment, each well was rinsed twice with 1 ml of Waymouth's Buffer (in mM: 135 NaCl, 13 Hepes, 2.5 $CaCl_2$-2·H_2O, 1.2 $MgCl_2$, 0.8 $MgSO_4$-7·H_2O, 5 KCl, 28 $C_6H_{12}O_6$). Uptake experiments were typically performed using 5 μM 6-CF as a substrate in the presence or absence of additional test compounds. To stop transport each well was rinsed three times with 2 ml of Waymouth's buffer containing 1 mM probenecid and resuspended in 0.800 ml of 0.5 N NaOH, 1.0% SDS. An aliquot of 0.750 ml was taken from each well and its fluorescence was measured in a Hitachi F-2000 Fluorescence Spectrophotometer. In a few experiments with OAT1-expressing cells, uptake of [^{3}H]PAH was measured. The results obtained were comparable to those of experiments that used 6-CF as a substrate. Uptakes were typically expressed as moles per cm^2 of the confluent cell monolayer.

III. DMPS Solutions and the Measurement of Free Thiol Groups

Reduced DMPS solutions were prepared immediately before experiments. Oxidized DMPS solutions were prepared by bubbling reduced DMPS at room temperature with pure oxygen for 24 hours. This solution was typically used within 24 hours. Ellman's Reagent was used to measure free thiol groups, employing a solution prepared by dissolving 39.6 mg of DTNB in 10 ml of Phosphate Buffer (100 mM, pH 7.4). Each test tube contained 33.3 μl of Ellman's Reagent, 2mL of Phosphate Buffer (100 mM, pH 8.0) and 3 ml of the oxidized DMPS solution or the glutathione standard. After two minutes the absorbance was measured at 412 nm. The concentration of free thiol groups in the

oxidized DMPS solution was determined by comparing the measured values to the glutathione standard curve.

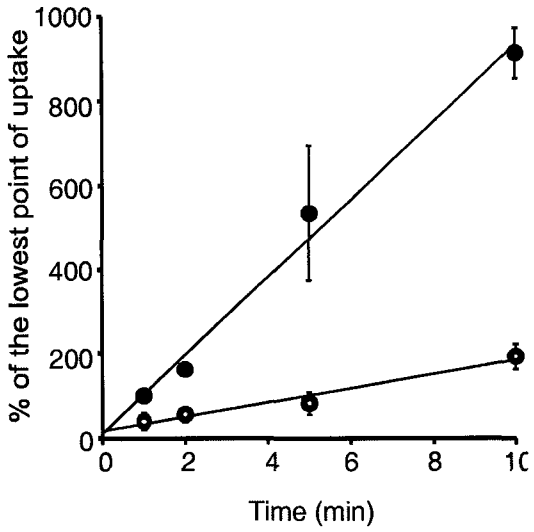
RESULTS

I. Time course of 6-CF uptake into Flp In CHO^{rbOAT1} and CHO^{rbOAT3} cells

The Flp In CHO^{rbOAT1} cells were exposed to 5 μM 6-CF at different times points: 1,2,5 and 10 minutes (Fig. 1a). Accumulation of fluorescence was approximately linear for 10 minutes, and the 2 minute point was chosen for future experiments. The same experiment was done on Flp In CHO^{rbOAT3}. Accumulation of fluorescence into Flp In CHO^{rbOAT3} cells was generally slower than that noted for the OAT1-expressing cells and a 5 min point of uptake was selected to represent the initial rate of 6-CF uptake into these cells (Fig. 1b).

In previous studies Fritzsch *et. al.* [10] characterized the "classical" OA transport pathway using a diverse set of compounds that differed in charge, length, hydrophobicity and aromaticity. We now know that this functionally defined pathway involves at least two distinct processes (i.e., OAT1 and OAT3). Consequently, the model developed by Ullrich that describes the basis of substrate selectivity for renal OA transport [10,31,32,33,34] represents an 'average' of the separate characteristics of (at least) two transporters. In my characterization of OAT1 and OAT3, I selected several sets of substrates that would serve to examine the influence of several parameters on selectivity of both transporters at the molecular level. The set of compounds included: dicarboxylates, monocarboxylates, aromatic anions, DMPS and molecules that were structurally similar to DMPS.

a.



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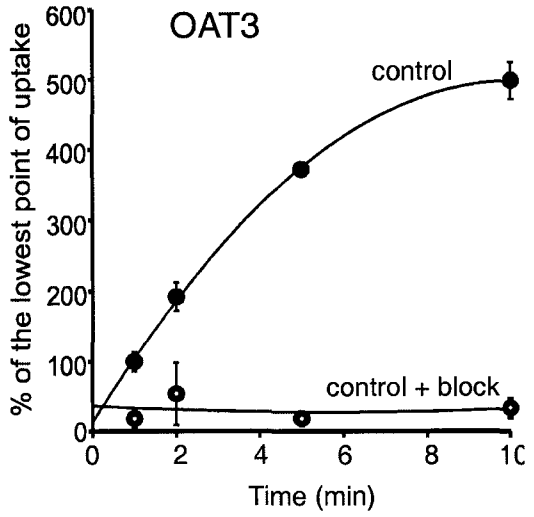


Fig. 1. *Concentration dependence of 6-CF uptake in CHO^{rbOAT1} and CHO^{rbOAT3}*. The closed circles (●) represent the control uptake and the open circles (○) represent the control plus the block. (a) The initial rate of rbOAT1 mediated transport of 6CF was found to be 2 minutes. (b) RbOAT3 had an initial rate of transport of 5 minutes.

II. Inhibition of 6-CF uptake by dicarboxylates

As exemplified by their ability to exchange α-ketoglutarate (αKG) for a wide range of OAs, OAT1 and OAT3 must interact with divalent anions as well as monovalent anions. Moreover, because DMPS can exist in both monovalent (i.e., reduced) and divalent (i.e., oxidized) forms, understanding the selectivity of these transporters for divalent anions is relevant to understanding their role in the transport of these metal chelators. In previous studies [10,33] it was observed that the PAH transport system and the dicarboxylate transport system were most likely to interact with substrates that had a charge separation of 6-7Å. It was also observed that the OA transport system was more likely to interact with the 5C dicarboxylate glutarate (identical to that for αKG). Compounds shorter or longer than glutarate were not as effective as inhibitors of renal OA transport. In my study, experiments were performed with dicarboxylic acids again ranging from 2 to 8 carbons in length.

As expected there were no detectable interactions of OAT1 and OAT3 with oxalic acid (2C) or malonic acid (3C) (Table 1). These compounds have a distance between the carboxyl residues of about 1.40 Å and 2.31 Å, respectively. Nevertheless, as the distance between the carbons containing the anionic charges got closer to 4.64 Å (i.e. glutaric acid), the compounds showed an increase in inhibition (Table 1). Succinic acid interacted weakly with both transporters. Glutaric acid had the lowest IC_{50} 2.4 ± 2.0 μM, n=2 in $rbOAT_1$ (Fig. 2a) and 34.6 ± 2.6 μM, n=3 in rbOAT3 (Fig. 2b). In general, as the distance between its charges decreased below or increased above approximately 5Å, a dicarboxylate's affinity for the transporter decreased slightly (Table 1; Fig. 2d-f); the

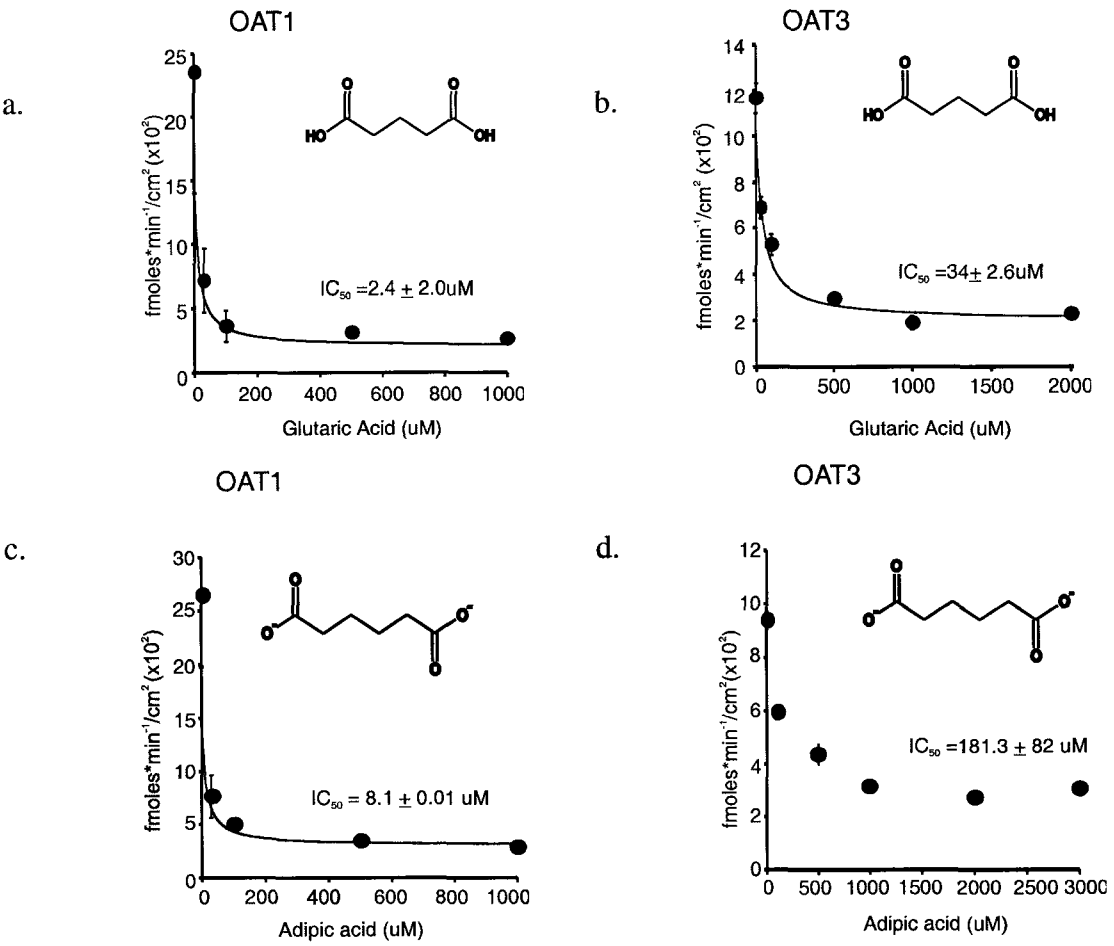


Fig. 2. *Inhibition of 6CF uptake by dicarboxylic anions.* (a) Glutaric acid is the best rbOAT1 inhibitor of the study, IC_{50} = 2.4 ± 2.0 μM. (b) Glutaric acid was also the best inhibitor for rbOAT3, IC_{50} = 34.0 ± 2.6 μM. Adipic acid (c,d) and suberic acid (e,f) inhibited both transporters to a lesser extent.

interactions with the transporters were the greatest at the distance of glutarate: succinate<<glutarate≥ adipate. This suggests that divalent molecules that structurally resemble glutaric acid are more likely to interact with the both transporters. Despite observing the same general trends, rbOAT3 had a weaker affinity for all of the compounds in this set compared to rbOAT1 (Fig. 2g,h), a trend noted for the other compounds studies, as noted below.

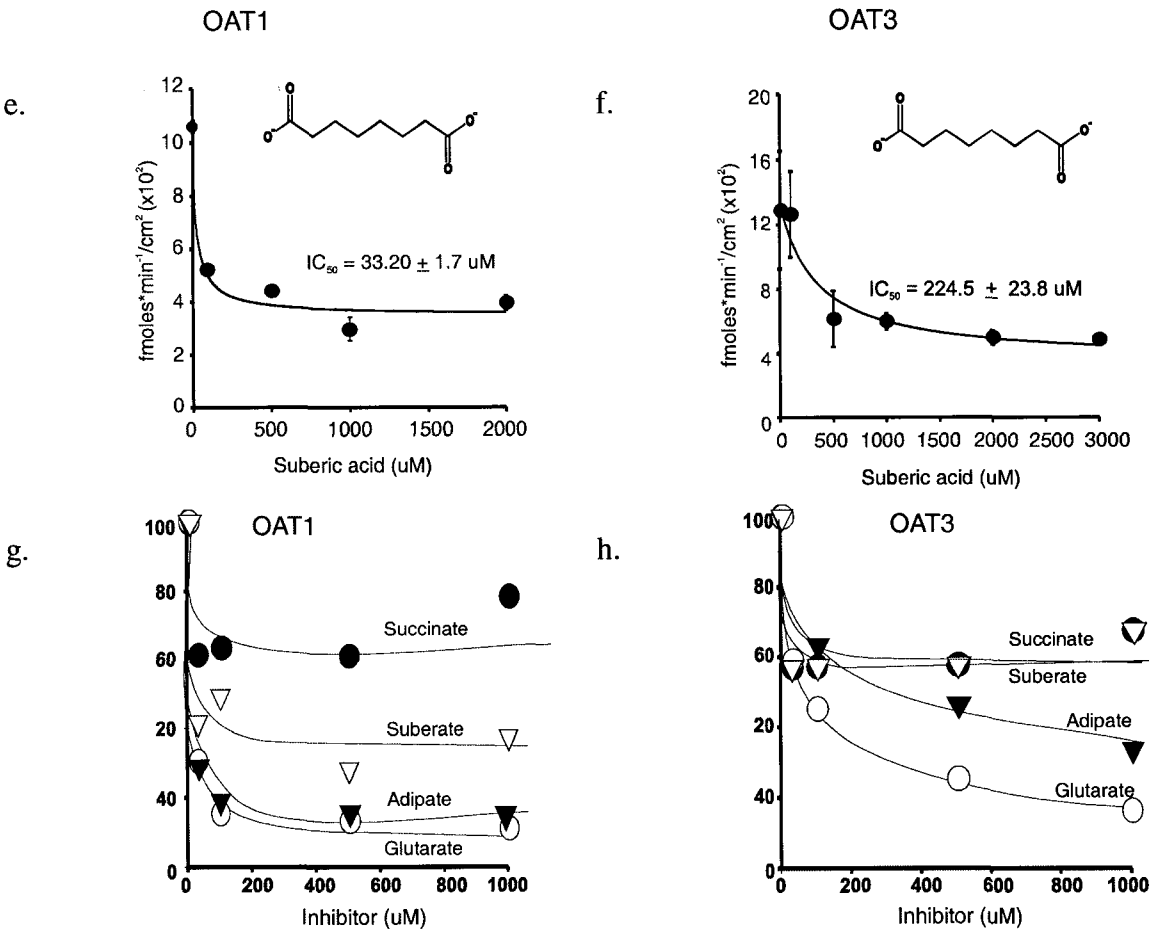


Fig. 2. *Inhibition of 6CF uptake by dicarboxylic anions.* Suberic acid (e,f) inhibited both transporters to a lesser extent. (g,h) The summary figures show that in general rbOAT1 is more likely to interact with the dicarboxylates tested. The x-axis has the concentrations used in μM and the y-axis has all values expressed as a % of the highest point of uptake.

III. Inhibition of 6-CF uptake by monocarboxylates

Previous studies have shown that short chain monocarboxylic acids are comparatively poor inhibitors of the OA transport pathway and that in order for monocarboxylates to interact favorably with the pathway, a hydrophobic core of at least 5Å needs to be present in the molecule. My next set of experiments examined the

inhibitory effect on activity of rbOAT1 and rbOAT3 of straight chain monocarboxylates ranging from acetic acid (2C) to octanoic acid (8C).

With the exception of octanoic acid, which inhibited with an IC_{50} of 5.95 ± 2.0 μM, n=2 in rbOAT1 (Fig. 3a) and 63.72 ± 91.60 μM, n=3 in rbOAT3 (Fig. 3b), the monocarboxylates were poor inhibitors of both transporters. Figure 3c,d shows how inhibition improved with increasing chain length of the monocarboxylates; the increase in chain length might have facilitated an electrostatic or temporary attraction between the molecule and the binding sites. Also evident in Figure 3c,d is the fact that, in general, monocarboxylates were more effective inhibitors of rbOAT1 than of rbOAT3. Presumably, structural differences within the binding site might be responsible for making OAT1 more likely to interact with the inhibitors than OAT3.

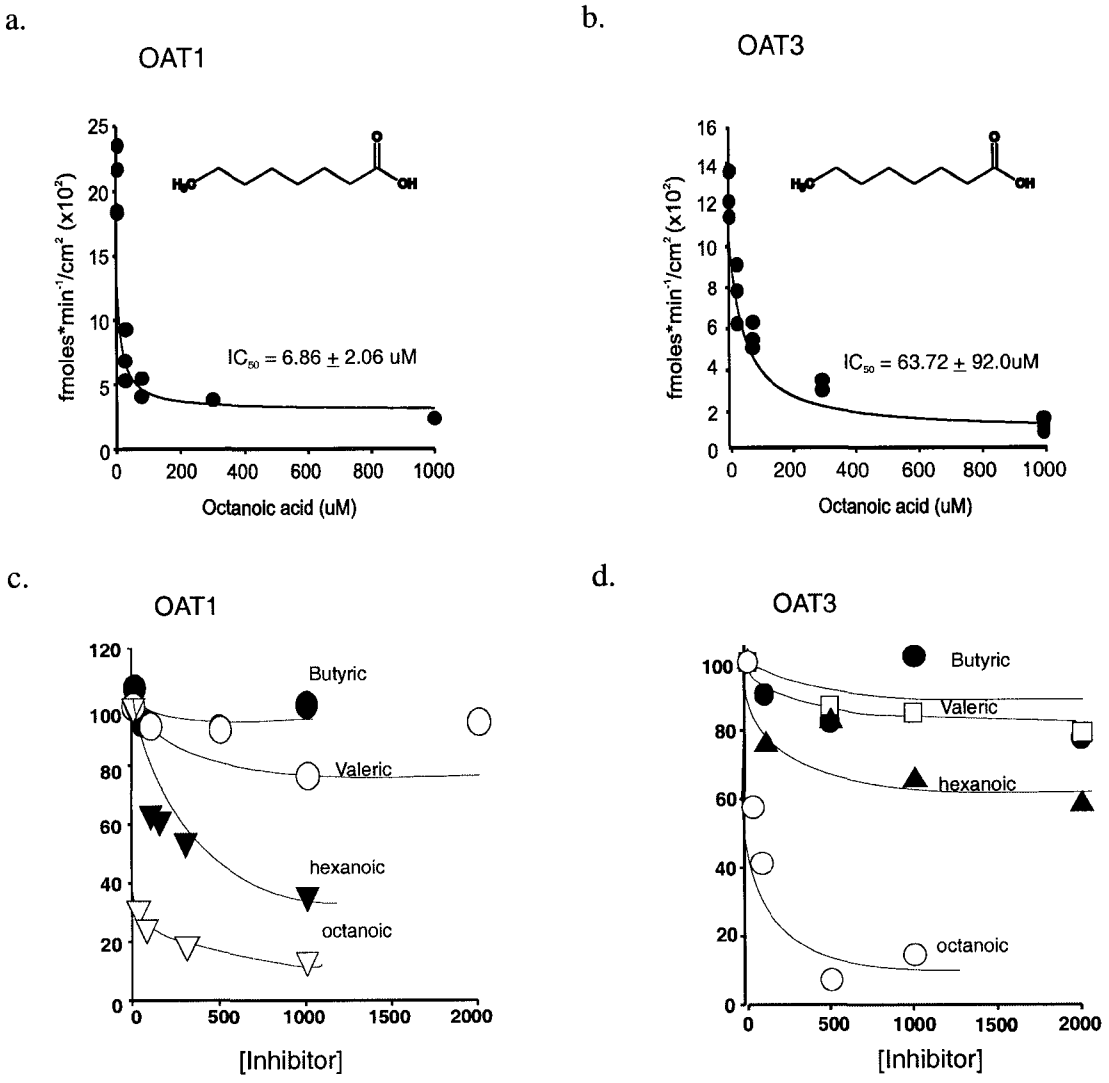


Fig. 3. *Inhibition of 6CF uptake by monocarboxylic anions.* (a) Octanoic acid was the best inhibitor of rbOAT1, IC_{50} = 6.86 ± 2.0 μM. (b) It also inhibited rbOAT3, IC_{50} = 63.72 ± 92.0 μM. Summary of all mono carboxylic anions tested on rbOAT1 (c) and rbOAT3 (d).

IV. Inhibition of 6-CF uptake by aromatic compounds

Already evident in this study is the observation that charge separation and hydrophobic interactions play an important role in substrate interaction with the transporter. To further assess the influence of hydrophobic moieties on binding of compounds to the transporters, I tested a series of compounds that featured benzene rings di-substituted with carboxyl residues (Table 1). Terephthalic acid (5.60 Å between carboxyl residues) had the highest affinity for both transporters with an IC_{50} of 3.46 ± 12.0 μM in rbOAT1 (based on inhibition of [^{3}H]PAH uptake) and an IC_{50} of 19.54 ± 6.3 μM in rbOAT3 (based on inhibition of 6-CF uptake). Isopthalic acid had no effect on 6-CF uptake mediated by rbOAT3 or PAH uptake in rbOAT1. Interestingly, the distance between carboxyl residues in terephthalic acid corresponds closely to that in 6-CF. The distance between carboxyl groups and the hydrophobic mass on both compounds helps explain how terephthalic acid and 6CF interact well with both transporters. Thus, it is relevant to note that a study with a stable CHO^{hOAT1} cell line [5] showed that uptake by 5-carboxyfluorescein occurred at much lower levels than the uptake of 6-CF. 5-CF and isophthalic acid have a similar distance between carboxyl residues, <5Å, and both appear to interact poorly with OATs.

I also tried to examine the interaction of compounds of greater hydrophobicity, such as naphthalene dicarboxylate, but found that they were only slightly soluble in water. Nevertheless, the findings in this study agree with those in the previous study [10,32] in that hydrophobic interactions and distance between negative charges seem to aid in binding to OAT1 and I can also extend this reasoning to OAT3.

V. Inhibition of 6-CF transport by reduced and oxidized DMPS

DMPS is a compound of clinical relevance as it has been shown to increase the clearance of heavy metals both experimentally and clinically [7,16,38]. Studies have also shown that this compound interacts with OAT1 [2,16]. Figures 4a through 4d, show the effect of increasing concentrations of reduced (DMPSH) and oxidized (DMPSS) DMPS on the rate of 6-CF uptake into CHO^{rbOAT1} and CHO^{rbOAT3} cells. Both compounds produced a concentration dependent inhibition of 6-CF uptake and appeared to have very similar affinities for both transporters (refer to Table 1 for the structures of these compounds). The IC_{50}s for rbOAT1 appeared to be similar at 52.4 ± 23 μM for DMPSH and 49.2 ± 13 μM for DMPSS. This was also true for the interactions of both compounds with rbOAT3; DMPSH had a constant of 75.9 ± 22 μM and the DMPSS had a constant of 69.5 ± 25 μM.

Despite having similar apparent inhibition constants, both transporters are likely to have a higher affinity for the oxidized, rather than reduced, species of DMPS. This conclusion stems from the method by which we calculated the apparent IC_{50} values for the oxidized form of DMPS. The concentrations used to calculate these IC_{50}s were based on the initial amount of reduced DMPS that was bubbled with pure O_2. Therefore, the final concentration of oxidized DMPS, which exists principally in the form of cyclic dimers [16], should have been approximately half of the starting amount of reduced DMPS. Consequently, the IC_{50}s calculated for the interaction of the oxidized species with both rbOAT1 and rbOAT3 were an overestimate of their true value.

The IC_{50} values for inhibition of rbOAT1 and OAT3 by DMPS were generally higher than those obtained in a study that evaluated the kinetics of DMPS inhibition of hOAT1 transfected into oocytes (Ki_{red}= 22.4 ± 8.4 μM, Ki_{oxi}= 66 ± 13.6 μM) [16]. These studies were performed in different experimental models (i.e., cell culture vs. oocytes), as well as with different transporters, therefore the difference in kinetic values was not unexpected.

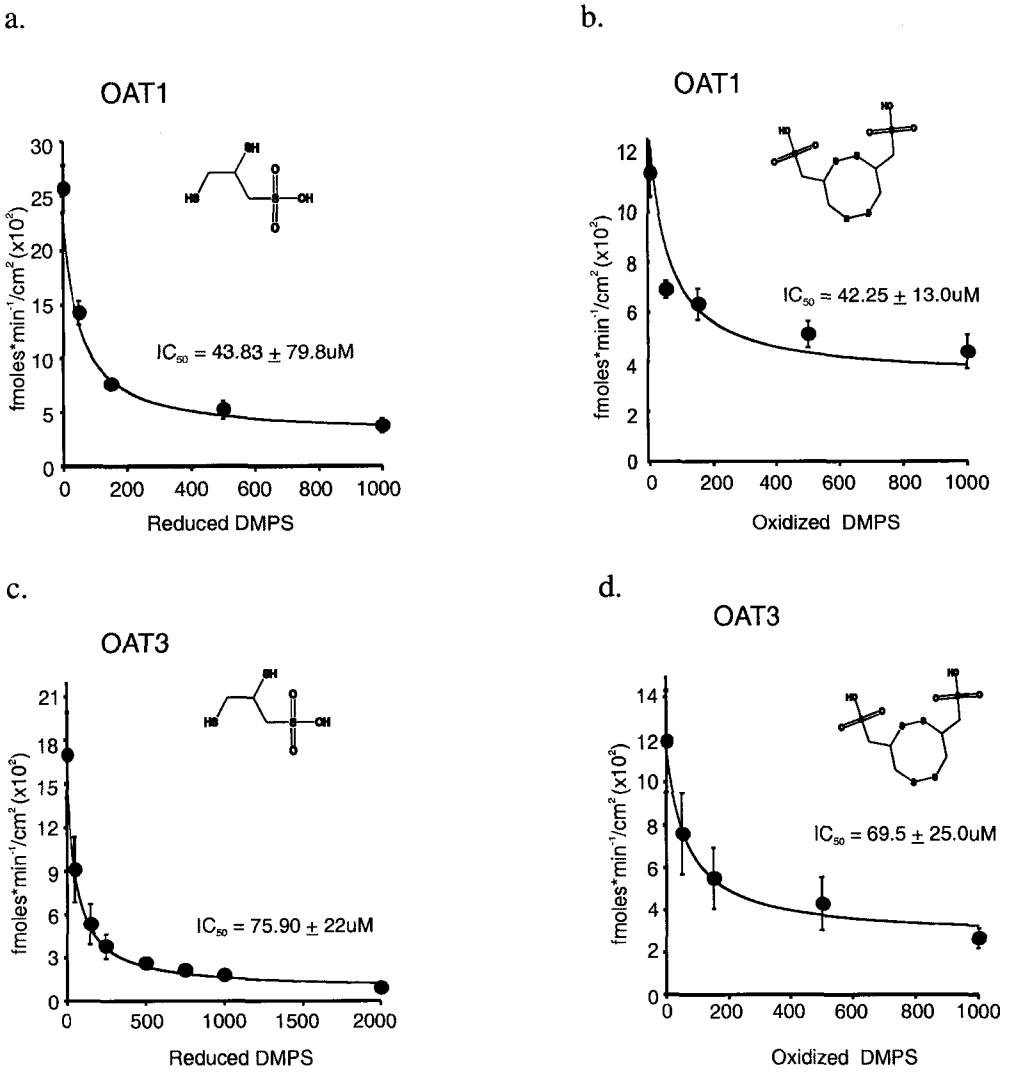


Fig. 4. *Inhibition of 6CF uptake by oxidized and reduced DMPS.* (a) Reduced DMPS inhibited rbOAT1 with an IC_{50}= 43.3 ± 79.8 μM. (b) Oxidized DMPS Inhibited the rbOAT1 transporter with an IC_{50}= 42.25 ± 13.0 μM. Nevertheless, the constant for the oxidized DMPS was calculated using the initial concentration of reduced DMPS present before its oxidation. Thus, the transporter is better inhibited by the oxidized DMPS. (c) Reduced DMPS inhibited the rbOAT3 with an IC_{50} = 75.9 ± 22 μM. (d) The oxidized species inhibited the transporter with an apparent constant similar to the reduced IC50, 59.5 ± 25 μM.

VI. Inhibition of 6-CF uptake by Sulfonic Acids

The acidic residue of DMPS is a sulfonyl residue rather than the carboxyl residue found in the test agents described to this point. To examine whether the presence of a sulfonyl residue exerts a systematic effect on binding to OAT1 or OAT3, I examined the effect of 1-heptanesulfonic acid, which corresponds in overall length to octanoic acid. The OAT3 transporter interacted with 1-heptanesulfonic acid with an IC_{50} of 84.9 ± 17.9 μM (Table 1) that was similar to the value obtained with octanoic acid, 63.7 ± 91.6 μM. This suggests that the chemical properties of the SO^{3-} group are similar to those of a COO^{-} group. However, a compound containing a sulfonyl group has a greater polar character and is perhaps more likely to react better in aqueous conditions. This might explain in part how a molecule as small as DMPS (length of approximately 5.04Å) is a better substrate for $rbOAT_3$ than propionate, butyrate, valerate, and isovalerate.

VII. Inhibition of 6-CF uptake by compounds that are structurally similar to DMPS

Compounds structurally similar to DMPS (see Table 1) were used in order to identify the properties that help DMPS have a favorable interaction with the transporters despite its relatively short chain length. Interestingly, isovaleric acid, 3-mercapto-propanesulfonate (MPS), and meso-2,3 dimercaptosuccinic acid (DMSA) had very little or no interaction with the rbOAT3 transporter. However, 3-MPS did interact with the rbOAT1 transporter with an IC_{50} = 385 ± 5 μM (Fig. 5). I believe that the SH group on MPS interacts with cationic residues within the OAT1 binding site that are harder to reach in the binding site of the OAT3 transporter. Perhaps a combination of strong acidity

(from the SO^{3-} group) and hydrogen bonding (to the SH groups) make reduced and oxidized DMPS good substrates. Nevertheless this experiment shows how the SH group on the second carbon of DMPS is necessary for its interaction with the rbOAT3 transporter.

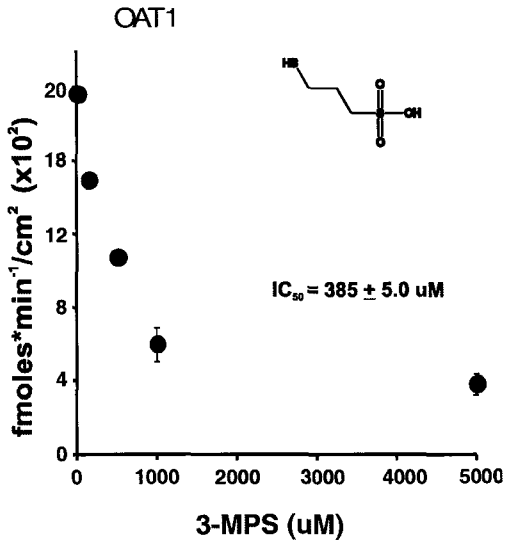


Fig. 5. *Inhibition of 6CF transport by 3-MPS in rbOAT1.* (a) 3-MPS inhibited rbOAT1 with an IC50 = 385 ± 5.0 μM while it failed to interact with rbOAT3 at high substrate concentrations (5 mM MPS).

DISCUSSION

The OA transport pathway has been extensively characterized in previous inhibition studies that employed microperfused rat tubules [10]. However the recent molecular cloning of individual transporters makes it possible to characterize OA transport at the molecular level. In this study it was my goal to test compounds that would help us better understand the kinds of substrates that interact with the OA-dicarboxylate exchangers OAT1 and OAT3. My hypothesis was that both transporters would interact with substrates that possess similar chemical properties. Previously, Fritzsch *et. al.* [10] described the general OA pathway by using a vast set of compounds that, among many properties, varied in charge, length and hydrophobicity. Due to time constraints, I chose a smaller set of compounds possessing similar properties and tested both transporters individually. Thus, I chose the following inhibitors: dicarboxylates, monocarboxylates, aromatic compounds, DMPS (oxidized and reduced) and other compounds that would help determine the basis of observed DMPS interactions with OAT1 and OAT3.

As expected, experimental observations showed that glutarate (5C dicarboxylate) had the best interaction with both transporters. Any compounds above or below this length had a less favorable interaction. From these results we can infer that a divalent anion with structural properties similar to glutarate should interact well with the binding site of both transporters. Aliphatic dicarboxylates longer than 5Å interact with the transporter perhaps due to rotations about sigma bonds and changes in conformation that

allow them to reach into a binding site pocket containing cationic residues. Monocarboxylates, however, interact better with the transporter as the carbon backbone or hydrophobic core increases. This was found to be true in both rbOAT1 and rbOAT3. A possible explanation is that hydrophobic interactions with the binding site help stabilize the molecule. Although both transporters share a high degree of homology, probable structural differences in the binding site permit gained stability due to these weak forces to differ between OAT1 and OAT3.

Because hydrophobicity was a factor in stabilizing the interactions of the monocarboxylates and dicarboxylates to both transporters, I tested a series of ring constrained dicarboxylic acids. There was a two-fold purpose: to test for the influence of hydrophobicity and to test for the steric effect of a planar molecule with a fixed charge distance. It was found that both transporters had a high affinity for terephthalic acid, which has a charge separation distance similar to that of glutarate. However, neither transporter interacted well with compounds of shorter charge separation: pthalic or isopthalic acid, both of which are isomers of terephthalic acid. The main conclusion drawn from this set of compounds is that the binding sits of both transporters probably contain positively charged residues separated by a fixed distance that is close to 5Å. This hypothesis helps explain how both the cis and trans forms of oxidized DMPS (separated by 6.33Å and 7.20Å, respectively) interact well with both OAT1 and OAT3.

DMPS is a dithiol compound that has been used clinically for the chelation of heavy metals. Nevertheless, the secretory transport pathway of this molecule remains unknown. It is important to discern which transport pathways are used for the secretion of

DMPS in order to better understand the renal handling of this compound, as well as that of similar drugs. There are numerous factors that influence the way in which a substrate interacts with transporters. For OAT1 and OAT3, the major players seem to be hydrophobicity and steric interactions that usually render a molecule more or less likely to interact with the binding site. DMPS is a highly acidic molecule that has a modest amount of hydrophobicity. Thus it is not only able to hydrogen bond but it is also able to interact with cationic residues and with "greasy" sidegroups (i.e., SH groups). The results obtained indicate that the reduced form interacts well with both transporters, although the oxidized form is likely to interact somewhat better.

Interestingly, structurally similar molecules such as DMSA and 3-MPS did not interact with rbOAT3. However, the absence of an interaction of 3-MPS indicates that both thiol groups of DMPS have a key role in stabilizing the molecule to the binding site of rbOAT3 and that only one thiol group is sufficient to have DMPS interact with OAT1. This explains how the second SH assures a better interaction and thus a slightly lower IC_{50}. Further characterization of the transport of DMPS was not possible since we had no way of quantifying the amount of substrate transported.

In general all of the compounds interacted better with rbOAT1 than with rbOAT3. Because both transporters have slight variations in the amino acid make-up of the protein, this difference was expected. Nevertheless, both exhibit the same trends with respect to the properties tested. From this study we can hypothesize that the binding site of both transporters should have cationic (probably arginine and/or lysine) residues separated by a distance of about 5Å. The binding site is likely to have a length and hydrophobicity

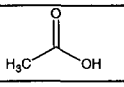
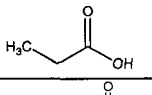
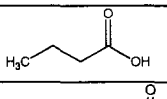
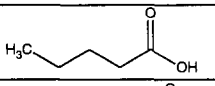
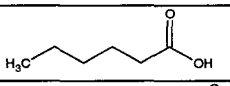
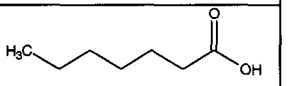
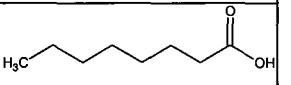
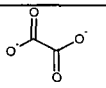
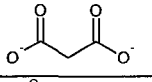
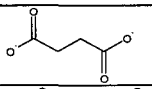
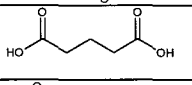
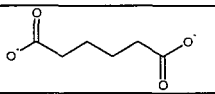
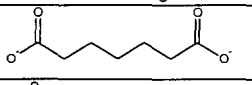
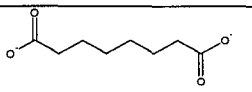
requirement associated with it as well. Lastly, several side chains with the following properties may also be present: atoms that can hydrogen bond, interact with the greasy "S" or contain a full or partial cationic charge that can interact with ionized SH groups.

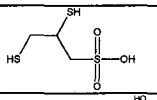
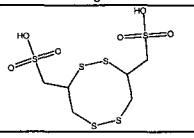
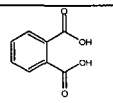
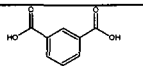
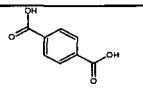
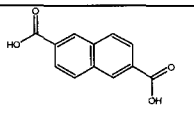
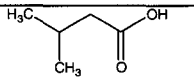
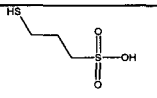
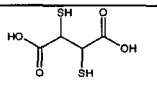
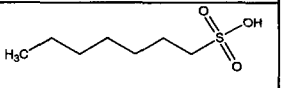
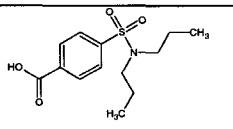
A limitation of this study was not ascertaining whether or not the compounds tested were transported. Therefore, further studies should include testing the transport of several compounds from this study along with DMPS and its heavy metal chelates. Testing the transport of radiolabeled DMPS into cells would confirm existing evidence that DMPSH is indeed transported via rbOAT1 and rbOAT3 and that DMPSS is only transported via rbOAT3 [16, Lungkaphin, et. al.]. Also, with this study there was no way to confirm the net activity of all organic anion transport processes. Thus, I propose that further experiments be done in rabbit renal proximal tubules. At the molecular biology level, it would be interesting to do site directed mutagenesis on conserved cationic amino acid residues of rbOAT1 and rbOAT3. Altering the function of the exchangers will help predict which cationic residues are involved in the anionic selectivity of the transporters. In a study by Gerhardt *et. al.* [37], 3 cationic amino acids present in all OATs but absent in the OCTs were selected and mutated. The three amino acids found in the flounder sequence were histidine (H34), lysine (K394) and arginine (R478). Replacing these residues with those found in OCTs did not result in organic cation transporter, however the transport of anions such as PAH was weakened, and the interaction with glutarate was eliminated. A similar study in rOAT3 confirmed these results [9].

In conclusion both rbOAT1 and rbOAT3 prefer the divalent anions over the monovalent anions. Although they both accept a wide variety of monovalent anions (i.e.,

PAH and probenecid), straight chain dicarboxylates are likely to inhibit the transporters if the backbone is long enough. Bulky anions such as the aromatic dicarboxylates are good inhibitors if the distance between the carboxyl groups is adequate (~5Å). Lastly both transporters are inhibited by compounds with very different properties, for instance DMPS, 6CF, and probenecid. Presumably chemical interactions other than the properties assessed in this study aid the compound in interacting with the transporter, thereby explaining the observation that PAH interacts more effectively with rbOAT1 than with rbOAT3 and estrone sulfate interacts more effectively with rbOAT3 than with rbOAT1. This is indicative of distinct yet related functions that each transporter has in the proximal tubule.

Table 1

Mono carboxylate	**Chain length**	**IC_{50} rbOAT1**	**IC50 rbOAT3**	**Structure**
Acetic acid	2	ND	>>2mM, n=3	
Propionic acid	3	ND	>>2mM, n=1	
Butyric acid	4	>>1mM, n=2	>>3mM, n=1	
Valeric acid	5	>>2mM, n=2	>>5mM, n=3	
Hexanoic acid	6	>>1mM, n=2	>>10mM, n=5	
Heptanoic acid	7	ND	>>5mM, n=4	
Octanoic acid	8	5.95 ± 2.05, n=2	63.72 ± 91.60, n=3	
Di carboxylate	**Chain length**	**Ic50 rbOAT1**	**Ic50 rbOAT3**	**Structure**
Oxalic acid	2	ND	ND	
Malonic acid	3	ND	ND	
Succinic acid	4	>>1.5 mM, n=2	>>5 mM, n=3	
Glutaric acid	5	2.40 ± 2, n=2	32.80± 2.04, n=2	
Adipic acid	6	9.10 ± 0.1, n=2	181.48 ± 91.9, n=3	
Pimelic acid	7	(^{3}H)	176.01± 15.41, n=3	
Suberic acid	8	33.2 ± 1.7, n=2	224.50 ± 23.8, n=3	
DMPS	**Chain length**	**Ic50 rbOAT1**	**Ic50 rbOAT3**	**Structure**

DMPS reduced	3	52.4 ± 23, n=2	43.83 ± 75.89, n=4	
DMPS oxidized	6	60 ± 21, n=2	114.56 ± 14, n=3	
Aromatic anions	**Chain length**	**Ic50 rbOAT1**	**Ic50 rbOAT3**	**Structure**
Pthalic acid (*o*)	4	>>1mM, n=3 (^{3}H)	>>5mM, n=3	
Isopthalic acid (*m*)	5	>>1mM n=3 (^{3}H)	>>350µM, n=2	
Terepthalic acid (*p*)	6	3.46 ± 2.0, n=3 (^{3}H)	19.54 ± 6.3, n=3	
2,6 Napthalene dicarboxylic acid	8	slightly soluble	slightly soluble	
Chelator like anions	**Chain length**	**Ic50 rbOAT1**	**Ic50 rbOAT3**	**Structure**
Isovaleric Acid	3	ND	5mM, n=3	
3-Mercapto-propanesulfonate	3	385 ± 5, n=2	>>5mM, n=3	
Meso-2,3 dimercaptosuccinic acid	4	ND	>>7.5mM, n=2	
other compounds	**# of C atoms**	**Ic50 rbOAT1**	**Ic50 rbOAT3**	**Structure**
1-Heptanesulfonic acid	7	ND	84.90 ± 17.90	
Probenecid	13	0.7 µM, n=1	5.0 µM, n=1	

REFERENCES

1. Babu E, Takeda M, Narikawa S, Kobabyashi Y, Enomoto A, Tojo A, Cha SH, Sekine T, Sakthisekaran D, Endou H: Role of human organic anion transporter 4 in the transport of ochratoxin A. *Biochim Biophys Acta* 1590: 64-75, 2002

2. Bahn A, Knabe M, Hagos Y, Rödiger M, Godehardt S, Graber-Neufeld DS, Evans KK, Buckhardt G, Wright SH: Interaction of the Metal Chelator 2,3-Dimercapto-1-propanesulfonate with the Rabbit Multispecific Organic Anion Transporter 1 (rbOAT1). *Mol Pharmacol* 62: 1128-1136, 2002

3. Cha SH, Sekine T, Fukushima J, Kanai Y, Kobayashi Y, Goya T, Endou H: Identification and Characterizatoin of Human Organic Anion Transporter 3 Expression Predominantly in the Kidney. *Mol Pharmacol* 59: 1277-1286, 2002

4. Cha SH, Sekine T, Kusuhara H, Yu E, Kim JY, Kim DK, Sugiyama Y, Kanai Y, Endou H: Molecular Cloning and Characterization of Multispecific Organic Anion Transporter 4 Expressed in the Placenta. *J Biol Chem* 275: 4507-4512, 2000

5.Cihlar T, Ho ES: Fluorescence-Based Assay for the Interaction of Small Molecules with the Human Renal Organic Anion Transporter 1. *Annalytical Biochemistry* 283: 49-55, 2000

6. Dantzler WH: Renal organic anion transport: a comparative and cellular perspective. *Biochim Biophys Acta.* Nov 13;1566(1-2): 169-81. Review. 2002

7. Dargan P, Giles LJ, Wallace CI, House IM, Thompson AH, Beale RJ, Jones AL: Case report: severe mercuric sulphate poisoning treated with 2,3-dimercaptopropane-1-sulphonate and haemodiafiltration. *Crit Care*. 2003 Jun;7(3):R1-6. Epub 2003 Feb 17.

8. Diamond GL, Klotzbach JM, Stewart JR: Complexing activity of 2,3-dimercapto-1-propanesulfonate and its disulfide auto-oxidation product in rat kidney. *J Pharmacol Exp Ther*. Jul;246(1):270-4, 1988

9. Feng B, Dresser MJ, Shu Y, Johns SJ, Giacomini KM: Arginine 454 and lysine 370 are essential for the anion specificity of the organic anion transporter, rOAT3. *Biochemistry.* May 8;40(18):5511-20, 2001

10. Fritzsch G, Rumrich G, Ullrich KJ: Anion transport through the contraluminal cell membrane of renal proximal tubule. The influence of hydrophobicity and molecular charge distribution on the inhibitory activity of organic anions. *Biochim Biophys Acta.* Jan 30;978(2):249-56, 1989

11. Gregg SD, Vincent AC, Roberg KJ, Huang Y, Iwanij V: Molecular cloning and characterization of a novel liver-specific transport protein. *J Cell Sci*: 1065-72 Apr; 107 (Pt 4), 1994

12. Groves CE, Morales MN: Chlorotrifluoroethylcysteine interaction with rabbit proximal tubule cell basolateral membrane organic anion transport and apical membrane amino acid transport. *J Pharmacol Exp Ther.* 1999 Nov;291(2):555-61.

13. Groves CE, Munoz L, Bahn A, Burckhardt G, Wright SH: Interaction of cysteine conjugates with human and rabbit organic anion transporter 1. *J Pharmacol Exp Ther.* 2003 Feb;304(2):560-6.

14. Hosoyamada M, Sekine T, Watanabe N, Kanai Y, Endou H: Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am J Physiol* 276: F122-F128, 1999

15. Keith RL, Setiarahardjo I, Fernando Q, Aposhian HV, Gandolfi AJ: Utilization of renal slices to evaluate the efficacy of chelating agents for removing mercury from the kidney. Toxicology. Jan 15;116(1-3):67-75, 1997

16. Islinger F, Gekle M, Wright SH: Interaction of 2,3 Dimercapto-1-propane Sulfonate with the Human Organic Anion Transporter hOAT1. *JPET* 299:741-747, 2001

17. Klotzbach JM, Diamond GL: Complexing activity and excretion of 2,3-dimercapto-1-propane sulfonate in rat kidney. *Am J Physiol.* Jun;254(6 Pt 2):F871-8, 1988

18. Koh A, Simmons-Willis TA, Pritchard JB, Grassl SM, Ballatori N: Identification of a Mechanism by Which the Methylmercury Antidotes N-Acetylcysteine and Dimercaptopropanesulfonate Enhance Unrinary Metal Excretion: Transport by the Renal Organic Anion Transporter-1. *Mol Pharmacol* 62:921-926, 2002

19. Kojima R, Sekine T, Kawachi M, Cha SH, Suzuki Y, Endou H: Immunolocalization of Multispecific Organic Anion Transporters, OAT1, OAT2, OAT3, in Rat Kidney. *J Am Soc Nephrol* 13: 848-857, 2002

20. Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y, Endou H: Molecular Cloning and Characterization of a New Multispecific Organic Anion Transporter from Rat Brain. *J Biol Chem* 274: 13675-13680, 1999

21. Lopez-Nieto CE, You G, Bush KT, Barros EJ, Beir 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* 272:6471-6478, 1997

22. Lu R, Chan BS, Schuster VL: Cloning of the human kidney PAH transporter: Narrow substrate specificity and regulation by protein kinase C. *Am J Physiol* 276: F295-F303, 1999

23. Race JE, Grassl S, Williams W, Holtzman EJ: Molecular Cloning and Characterization of Two Novel Human Renal Organic Anion Transporters (hOAT1 and hOAT3). *Biochem Biophys Res Commun* 255: 508-514, 1999

24. Sekine T, Seok HC, Tsuda M, Apiwattanakul N, Nakajuma N, Kanai Y, Endou H: Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. *FEBS Letters* 429: 179-182, 1998

25. Sekine T, Watanabe N, Hosoyamada M, Kanai Y, Endou H: Expression Cloning and characterization of a novel multispecific organic anion transporter. *J. Biol Chem* 272: 18526-18529, 1997

26. Sheridan E, Ullrich K, Rumrich G: Reabsorption of dicarboxylic acids from the proximal convolution of rat kidney. *Pflügers Arch* 399:18-28, 1983

27. Shimomura A, Chonko AM and Grantham JJ: Basis for heterogeneity of *para*-aminohippurate secretion in rabbit proximal tubules. *Am J Physiol* 240: F430-F436, 1981

28. Sweet DH, Wolff NA, Pritchard JB: Expression Cloning and characterization of ROAT1. The basolateral organic anion transporter in rat kidney. *J. Biol. Chem* 272: 30088-30095, 1997

29. Sweet DH, Chan LM, Walden R, Yang XP, Miller DS, Pritchard JB: Organic anion transporter 3 (Slc22a8) is a dicarboxylate exchanger indirectly coupled to the Na+ gradient. *Am J Physiol Renal Physiol.* 284(4):F763-9, 2003

30. Sweet DH, Bush KT, Nigam SK: The organic anion transporter family: from physiology to ontogeny and the clinic. *Am J Physiol Renal Physiol.* 281: F197-205, 2001

31. Ullrich K, Rumrich G, Klöss S: Reabsorption of Monocarboxylic Acids in the Proximal Tubule of the Rat Kidney. I Transport Kinetics of D-Lactate, Na+-Dependence and Effect of Inhibitors. *Pflügers Arch* 395:212-219, 1982

32. Ullrich K, Rumrich G, Klöss S, Fasold H: Reabsorption of Monocarboxylic Acids in the Proximal Tubule of the Rat Kidney. III. Specificity for Aromatic Compounds. *Pflügers Arch* 395:227-231, 1982

33. Ullrich KJ, Fasold H, Rumrich G, Klöss S: Secretion and Contraluminal uptake of dicarboxylic acids in the proximal convolution of rat kidney. *Pflügers Arch* 400:241-249, 1984

34. Ullrich KJ, Rumrich G, David C, Fritzsch G: Bistubstrates: substances that interact with both, renal contraluminal organic anion and organic cation transport systems. II. Zwiterionic substrates: dipeptides, cephalosporins, quinolone-carboxylate gyrase inhibitors and phosphamide thiazine carboxylate, nonioniable substrates: steroid hormones and cyclophosphamides. *Pflugers Arch* 425: 300-312, 1993

35. Ullrich KJ, Rumrich G, David C, Fritzsch G : Bistubstrates: substances that interact with both, renal contraluminal organic anion and organic cation transport systems. I.

Amines, piperidine, piperazines, azepines, pyridines, quinolines, imidazoles, thiazoles, guanidines and hydrazines. *Pflugers Arch* 425: 280-299, 1993

36. Wolff NA, Werner A, Burkhardt A, Burkhardt G: Expression cloning and characterization of a renal organic anion transporter from winter flounder. *FEBS Letters* 417: 287-291

37. Wolff N, Grunwald B, Friedrich B, Lang F, Godehardt S, Gerhardt G: Molecular Cationic amino acids involved in dicarboxylate binding of the flounder renal organic anion transporter. *J Am Soc Nephrol.* 12(10):2012-8, 2001

38. Zalups RK, Parks LD, Cannon VT, Barfuss DW: Mechanisms of action of 2,3-Dimercaptopropane-1-sulfonate and the Transport, Disposition, and Toxicity of Inorganic Mercury in isolated Perfused Segments of Rabbit Proximal Tubules. *Mol Pharmacol.* Aug;54(2):353-63, 1998

39. Zalups RK: Influence of 2,3-dimercaptopropane-1-sulfonate (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA) on the renal disposition of mercury in normal and uninephrectomized rats exposed to inorganic mercury. *J Pharmacol Exp Ther.* Nov; 267(2):791-800, 1993

40. Zalups RK, Gelein RM, Cernichiari E: DMPS as a rescue agent for the nephropathy induced by mercuric chloride. *J Pharmacol Exp Ther.* Jan;256(1):1-10, 1991

41. Zalups RK, Lash LH: Binding of mercury in renal brush-border and basolateral membrane-vesicles. *Biochem Pharmacol.* Jun 15;53(12):1889-900, 1997