Published in final edited form as:
Am J Physiol Renal Physiol. 2006 August ; 291(2): F314–F321. doi:10.1152/ajprenal.00497.2005.

Transport of estrone sulfate by the novel organic anion transporter Oat6 (*Slc22a20*)

Gloriane W. Schnabolk, **Geri L. Youngblood**, and **Douglas H. Sweet**
Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, South Carolina

Abstract

Recently, a novel *Slc22* gene family member expressed in murine olfactory mucosa was identified and based on sequence homology proposed to be an organic anion transporter [Oat6 (*Slc22a20*); J. C. Monte, M. A. Nagle, S. A. Eraly, and S. K. Nigam. *Biochem Biophys Res Commun* 323: 429–436, 2004]. However, no functional data for Oat6 was reported. In the present study, we demonstrate that murine Oat6 mediates the inhibitable transport of estrone sulfate using both *Xenopus* oocyte expression assay and Chinese hamster ovary (CHO) cells stably transfected with mOat6 (CHO-mOat6). Uptake was virtually eliminated by probenecid and the anionic herbicide 2,4-dichlorophenoxyacetate. The organic anions ochratoxin A, salicylate, penicillin G, *p*-aminohippurate, and urate inhibited mOat6-mediated accumulation to varying degrees. Transport of estrone sulfate by mOat6 was demonstrated to be saturable, and K_m estimates of 109.8 ± 22.6 μM in oocytes and 44.8 ± 7.3 μM in CHO-mOat6 cells were obtained. Inhibitory constants for 2,4-dichlorophenoxyacetate (15.7 ± 2.0 μM), salicylate (49.0 ± 4.4 μM), probenecid (8.3 ± 2.5 μM), and penicillin G (1,450 ± 480 μM) were also determined. Accumulation of estrone sulfate mediated by mOat6 was significantly *trans*-stimulated by glutarate, indicating that mOat6 functions as an organic anion/dicarboxylate exchanger. These data demonstrate for the first time that the novel murine gene *Oat6* (*Slc22a20*) encodes a functional organic anion transporter and mOat6 is indeed the newest member of the OAT gene family.

Keywords

drug transport; olfactory mucosa; oocytes; *Xenopus laevis*

Delivery of drugs via intranasal administration offers several advantages, including rapid absorption, bypassing of gut efflux transporters that limit bioavailability, avoidance of hepatic first-pass effects after oral administration, and a direct route in the central nervous system (CNS), circumventing the need to cross the blood-brain barrier. Indeed, the direct transfer of drugs, hormones, neurotransmitters, and carboxylic acids into the CNS after administration to the nasal cavity has been observed (7–9,11,12,17). Transport of these substances across olfactory mucosa, coupled with microarray data that detected the expression of a wide variety of transporters in murine olfactory mucosa (e.g., *Abca2*, *Abcc1*, *Abcg1*, *Slc4a1*, *Slc5a1*, *Slc27a2*), have refueled an interest in this tissue as a potential drug delivery route to treat infections and disorders of the CNS (18). Recently, a novel *Slc22* gene family member expressed in murine olfactory mucosa was identified in the Ensembl mouse genome database and proposed to be an organic anion transporter, Oat6 (*Slc22a20*; see Ref. 31).

Address for reprint requests and other correspondence: D. H. Sweet, Dept. of Pharmaceutical Sciences, Medical Univ. of South Carolina, 280 Calhoun St. (Rm. QE218), P.O. Box 250140, Charleston, SC 29425 sweetd@musc.edu.

The organic anion transporter (OAT) family plays a critical role in the excretion and detoxification of anionic drugs, herbicides, toxins, sulfated steroid hormones, and neurotransmitter metabolites. The OATs, along with the organic cation transporters, comprise the amphiphilic solute transporter (*Slc22a*) family, a branch of the solute carrier gene superfamily (39,40,50). Six members of the OAT family have been functionally characterized; Oat1, Oat2, Oat3, OAT4 (which has thus far only been identified in humans), Oat5 (which has rat and mouse orthologs, but as of yet no human ortholog), and Urat1 (39,50). All of these transporters (Oat1–5 and Urat1) are expressed in the kidney where they play a central role as determinants of toxicity, in the therapeutic action of drugs, and in the excretion and reabsorption of a variety of endogenous substances. OATs are also expressed in other barrier epithelia, such as liver (Oat2; see Refs. 36 and 38), placenta (OAT4; see Ref. 6), choroid plexus (Oat1, Oat2, and Oat3; see Ref. 43), and brain capillary endothelium (Oat3; see Ref. 32). Thus a more detailed understanding of OAT function and specificity at the molecular level will aid in the pharmacokinetic modeling of substrate distribution within body compartments and in the prediction of pharmacodynamic interactions and toxicity. Increased knowledge of OAT expression and function in olfactory mucosa may improve our ability to exploit these transporters as drug targets and improve the efficacy of drugs delivered to the CNS via intranasal administration.

Interestingly, Oat6 mRNA was only strongly expressed in olfactory mucosa, and to a lesser extent in testis, with no expression in kidney or liver (31). This unique distribution may reflect an as yet unknown role for Oat6 in odorant detection and detoxification. Detailed understanding of the mechanism of action and substrate specificity of Oat6 compared with Oat1, also found in olfactory mucosa (31), will be essential for accurate modeling of the basic physiology and toxicology of this barrier tissue. This information, combined with what is known about those OATs already found to be expressed in the brain capillary endothelium and/or choroid plexus (Oat1–3), could aid the design of future therapeutic strategies aimed at the successful delivery of substances to the CNS through the olfactory mucosa. Toward this end, in the current work we sought to establish the function of the putative organic anion transporter Oat6 (*Slc22a20*) previously identified by in silico analysis (31). We have demonstrated for the first time that the novel murine gene *Oat6* encodes a functional organic anion transporter.

MATERIALS AND METHODS

Murine Oat6 cDNA clone

The DNA sequence ENST00000324623 identified in the Ensembl mouse genome database and designated as Oat6 (31) was used to perform a BLAST search (1) of the GenBank database to find a corresponding cDNA clone (GenBank accession nos. BC046588 and NM198650). The corresponding clone was then selected from a cDNA library of the Integrated Molecular Analysis of Genomes, and their Expression Consortium (cDNA clone MGC: 54868; IMAGE:6309674) and obtained from the American Type Culture Collection (Manassas, VA). The Oat6 plasmid was transformed into *Escherichia coli*, amplified, and purified according to standard laboratory protocols (35). All manipulations were done in accordance with the procedures outlined by the Medical University of South Carolina Institutional Biosafety Committee registration for infectious agents and recombinant DNA (IBC#2123–3 and IBC#2123–4, reapproved 5/17/2005).

Xenopus oocyte expression assay

Xenopus oocyte isolation and expression assay procedures were conducted as reported previously (41,43,51). Tricaine methanesulfonate (6 mg/ml; Sigma, St. Louis, MO) anesthetized adult female *Xenopus laevis* were decapitated, and the ovaries were removed.

Ovaries were subjected to collagenase A treatment (5 mg/ml; Roche, Nutley, NJ) to produce follicle-free stage V and VI oocytes. The following day, oocytes were injected with 20 ng of capped cRNA synthesized from linearized Oat6 cDNA using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). After 3 days, oocytes were randomly divided into experimental groups (n = 5–10 oocytes/group) and incubated for 1 h at room temperature in oocyte Ringer 2 (OR-2; in mM: 82.5 NaCl, 2.5 KCl, 1 Na_2PO_4, 3 NaOH, 1 $CaCl_2$, 1 $MgCl_2$, 1 pyruvic acid, and 5 HEPES, pH 7.6) containing 2–400 μM [^{3}H]estrone sulfate (ES; 0.5 μCi/ml). Inhibitors were added as indicated in the legends for Figs. 1–5. For *trans*-stimulation studies, oocytes were preloaded by 90 min incubation in OR-2 containing 2.5 mM glutarate and quickly rinsed in ice-cold glutarate-free medium before initiation of uptake. Water-injected or uninjected control oocytes were included in every experiment; no differences were observed between the two treatments. Individual oocyte radioactivity was measured in a Packard Tri-Carb 2900TR liquid scintillation counter with external quench correction. All experiments were done in three to five animals to confirm results.

The program for laboratory animal care at the Medical University of South Carolina has an assurance statement on file with NIH Office for the Protection from Research Risks (OPRR)/Department of Health and Human Services and full American Association for the Accreditation of Laboratory Animal Care accreditation (ongoing since 11/05/1987). Animal procedures reported in this manuscript were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (AR#2080, reapproved 9/1/2005).

Isolation of stably transfected cell lines

Chinese hamster ovary (CHO)-FlpIn cells were purchased from Invitrogen (Carlsbad, CA) and maintained in Ham's F-12 medium (Mediatech, Herndon, VA) containing 10% SerumSupreme (Cambrex, Walkersville, MD) and 100 μg/ml zeocin (Invitrogen). Two stably transfected cell lines were produced [a control "mock" transfected cell line stably transformed with the empty pcDNA5/FRT vector (CHO-FRT cells) and the experimental line stably transformed with a pcDNA5/FRT-mouse (m) Oat6 vector (CHO-mOat6 cells)]. The pcDNA5/FRT-mOat6 vector was constructed by gel isolating the 2,058-bp full-length *Xho*I-*Kpn*I fragment from the pCMVSPORT6/mOat6 vector (MGC:54868; IM-AGE: 6309674) and ligating it into *Xho*I-*Kpn*I cut pcDNA5/FRT vector. Before transfection (1 day), 2×10^5 cells were plated in individual wells of a six-well culture plate. Cells were transfected with 1 μg pcDNA5/FRT or pcDNA5/FRT-mOat6 DNA and 8 μg pOG44 DNA overnight at 37°C using Transfectin Lipid Reagent (2 μl transfectin/μg DNA; Bio-Rad, Hercules, CA). The next day, transfected cells were given fresh medium containing 500 μg/ml hygromycin B (Invitrogen) and maintained at 37°C with 5% CO_2. Transfected cell clones were selected with 500 μg/ml hygromycin B for 4 wk and maintained in 125 μg/ml hygromycin B after testing positive for organic anion transport activity.

Cell transport assays

Before uptake assay (2 days), 2×10^5 cells were seeded per well in 24-well tissue culture plates. Antibiotics were removed from the culture medium ~24–36 h before assays were performed. Before transport experiments, the culture medium was removed, and the cells were washed with 500 μl of transport buffer [Hanks' balanced salt solution (Sigma) containing 10 mM HEPES, pH 7.4] with a final application of 500 μl transport buffer containing 5–500 μM [^{3}H]ES (0.5 μCi/ml) in the absence or presence of inhibitors as indicated. After incubation at room temperature (2 or 15 min), the medium was removed, and the cells were rinsed rapidly three times with ice-cold transport buffer. The cells were dissolved in 500 μl of 1 M NaOH and neutralized with 50 μl of 10 M HCl. Aliquots were removed for protein assay using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories) and for liquid scintillation counting using Ecoscint H (National Diagnostics, Atlanta, GA). Uptake

was calculated as picomoles of substrate per microgram protein. For *trans*-stimulation studies, cells were preloaded by 90 min incubation in transport buffer containing 2.5 mM glutarate and quickly rinsed in ice-cold glutarate-free transport buffer before initiation of uptake. For inhibitory constant (K_i) experiments accumulation was corrected for background using CHO-FRT cells allowing analysis to focus on the mediated fraction of uptake. The concentration of ES (5 μM) was well below the measured Oat6 K_m for ES of 45 μM to ensure the validity of the calculations. Data were analyzed by nonlinear regression (using GraphPad Prism software), and K_i values were estimated by the Cheng-Prusoff equation assuming competitive inhibition. In support of this assumption, a number of studies have determined that probenecid, 2,4-dichlorophenoxyacetate (2,4-D), and penicillin G interact with OAT family members in a competitive manner (15,22,33). All experiments were repeated three to four times in triplicate (3 wells of cells/experimental manipulation) to confirm results.

Statistics

Data are presented as means ± SE. Comparisons were made using unpaired Student's *t*-test, and differences in mean values were considered significant at $P \leq 0.05$.

Chemicals

All chemicals were of reagent grade. [^{3}H]ES (50 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled ES, *p*-aminohippurate (PAH), 2,4-D, ochratoxin A, penicillin G, urate, and probenecid were obtained from Sigma; salicylate was obtained from Fisher (Fair Lawn, NJ).

RESULTS

Organic anion transport by mOat6 (Slc22a20)

Two heterologous expression systems were used to verify that Oat6 is a functional transporter [*Xenopus laevis* oocyte expression assay and transport in stably transfected mammalian cell lines (cell line transport assay)]. Oocytes expressing Oat6 exhibited an approximately sixfold increase in accumulation of the organic anion ES over that measured in nonexpressing control oocytes (Fig. 1). These results were confirmed using a CHO cell line stably transfected with mOat6 (CHO-mOat6), which accumulated fivefold greater ES than a cell line stably transfected with empty vector (CHO-FRT; Fig. 1). Oat6-mediated transport of ES was completely inhibited by the organic anion transport inhibitor probenecid in both the oocyte expression assay and the cell line transport assay (Fig. 1). The time course of mediated ES accumulation in the CHO-mOat6 cells was examined and found to be linear for at least 15 min (data not shown). Therefore, 2 min was chosen as an approximation of initial rates of uptake in the kinetic studies.

Saturation kinetics

Further analysis demonstrated that Oat6-mediated ES transport was a saturable process. Uptake of ES was determined in mOat6-and water-injected oocytes exposed to increasing concentrations of ES (Fig. 2, *A* and *B*). Uptake data from Oat6-expressing oocytes were corrected for diffusion at each ES concentration (Fig. 2*A*) and then subjected to double-reciprocal analysis (Fig. 2*B*). The kinetic analysis was repeated in four different animals, and an estimated K_m value for ES of 110 ± 23 μM was obtained. Saturable uptake of ES was also observed in the CHO-mOat6 cells (Fig. 2, *C* and *D*). Uptake was corrected for diffusion by subtracting background accumulation in CHO-FRT control cells (Fig. 2*C*), and a Lineweaver-Burke plot yielded an estimated K_m value for ES of 45 ± 7 μM (Fig. 2*D*).

Inhibition profile

Members of the OAT family exhibit overlapping specificities for many substrates. Therefore, we examined the inhibitory effect of a number of known OAT family substrates on Oat6-mediated ES transport using both the oocyte expression assay and cell line transport assay (Fig. 3). Probenecid, a compound that inhibits transport mediated by every member of the OAT family, completely blocked ES transport by Oat6. The anionic herbicide 2,4-D also virtually eliminated Oat6 transport of ES in both oocytes and CHO-mOat6 cells (Fig. 3). Significant inhibition of Oat6-mediated ES transport was also observed in each model system for the OAT substrates ochratoxin A, salicylate, and penicillin G. In mOat6-expressing oocytes, PAH produced only a modest inhibition of ES transport that was not found to be significant, whereas urate had little to no effect (Fig. 3). In contrast, inhibition of ES transport by PAH and urate in CHO-mOat6 cells was significant even though roughly similar levels of inhibition were observed in the two systems (Fig. 3). Such differences in observations made in oocytes and cell lines have been observed (21,27,28,45).

Further experiments were conducted in the CHO-mOat6 cells to examine the kinetics of inhibition for four of the common OAT substrates shown to inhibit mOat6 [probenecid, salicylate, 2,4-D, and penicillin G (Fig. 4)]. Inhibition of mOat6-mediated ES (5 μM) transport by increasing concentrations (10^{-6} to 10^{-1} M) of unlabeled test compound was examined, and the K_i values were calculated from the data using the Cheng-Prusoff equation and GraphPad Prism software assuming competitive inhibition (Fig. 4 and Table 1). Probenecid (K_i = 8.3 ± 2.5 μM) and 2,4-D (K_i = 15.7 ± 2.0 μM) displayed similar apparent affinities for mOat6 and had the highest affinity for mOat6 of the four compounds tested. Salicylate (K_i = 49.0 ± 4.4 μM) also had high affinity for mOat6, albeit slightly less than that of probenecid and 2,4-D. Finally, the data indicated that penicillin G (K_i = 1,450 ± 480 μM) interacts very poorly with mOat6, with its affinity being two to three orders of magnitude less than the other compounds (Fig. 4 and Table 1).

trans-Stimulation

The mOat6 protein is most similar to mOat1 and mOat3 in comparison with all the other OATs (31). Oat1 and Oat3 are organic anion/dicarboxylate exchangers and transport organic anions into the cell in exchange for α-keto-glutarate and glutarate (41,46). If mOat6, like Oat1 and Oat3, functions as an exchanger then preloading the oocyte or cell with the dicarboxylate glutarate would increase the cellular concentration of the available counterion and thus stimulate ES transport into the oocyte or cell. The results of such a *trans*-stimulation experiment are shown in Fig. 5. Preloading the mOat6-expressing oocytes with glutarate produced a threefold increase in ES transport over oocytes that were not preloaded before uptake. Control oocytes showed no active ES transport nor any increase related to preloading the oocytes with glutarate. Similar results were generated using the CHO-mOat6 cells (Fig. 5). A greater than twofold increase in ES accumulation was observed in CHO-mOat6 preloaded with glutarate compared with nonpreloaded cells. The CHO-FRT control cells confirmed that the increase in ES accumulation was a result of the expression of mOat6 and not a nonspecific effect of glutarate on the integrity of the cells.

DISCUSSION

Genome sequencing and bioinformatics have identified a multitude of putative genes for which no function has been assigned. Sequence homology to known gene families can indicate a potential activity for a gene product, but actual demonstration of this function is necessary for the accurate modeling of complex biochemical processes. Therefore, after Monte et al. (31) suggested that Oat6 was a new member of the OAT gene family, we sought to identify an organic anion substrate for Oat6 and thus confirm its predicted organic

anion transporter function. Using *Xenopus laevis* oocyte expression assay and cultured cell transport assay, we demonstrated Oat6-mediated accumulation of the organic anion ES (Figs. 1–5).

Like all OAT family members, mOat6 is multispecific and its function is impacted by a number of known substrates and inhibitors of Oat1–5, including ochratoxin A, salicylate, and penicillin G (2,3,5,6,26,28,36,37,48,51). Despite these similarities, some differences in substrate specificity were noted. For example, although mOat6 shares the greatest sequence homology with murine Oat1, and both are expressed in olfactory mucosa (31), ES is a high-affinity substrate for mOat6 but is not transported by mOat1 (43). Conversely, PAH is a high-affinity substrate for Oat1 (37,43,46), but despite producing a modest inhibition of ES uptake in CHO-mOat6 cells (Fig. 3), no significant Oat6-mediated accumulation of PAH was directly observed (data not shown). Furthermore, mOat3, which is nearly as similar to mOat6 as mOat1, transports both PAH and ES (43). However, when examined by PCR, mOat3 was not found to be expressed in olfactory mucosa (31). Thus the olfactory mucosa is the first barrier epithelium for which it has been demonstrated that Oat1 and Oat3, which exist as a gene pair (16), are not expressed together. Perhaps this unique expression profile of Oat1 and Oat6 in olfactory mucosa, in combination with their somewhat different substrate specificities, serves to make organic anion uptake in this tissue distinct from other barrier epithelia in which Oat1 and Oat3 are coexpressed (e.g., renal proximal tubule or choroid plexus).

Comparison of kinetic constants determined for the OATs is somewhat tenuous due to the variety of substrates, heterologous expression systems, and transporter species used in their determination. Estimates of K_m for ES on rat and human OAT3 (2 and 3 μM, respectively), human OAT4 (1 μM), and murine and rat Oat5 (2 and 19 μM, respectively) have been reported and are comparable to the K_m of 45 μM reported here for mOat6 (2,5,6,28,29). Determination of K_i for probenecid, 2,4-D, salicylate, and penicillin G on mOat6 indicate a rank order of affinity of probenecid = 2,4-D > salicylate ⋙ penicillin G (Table 1). Estimates of K_i for probenecid on the exchangers human (h) OAT1 (12.1 μM) and hOAT3 (9 μM) are most similar to the K_i determined for mOat6 (8.3 μM); the K_i for hOAT4 (54.9 μM) is fairly similar; and the K_i reported for hOAT2 of 766 μM is approximately two orders of magnitude higher (15,47). A K_m of 20 μM for 2,4-D on rat (r) Oat3 has been reported and is similar to the K_i of 15.7 μM determined here for 2,4-D inhibition of ES transport by mOat6 (33). The mOat6 K_i of 49 μM for salicylate is close to the K_m of 88.9 μM reported for the transport of salicylate by rOat2 and approximately sevenfold lower (i.e., indicating higher affinity) than the K_i of 341 μM measured for hOAT1 (3,36). Last, the K_i for penicillin G on mOat6 of 1.5 mM is similar to that determined for rOat1 (1.7 mM) and 30-fold higher (i.e., indicating lower affinity) than that for rOat3 (52.8 μM; see Refs. 21 and 23). Thus, for probenecid, Oat6 is like Oat1 and Oat3; for 2,4-D like Oat3; for salicylate like Oat2; and for penicillin G like Oat1 and vastly different from Oat3 (Table 1).

Like Oat6, Oat1, Oat3, and Oat5 are known to either transport or be inhibited by 2,4-D (10,33,34,39). Stupor, coma, and even death have been documented as endpoints of exposure to 2,4-D (13,14), and it has previously been demonstrated that the basolateral organic anion exchangers Oat1 and Oat3 transport 2,4-D (33,39). Many common household herbicides that are applied by spraying contain 2,4-D, and thus exposure to the olfactory mucosa through inhalation of aerosols and subsequent translocation to the CNS mediated by Oat1 and/or Oat6 could readily occur.

Similar to the renal proximal tubule and the choroid plexus, the olfactory mucosa is a polarized epithelium. It covers the ceiling of the nasal cavity and consists of olfactory receptors (neurons), sustentacular cells, and basal cells (4). The polarized olfactory receptors

and sustentacular cells have their apical membranes, which possess cilia or microvilli (respectively), oriented toward the nasal cavity. Recent studies have indicated the presence of a number of transporters in the olfactory mucosa and confirmed that they support active solute transport across this epithelium (19,20,24,25,31,49). Currently, the membrane targeting of Oat1 and Oat6 in olfactory mucosa is unknown. Tissue-specific membrane targeting of OATs has been observed. For example, the exchanger Oat1 is targeted to the basolateral membrane in kidney and the apical membrane in choroid plexus. If Oat1 were targeted to the apical membrane of olfactory mucosa cells, its localization and mechanism of action (organic anion exchanger) would be consistent with it playing a role in the absorption of xenobiotic molecules from the mucus. This situation would be analogous to the targeting of Oat1 to the brush-border membrane in choroid plexus where it mediates the removal of organic anions from the cerebrospinal fluid (34). Similarly, if Oat6 is localized to the apical membrane of cells in the olfactory mucosa, it will be poised to mediate the cellular uptake of organic anions dissolved in the mucus.

However, if Oat1 is localized to the basolateral membrane, it could potentially mediate the removal of organic anions and toxicants from the richly vascularized lamina propria underlying the olfactory mucosa. Subsequent excretion in the mucus would be accomplished by an apically targeted transporter such as one of the multidrug resistance-associated proteins (18,24,30). This situation would be analogous to renal proximal tubule cells where Oat1 is targeted to the basolateral membrane and it mediates the removal of organic anions from the blood for eventual secretion in urine (42,46). Thus targeting of Oat6 to the basolateral membrane of the olfactory mucosa would allow it to play a role in clearance/detoxification from the lamina propria.

In summary, we have provided functional evidence that the novel OAT family member Oat6 (*Slc22a20*) does mediate the transport of the organic anion ES. This transport is inhibited by several classic OAT substrates and inhibitors. The transport of ES by mOat6 was also shown to be *trans*-stimulated by glutarate. This indicates mOat6 can function as an exchanger and suggests mOat6 may be mechanistically similar to Oat1 and Oat3, being indirectly coupled to cellular energy and the Na^+ gradient through the tertiary active transport process (39,40). Identification of the substrate specificity of Oat6 increases our understanding of organic anion transport in the olfactory epithelium and will allow us to accurately model the mechanisms contributing to the absorption of drugs and toxic substances via the olfactory mucosa. Further work is needed to elucidate the importance of Oat6 function in the olfactory epithelium and its potential exploitation for the targeted delivery of drugs to treat infections and disorders of the CNS as well as the repercussions of inhaling toxic organic anions. Future studies will also center on further delineating the energetics driving Oat6-mediated transport. It is well established that the physiological properties of organic anion transport across the basolateral and apical membranes of polarized cells are distinct (39,41,44,46). Thus knowing that a transporter functions as an OAT is insufficient to determine its placement in the organic anion transport model or to define its role in the transcellular flux of organic anions. Key to understanding any potential role for Oat6 in drug delivery, odorant detection, or detoxification in olfactory mucosa will be determining its membrane distribution in the cells that express it.

Acknowledgments

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-067216.

References

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389–3402. [PubMed: 9254694]
2. Anzai N, Jutabha P, Enomoto A, Yokoyama H, Nonoguchi H, Hirata T, Shiraya K, He X, Cha SH, Takeda M, Miyazaki H, Sakata T, Tomita K, Igarashi T, Kanai Y, Endou H. Functional characterization of rat organic anion transporter 5 (slc22a19) at the apical membrane of renal proximal tubules. J Pharmacol Exp Ther. 2005; 315:534–544. [PubMed: 16079298]
3. Apiwattanakul N, Sekine T, Chairoungdua A, Kanai Y, Nakajima N, Sophasan S, Endou H. Transport properties of nonsteroidal anti-inflammatory drugs by organic anion transporter 1 expressed in Xenopus laevis oocytes. Mol Pharmacol. 1999; 55:847–854. [PubMed: 10220563]
4. Carola, R.; Harley, JP.; Noback, CR. Human Anatomy and Physiology. New York: McGraw Hill; 1992.
5. Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, Endou H. Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. Mol Pharmacol. 2001; 59:1277–1286. [PubMed: 11306713]
6. 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. 2000; 275:4507–4512. [PubMed: 10660625]
7. Chou KJ, Donovan MD. Lidocaine distribution into the CNS following nasal and arterial delivery: a comparison of local sampling and microdialysis techniques. Int J Pharm. 1998; 171:53–61.
8. Chow HH, Anavy N, Villalobos A. Direct nose-brain transport of benzoylecgonine following intranasal administration in rats. J Pharm Sci. 2001; 90:1729–1735. [PubMed: 11745730]
9. Chow HS, Chen Z, Matsuura GT. Direct transport of cocaine from the nasal cavity to the brain following intranasal cocaine administration in rats. J Pharm Sci. 1999; 88:754–758. [PubMed: 10430537]
10. Cihlar T, Lin D, Pritchard JB, Fuller MD, Mendel DB, Sweet DH. The antiviral nucleoside phosphonates cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. Mol Pharm. 1999; 56:570–580.
11. Dahlin M, Bergman U, Jansson B, Bjork E, Brittebo E. Transfer of dopamine in the olfactory pathway following nasal administration in mice. Pharm Res. 2000; 17:737–742. [PubMed: 10955850]
12. Derad I, Willeke K, Pietrowsky R, Born J, Fehm HL. Intranasal angiotensin II directly influences central nervous regulation of blood pressure. Am J Hypertens. 1998; 11:971–977. [PubMed: 9715790]
13. Dudley AW Jr, Thapar NT. Fatal human ingestion of 2,4-D, a common herbicide. Arch Pathol. 1972; 94:270–275. [PubMed: 5051650]
14. Elo HA, Ylitalo P. Distribution of 2-methyl-4-chlorophenoxyacetic acid and 2,4dichlorophenoxyacetic acid in male rats: evidence for the involvement of the central nervous system in their toxicity. Toxicol Appl Pharmacol. 1979; 51:439–446. [PubMed: 538756]
15. Enomoto A, Takeda M, Shimoda M, Narikawa S, Kobayashi Y, Yamamoto T, Sekine T, Cha SH, Niwa T, Endou H. Interaction of human organic anion transporters 2 and 4 with organic anion transport inhibitors. J Pharmacol Exp Ther. 2002; 301:797–802. [PubMed: 12023506]
16. Eraly SA, Hamilton BA, Nigam SK. Organic anion and cation transporters occur in pairs of similar and similarly expressed genes. Biochem Biophys Res Commun. 2003; 300:333–342. [PubMed: 12504088]
17. Eriksson C, Bergman U, Franzen A, Sjoblom M, Brittebo EB. Transfer of some carboxylic acids in the olfactory system following intranasal administration. J Drug Target. 1999; 7:131–142. [PubMed: 10617298]
18. Genter MB, Van Veldhoven PP, Jegga AG, Sakthivel B, Kong S, Stanley K, Witte DP, Ebert CL, Aronow BJ. Microarray-based discovery of highly expressed olfactory mucosal genes: potential roles in the various functions of the olfactory system. Physiol Genomics. 2003; 16:67–81. [PubMed: 14570983]

19. Graff CL, Pollack GM. Functional evidence for P-glycoprotein at the nose-brain barrier. Pharm Res. 2005; 22:86–93. [PubMed: 15771234]
20. Graff CL, Pollack GM. P-Glycoprotein attenuates brain uptake of substrates after nasal instillation. Pharm Res. 2003; 20:1225–1230. [PubMed: 12948020]
21. Hasegawa M, Kusuhara H, Sugiyama D, Ito K, Ueda S, Endou H, Sugiyama Y. Functional involvement of rat organic anion transporter 3 (rOat3; Slc22a8) in the renal uptake of organic anions. J Pharmacol Exp Ther. 2002; 300:746–753. [PubMed: 11861777]
22. Hashimoto T, Narikawa S, Huang XL, Minematsu T, Usui T, Kamimura H, Endou H. Characterization of the renal tubular transport of zonampanel, a novel alpha-amino-3hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist, by human organic anion transporters. Drug Metab Dispos. 2004; 32:1096–1102. [PubMed: 15377641]
23. Jariyawat S, Sekine T, Takeda M, Apiwattanakul N, Kanai Y, Sophasan S, Endou H. The interaction and transport of beta-lactam antibiotics with the cloned rat renal organic anion transporter 1. J Pharmacol Exp Ther. 1999; 290:672–677. [PubMed: 10411577]
24. Kandimalla KK, Donovan MD. Carrier mediated transport of chlorpheniramine and chlorcyclizine across bovine olfactory mucosa: implications on nose-to-brain transport. J Pharm Sci. 2005; 94:613–624. [PubMed: 15666293]
25. Kandimalla KK, Donovan MD. Localization and differential activity of P-glycoprotein in the bovine olfactory and nasal respiratory mucosae. Pharm Res. 2005; 22:1121–1128. [PubMed: 16028013]
26. Kobayashi Y, Ohshiro N, Shibusawa A, Sasaki T, Tokuyama S, Sekine T, Endou H, Yamamoto T. Isolation, characterization and differential gene expression of multispecific organic anion transporter 2 in mice. Mol Pharmacol. 2002; 62:7–14. [PubMed: 12065749]
27. Kumar CK, Nguyen TT, Gonzales FB, Said HM. Comparison of intestinal folate carrier clone expressed in IEC-6 cells and in *Xenopus* oocytes. Am J Physiol Cell Physiol. 1998; 274:C289–C294.
28. 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. 1999; 274:13675–13680. [PubMed: 10224140]
29. Kwak JO, Kim HW, Oh KJ, Ko CB, Park H, Cha SH. Characterization of mouse organic anion transporter 5 as a renal steroid sulfate transporter. J Steroid Biochem Mol Biol. 2005; 97:369–375. [PubMed: 16150593]
30. Manzini I, Schild D. Multidrug resistance transporters in the olfactory receptor neurons of Xenopus laevis tadpoles. J Physiol. 2003; 546:375–385. [PubMed: 12527725]
31. Monte JC, Nagle MA, Eraly SA, Nigam SK. Identification of a novel murine organic anion transporter family member, OAT6, expressed in olfactory mucosa. Biochem Biophys Res Commun. 2004; 323:429–436. [PubMed: 15369770]
32. Mori S, Takanaga H, Ohtsuki S, Deguchi T, Kang YS, Hosoya K, Terasaki T. Rat organic anion transporter 3 (rOAT3) is responsible for brain-to-blood efflux of homovanillic acid at the abluminal membrane of brain capillary endothelial cells. J Cereb Blood Flow Metab. 2003; 23:432–440. [PubMed: 12679720]
33. Nagata Y, Kusuhara H, Imaoka T, Endou H, Sugiyama Y. Involvement of rat organic anion transporter 3 in the uptake of an organic herbicide, 2,4dichlorophenoxyacetate, by the isolated rat choroid plexus. J Pharm Sci. 2004; 93:2724–2732. [PubMed: 15389674]
34. Pritchard JB, Sweet DH, Miller DS, Walden R. Mechanism of organic anion transport across the apical membrane of choroid plexus. J Biol Chem. 1999; 274:33382–33387. [PubMed: 10559217]
35. Sambrook, J.; Russell, DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.
36. Sekine T, Cha SH, Tsuda M, Apiwattanakul N, Nakajima Y, Endou H. Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. FEBS Lett. 1998; 429:179–182. [PubMed: 9650585]
37. Sekine T, Watanabe N, Hosoyamada M, Kanai Y, Endou H. Expression cloning and characterization of a novel multispecific organic anion transporter. J Biol Chem. 1997; 272:18526–18529. [PubMed: 9228014]

38. Simonson GD, Vincent AC, Roberg KJ, Huang Y, Iwanij V. Molecular cloning and characterization of a novel liver-specific transport protein. J Cell Sci. 1994; 107:1065–1072. [PubMed: 8056831]
39. Sweet DH. Organic anion transporter (Slc22a) family members as mediators of toxicity. Toxicol Appl Pharmacol. 2005; 204:198–215. [PubMed: 15845414]
40. Sweet DH, Bush KT, Nigam SK. The organic anion transporter family: from physiology to ontogeny and the clinic. Am J Physiol Renal Physiol. 2001; 281:F197–F205. [PubMed: 11457711]
41. Sweet DH, Chan LMS, Walden R, Yang X, Miller DS, Pritchard JB. Organic anion transporter 3 [Slc22a8] is a dicarboxylate exchanger indirectly coupled to the Na^+ gradient. Am J Physiol Renal Physiol. 2003; 284:F763–F769. [PubMed: 12488248]
42. Sweet DH, Miller DS, Pritchard JB. Localization of an organic anion transporter-GFP fusion construct (rROAT1-GFP) in intact proximal tubules. Am J Physiol Renal Physiol. 1999; 276:F864–F873.
43. Sweet DH, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, Nigam SK. Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (*Oat3* [*Slc22a8*]) knockout mice. J Biol Chem. 2002; 277:26934–26943. [PubMed: 12011098]
44. Sweet DH, Pritchard JB. The molecular biology of renal organic anion and organic cation transporters. Cell Biochem Biophys. 1999; 31:89–118. [PubMed: 10505670]
45. Sweet DH, Pritchard JB. rOCT2 is a basolateral potential-driven carrier, not an organic cation/proton exchanger. Am J Physiol Renal Physiol. 1999; 277:F890–F898.
46. Sweet DH, Wolff NA, Pritchard JB. Expression cloning and characterization of ROAT1. The basolateral organic anion transporter in rat kidney. J Biol Chem. 1997; 272:30088–30095. [PubMed: 9374486]
47. Takeda M, Narikawa S, Hosoyamada M, Cha SH, Sekine T, Endou H. Characterization of organic anion transport inhibitors using cells stably expressing human organic anion transporters. Eur J Pharmacol. 2001; 419:113–120. [PubMed: 11426832]
48. Tsuda M, Sekine T, Takeda M, Cha SH, Kanai Y, Kimura M, Endou H. Transport of ochratoxin A by renal multispecific organic anion transporter 1. J Pharmacol Exp Ther. 1999; 289:1301–1305. [PubMed: 10336520]
49. Wang F, Jiang X, Lu W. Profiles of methotrexate in blood and CSF following intranasal and intravenous administration to rats. Int J Pharm. 2003; 263:1–7. [PubMed: 12954175]
50. Wright SH, Dantzler WH. Molecular and cellular physiology of renal organic cation and anion transport. Physiol Rev. 2004; 84:987–1049. [PubMed: 15269342]
51. Youngblood GL, Sweet DH. Identification and functional assessment of the novel murine organic anion transporter Oat5 (Slc22a19) expressed in kidney. Am J Physiol Renal Physiol. 2004; 287:F236–F244. [PubMed: 15068970]

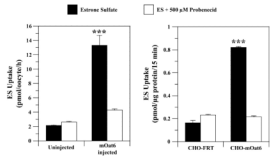

Fig. 1.

Mediated organic anion transport by murine organic anion transporter (Oat) 6. Uptake of estrone sulfate (ES) was measured in mOat6-expressing *Xenopus* oocytes and in Chinese hamster ovary (CHO) cells stably transfected with mOat6 (CHOmOat6). *Left*: 3 days after injection with Oat6 cRNA or water, 60 min ES uptake was measured by *Xenopus* oocyte expression assay. Inhibitor-sensitive (+500 μM probenecid) accumulation of [^{3}H]ES (5 μM) was observed in Oat6-expressing oocytes, demonstrating that Oat6 does function as an organic anion transporter. *Right*: significant probenecid-sensitive [^{3}H]ES (5 μM) accumulation was also observed in CHO-mOat6 cells. The presence of 500 μM probenecid reduced ES levels to that observed in the CHO-FRT control cells. Data shown are mean values ± SE from representative experiments. ***$P \leq 0.0001$ compared with corresponding control.

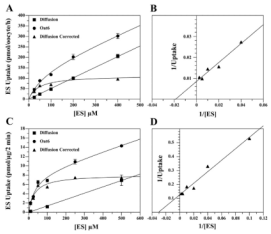

Fig. 2.

Kinetic analysis of mOat6-mediated ES transport. Kinetic parameters of Oat6 transport were measured in *Xenopus* oocytes and in CHO-mOat6 cells exposed to 5–500 μM ES. *A*: saturation analysis. Total ES uptake in Oat6-expressing oocytes (Oat6) was corrected for diffusion by subtracting the ES uptake in water-injected oocytes (Diffusion) at each concentration. *B*: Lineweaver-Burke plot of the diffusion-corrected data with linear regression line. K_m was estimated to be 110 ± 23 μM (n = 4 animals). Data shown are mean values ± SE from a representative animal. *C*: saturation analysis in CHO-mOat6 cells. Total ES uptake in CHO-mOat6 cells (Oat6) was corrected for diffusion by subtracting the calculated ES value from the diffusion line determined by measuring uptake of ES at 10, 100, and 500 μM (Diffusion) in CHO-FRT control cells. *D*: Lineweaver-Burke plot of the diffusion-corrected CHO-mOat6 data with linear regression line. K_m was estimated to be 45 ± 7 μM (n = 3 experiments performed in triplicate). Data are mean values ± SE from a representative experiment. [ES], ES concentration.

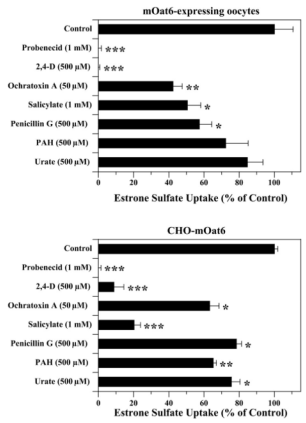

Fig. 3.

Inhibition profile. Inhibition of Oat6 transport of [^{3}H]ES (5 μM) by probenecid (1 mM), 2,4-dichlorophenoxyacetate (2,4-D; 500 μM), ochratoxin A (50 μM), salicylate (1 mM), penicillin G (500 μM), *p*-aminohippurate (PAH; 500 μM), and urate (500 μM) was measured in *Xenopus* oocytes (60 min) and CHO-mOat6 cells (15 min). Oocyte data were corrected for background ES levels measured in water-injected oocytes under each condition and are mean values ± SE from a representative animal; CHOmOat6 data were corrected for background ES level measured in CHO-FRT control cells and are mean values ± SE from a representative experiment. *$P \leq 0.01$, **$P \leq 0.001$, and ***$P \leq 0.0001$ compared with no inhibitor control.

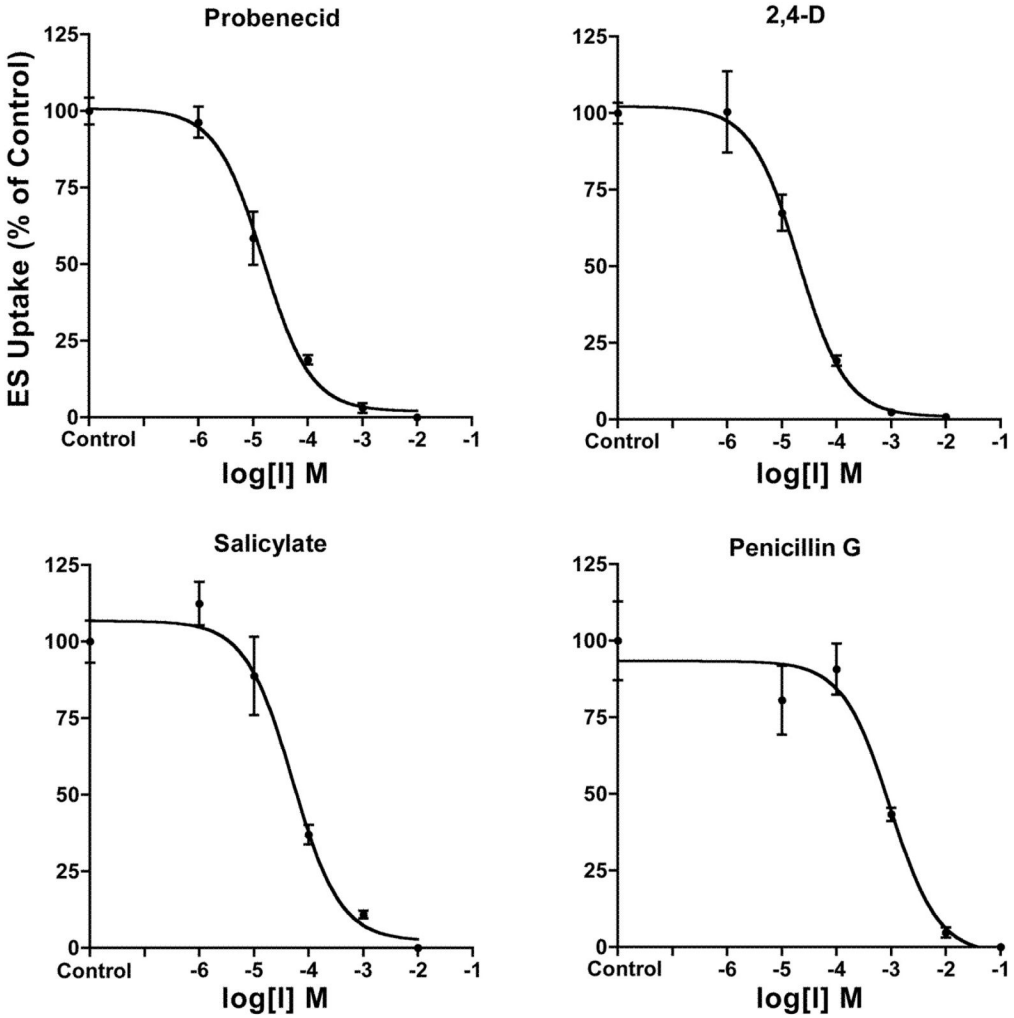

Fig. 4.

Determination of inhibition constant (K_i) values for mOat6. Two-minute uptake of [^{3}H]ES (5 μM) in the presence of increasing concentrations (10^{-6} to 10^{-1} M) of 2,4-D, salicylate, probenecid, and penicillin G was measured in CHO-mOat6 cells. Accumulation was corrected for background using CHO-FRT cells. Results were plotted and analyzed by nonlinear regression, and K_i values were estimated by the Cheng-Prusoff equation using GraphPad Prism software. Estimated K_i values are given in Table 1. Experiments were repeated three to four times in triplicate, and the data shown are mean values ± SE from representative experiments.

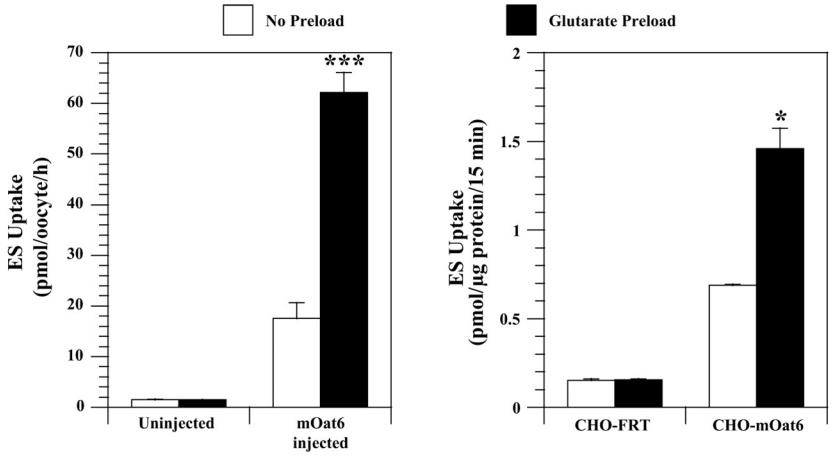

Fig. 5.

Ability of glutarate to *trans*-stimulate mOat6. Uptake of ES in oocytes and CHO cell lines under conditions of unaltered (No Preload) and increased (Glutarate Preload) internal glutarate was examined. Oocytes and CHO cells were preloaded with glutarate by incubation with 2.5 mM glutarate for 90 min followed by a rapid rinse with ice-cold transport buffer before undergoing transport assay. The level of uptake was compared with that of the appropriate controls that did not undergo the glutarate preload. Results indicate that mOat6-mediated uptake is *trans*-stimulated by glutarate. *$P \leq 0.01$ and ***$P \leq 0.0001$ compared with corresponding no preload control.

Table 1

Comparison of estimated K_i (μM) values for OAT family members

Substrate	Oat6	Oat1*	Oat2*	Oat3*	OAT4*	Oat5*	Refs.
Probenecid	8.3±2.5	12.1 (h, PAH)†	766 (h, $PGF_{2\alpha}$)†	9.0; 1.7 (h, ES; r, IS)†	54.9 (h, ES)†	ND	15,47
2,4-D	15.7±2.0	ND	Unknown	20 (K_m) (r, 2,4-D)†	Unknown	ND	33
Salicylate	49.0±4.4	341 (r, PAH)†	88.9 (K_m) (r, salicylate)†	ND	ND	ND	3,36
Penicillin G	1,450±480	1,700 (r, PAH)†	ND	52.8 (r, pravastatin)†	ND	ND	21,23

Oat6 values are reported as means ± SE; PAH, p-aminohippurate; OAT, organic anion transporter; h, human; r, rat; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; ES, estrone sulfate; IS, indoxyl sulfate; ND, inhibitory constant or K_m value not determined; Unknown, interaction never investigated.

*Values were taken from the listed references;

†transporter species and substrate used in determining values were taken from the listed references.