Role of Glycosylation in the Organic Anion Transporter OAT1*

Kunihiko Tanaka, Wen Xu, Fanfan Zhou, and Guofeng You†

From the Department of Pharmaceutics, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854

Organic anion transporters (OAT) play essential roles in the body disposition of clinically important anionic drugs, including antiviral drugs, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories. We reported previously (Kuze, K., Graves, P., Leahy, A., Wilson, P., Stuhlmann, H., and You, G. (1999) J. Biol. Chem. 274, 1519–1524) that tunicamycin, an inhibitor of asparagine-linked glycosylation, significantly inhibited organic anion transport in COS-7 cells expressing a mouse organic anion transporter (mOAT1), suggesting an important role of glycosylation in mOAT1 function. In the present study, we investigated the effect of disrupting putative glycosylation sites in mOAT1 as well as its human counterpart, hOAT1, by mutating asparagine to glutamine and assessing mutant transporters in HeLa cells. We showed that the putative glycosylation site Asp-39 in mOAT1 was not glycosylated but the corresponding site (Asp-39) in hOAT1 was glycosylated. Disrupting Asp-39 resulted in a complete loss of transport activity in both mOAT1 and hOAT1 without affecting their cell surface expression, suggesting that the loss of function is not because of deglycosylation of Asp-39 but rather is likely because of the change of this important amino acid critically involved in the substrate binding. Single replacement of asparagines at other sites had no effect on transport activity indicating that glycosylation at individual sites is not essential for OAT function. In contrast, a simultaneous replacement of all asparagines in both mOAT1 and hOAT1 impaired the trafficking of the transporters to the plasma membrane. In summary, we provided the evidence that 1) Asp-39 is crucially involved in substrate recognition of OAT1, 2) glycosylation at individual sites is not required for OAT1 function, and 3) glycosylation plays an important role in the targeting of OAT1 onto the plasma membrane. This study is the first molecular identification and characterization of glycosylation of OAT1 and may provide important insights into the structure-function relationships of the organic anion transporter family.

Organic anion transporters (OAT) play essential roles in the body disposition of clinically important anionic drugs including anti-human immunodeficiency virus therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatory drugs. Several OAT isoforms have been identified by us and others (2). OAT1 and -3 are predominantly expressed in the kidney and brain. In the kidney, these transporters utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit/elimination across the apical membrane into urine. Through this tertiary transport mechanism, Na\(^+\)-K\(^+\)-ATPase maintains an inwardly directed (blood to cell) Na\(^+\) gradient. The Na\(^+\) gradient then drives a Na\(^+\)-dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion (OA) exchanger to move the OA substrate into the cell. This cascade of events indirectly links OA transport to metabolic energy and the Na\(^+\) gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell. OAT2 is predominantly expressed in the liver. OAT4 is present mainly in the placenta and kidney. The transport mechanisms for OAT2 and -4 remain to be elucidated.

All cloned OATs share a common predicted structure comprising 12 putative transmembrane domains and a large hydrophilic loop between transmembrane domains 1 and 2, which contains several potential N-glycosylation sites. The N-glycosylation of proteins has been demonstrated to play a variety of roles including modulation of biological activity, regulation of intracellular targeting, protein folding, and maintenance of protein stability. We have shown previously (3) that tunicamycin, an inhibitor of asparagine-linked glycosylation, significantly inhibited organic anion transport in COS-7 cells expressing a mouse organic anion transporter (mOAT1), suggesting that glycosylation is essential for mOAT1 function. Despite this initial characterization, the direct molecular evidence on the sites, and the roles of glycosylation in OATs is not yet available. In the present study, we investigated the consequence of disruption of the potential N-glycosylation sites in mOAT1 on its function and cellular localization. Because hOAT1, the human counterpart of mOAT1, is the ultimate target for improving human therapy, the glycosylation of hOAT1 was also investigated.

EXPERIMENTAL PROCEDURES

Materials

\[^{14}\text{C}]\text{Clp-Ammoniophosphoric acid (PAH) was purchased from PerkinElmer Life Sciences. NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce. All other reagents were purchased from Sigma.}

Methods

Site-directed Mutagenesis—Mutant transporters were generated by site-directed mutagenesis of asparagine to glutamine of mOAT1-3myc and hOAT1-myc. Both mOAT1-myc and hOAT1-myc contain a 10-amino acid e-myc tag at the C terminus of mOAT1 and hOAT1. Previous studies from our laboratory (3, 4) showed that the myc-tagged proteins retained the functional properties of the native (unmodified) structure. The mutant sequences were confirmed by the dideoxy chain-termination method.

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† To whom correspondence should be addressed: Dept. of Pharmaceutics, Rutgers, The State University of New Jersey, Piscataway, NJ 08854. Tel.: 732-445-3831 (ext. 218); E-mail: gyou@cop.rutgers.edu

‡ The abbreviations used are: OAT, organic anion transporter; OA, organic anion; mOAT, mouse OAT; hOAT, human OAT; PAH, \[^{14}\text{C}]\text{Clp-ammoniophosphoric acid; PBS, phosphate-buffered saline; NHS-SS-biotin, sulfo-\text{NHS-biotinyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; PNGase F, peptide N-glycosidase F; CM, Ca\(^{2+}\)Mg\(^{2+}\) .}
Expression in HeLa Cells—HeLa cells were grown at 37 °C and 5% CO₂ in minimum Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Confluent HeLa cells were infected with recombinant vTF-7 vaccinia virus and then transfected with DNA plasmid using LipofectAMINE 2000 reagent (Invitrogen) following the established protocol (5). Transfected cells were incubated for 14–20 h at 37 °C and then used for transport assay and cell surface biotinylation.

Transport Measurements—For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/CM (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 0.1 mM CaCl₂, and 1 mM MgCl₂, pH 7.3) and [¹⁴C]PAH. At the times indicated in the figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS solution. The cells were then solubilized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (n = 3).

Isolation of Plasma Membrane Proteins by Cell Surface Biotinylation—Cell surface expression levels of mOAT1, hOAT1, and their mutants were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin. The transporters were expressed in HeLa cells in 6-well plates using vaccinia T7/LipofectAMINE 2000 as described above. After 20 h, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS/CM, pH 8.0. The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 200 mg/ml protease inhibitor phenylmethylsulfonyl fluoride, 3 mg/ml leupeptin, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4 °C. 50 ml of streptavidin-agarose beads were then added to the supernatant to isolate the cell membrane protein. mOAT1 and hOAT1 were detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-myc antibody.

Deglycosylation of Plasma Membrane Proteins with Peptide N-Glycosidase F—For deglycosylation, cell surface proteins were denatured in 0.5% SDS and 1% mercaptoethanol, heated at 80 °C for 10 min, and then incubated in 50 mM sodium phosphate buffer, pH 7.5, 1% Nonidet P-40, and 1 µl of peptide N-glycosidase F (PNGase F, 500 units/µl, New England Biolabs) at 37 °C for 1 h. The samples were then used for immunoblotting.

Electrophoresis and Immunoblotting—Protein samples (with equal amount) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween, washed, and incubated for 1 h at room temperature with monoclonal anti-myc antibody (1:1000). The membranes were washed and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:20,000) and signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce).

Immunofluorescence of Transfected Cells—Twenty hours after transfection, HeLa cells were washed three times in phosphate-buffered saline, fixed for 15 min at room temperature in 4% paraformaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. The cells were incubated for 30 min at room temperature in PBS containing 5% goat serum and then incubated for 1 h in the same medium containing anti-myc antibody (1:100). The cells were washed, and bound primary antibodies were detected by reaction with fluorescein isothiocyanate-coupled goat anti-mouse IgG (Chemicon) diluted to 1:100 for 1 h. Cells were thoroughly washed, and the cover glasses were mounted in GEL/MOUNT™ (Biomeda Corp., Foster City, CA). Samples were visualized on a fluorescence microscope.

RESULTS

N-Glycosylation of mOAT1 and hOAT1—We and others have shown previously (6, 7) that mature OATs are heavily glycosylated proteins in vivo. We showed here (Fig. 1) that both mOAT1 and hOAT1 are also glycosylated in HeLa cells. Immunoblot analysis of plasma membrane proteins isolated from HeLa cells expressing mOAT1 (Fig. 1a) and hOAT1 (Fig. 1b) revealed a single band with an apparent molecular mass of ~90 kDa. When these proteins were treated with PNGase F, an enzyme removing the N-linked carbohydrate groups from glycoproteins, the molecular mass shifted to a smaller size of ~55 kDa, consistent with the predicted (unglycosylated) sizes for mOAT1 and hOAT1. These results showed clearly that both transporters are glycosylated in HeLa cells.

Immunoblot Analysis of the Effect of Single Disruption of Putative Glycosylation Sites—Based on the deduced amino acid sequences of OATs and their secondary structure model, multiple consensus sites (NX(S/T) for N-glycosylation were identified...
Mutant transporters were generated by individually replacing asparagine (N) with glutamine (Q). Western blot analysis of plasma membrane proteins from cells expressing mOAT1 mutants (Fig. 3a) showed that in the absence of PNGase F, N56Q, N86Q, N91Q, and N107Q had molecular masses smaller than that of fully glycosylated (wild type) but larger than that of unglycosylated mOAT1. N39Q had a molecular mass that was the same size as that of wild type mOAT1. Treating these mutant proteins with PNGase F reduced their molecular mass to the same size as that of unglycosylated mOAT1, suggesting that in mOAT1 Asp-56, Asp-86, Asp-91, and Asp-107 are glycosylated, whereas Asp-39 is not utilized for glycosylation. Western blot analysis of plasma membrane proteins from cells expressing hOAT1 mutants (Fig. 3b) showed that in the absence of PNGase F N39Q, N56Q, N92Q, and N97Q had molecular masses smaller than that of fully glycosylated (wild type) but larger than that of unglycosylated mOAT1. N113Q had a molecular mass the same size as that of wild type hOAT1. Treating these mutant proteins with PNGase F reduced their molecular masses to the same as that of unglycosylated hOAT1, suggesting that in hOAT1, Asp-39, Asp-56, Asp-92, and Asp-97 are glycosylated, whereas Asp-113 is not utilized for glycosylation.

Functional Analysis of the Effect of Single Disruption of Putative Glycosylation Sites—We then examined the functional consequence of disrupting these putative glycosylation sites by measuring the uptake of [14C]PAH (a prototypical substrate for OAT) in mutant-transfected cells. As shown in Fig. 4, the single replacement of Asp-39 resulted in a complete loss of transport activity in both mOAT1 (Fig. 4a) and hOAT1 (Fig. 4b). Interestingly, Asp-39 is not glycosylated in mOAT1 (Fig. 3a) but is glycosylated in hOAT1 (Fig. 3b). Therefore, the loss of transport activity of N39Q in both mOAT1 and hOAT1 may not be because of deglycosylation of Asp-39 per se but may rather be because of the change in amino acid sequence. Because the cell surface abundance of N39Q is comparable with that of both wild type mOAT1 (Fig. 3a) and wild type hOAT1 (Fig. 3b), the loss of function of this mutant is not because of the defective targeting of the transporters to the plasma membrane but is rather because of the poor substrate binding/recognition. Replacing asparagines at other sites had no effect on transport activity for both mOAT1 (Fig. 3a) and wild type hOAT1 (Fig. 3b), suggesting that glycosylation at individual sites is not required for the transport function of both transporters.

Immunoblot Analysis of the Effect of Multiple Disruptions of Putative Glycosylation Sites—We next investigated the effect of disruption of all of the glycosylation sites. In mOAT1, simultaneous replacement of Asp-56, Asp-86, Asp-91, and Asp-107 (N56Q/N86Q/N91Q/N107Q) produced an ~55 kDa protein with or without PNGase F treatment (Fig. 5a). This molecular mass is the same as that of unglycosylated mOAT1, indicating that there are no additional sites for N-glycosylation. It is important to note that when all of the glycosylation sites were removed, the expression level of the unglycosylated mOAT1 was dramatically reduced as compared with that of wild type mOAT1 (although the total cellular protein of the quadruple mutant was similar to that of wild type, data not shown), suggesting that glycosylation is critical for the proper trafficking of the transporter onto the plasma membrane. In hOAT1, Asp-39,
N56Q, N92Q, and N97Q are normally glycosylated, whereas Asp-113 was not utilized for glycosylation (Fig. 3b). If this observation is true, then one might predict that the quadruple mutant N39Q/N56Q/N92Q/N97Q should have a molecular mass of ~55 kDa, the size of unglycosylated hOAT1, and PNGase F should fail to produce a molecular mass shift. Interestingly, as shown in Fig. 5b, in the absence of PNGase F N39Q/N56Q/N92Q/N97Q produced a protein of ~65 kDa. Treatment of N39Q/N56Q/N92Q/N97Q with PNGase F further reduced the molecular mass to the size of unglycosylated hOAT1. This result implies that an alternative glycosylation site may be used when the four normal glycosylation sites are eliminated.

To test the hypothesis that Asp-113 is the alternative glycosylation site, we created quintuple mutant N39Q/N56Q/N92Q/N97Q/N113Q. Indeed, this mutant yielded a protein ~55 kDa, the expected molecular mass of the unglycosylated hOAT1, both in the absence and presence of PNGase F. This result proved that Asp-113 became glycosylated when other sites were mutated. Again it is important to note that hOAT1 arrived to the plasma membrane with progressively more difficulties as the degree of glycosylation decrease. Whereas the total amount of cellular proteins of the quadruple (N39Q/N56Q/N92Q/N97Q) and the quintuple (N39Q/N56Q/N92Q/N97Q/N113Q) was similar to that of the wild type (data not shown), the membrane abundance of the quadruple mutant was only ~10% of the wild type hOAT1, and the membrane abundance of the quintuple mutant was almost undetectable.

**Immunofluorescence Analysis of the Effect of Multiple Disruptions of Putative Glycosylation Sites**—Further evidence of this difficulty of deglycosylated protein to be transported to the plasma membrane was obtained by immunofluorescence (Fig. 6). Whereas the plasma membrane was clearly labeled (shown as green fluorescence) in cells transfected with wild type mOAT1 and hOAT1, fluorescence remained mainly in the intracellular compartment for the unglycosylated mOAT1 (N56Q/N86Q/N91Q/N107Q) and the unglycosylated hOAT1 (N39Q/N56Q/N92Q/N97Q/N113Q). Phase contrast images showed that the cells were fully attached to the culture dishes under all conditions. Considering all of these data together, it can be concluded that glycosylation is necessary for proper targeting of both mOAT1 and hOAT1 to the plasma membrane.

**Functional Analysis of the Effect of Multiple Disruption of Putative Glycosylation Sites**—The impact of multiple replacements of glycosylation sites on the function of mOAT1 and hOAT1 were examined (Fig. 7). Although no individual glycosylation site is critical for mOAT1 function, simultaneously removing all of the glycosylation sites resulted in a dramatic reduction in transport activity (Fig. 7a). The unglycosylated hOAT1 also showed no transport activity (Fig. 7b).

Wild type OAT1 is known to function as an exchanger (1) with one organic anion transported into the cells to be exchanged for another organic anion effluxed out of the cells. To determine whether deglycosylation of mOAT1 affects such functional characteristic, cells expressing the quadruple mutant (N56Q/N86Q/N91Q/N107Q) were preloaded with [14C]PAH followed by exposure to the medium with or without α-ketoglutarate, another substrate for OAT1. As shown in Fig. 8, significant efflux of intracellular [14C]PAH was observed with both wild type mOAT1 and its quadruple mutant when cells were exposed to medium containing an exchangeable substrate α-ketoglutarate, as compared with medium without α-ketoglutarate (Fig. 8a). The quadruple mutant was also equally sensitive to the inhibition by probenecid, an inhibitor for OAT, to that of wild type mOAT1 (Fig. 8b). These results suggest that the unglycosylated mOAT1 preserves the basic functional characteristics of the wild type mOAT1.

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**Fig. 5.** Western blot analysis of the effect of multiple disruptions of putative glycosylation sites. Mutant transporters were generated by simultaneously replacing all the asparagines (N) with glutamine (Q) in mOAT1 (a) and hOAT1 (b). The wild type (WT) and mutants were expressed in HeLa cells. Plasma membrane proteins were isolated through bionylation from these cells. The proteins were then treated with (+) or without (−) PNGase F followed by separation on 7.5% SDS-PAGE, transfer onto polyvinylidene difluoride membrane, and probing with anti-myc antibody.

**Fig. 6.** Immunofluorescence study of the effect of deglycosylation of mOAT1 (a) and hOAT1 (b) on their cellular expression. Wild type mOAT1, wild type hOAT1, and their deglycosylated mutants were expressed in HeLa cells. The cells were then stained with anti-myc antibody and fluorescein isothiocyanate-coupled goat anti-mouse IgG. Specific immunostaining appears as green fluorescence. Phase contrast images showed that cells were fully attached to the culture dishes under all conditions.
DISCUSSION

OATs play essential roles in the body disposition of clinically important anionic drugs, including antiviral drugs, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories. One common structure feature shared among all of the cloned OATs is the presence of consensus sites for N-linked glycosylation in the first extracellular loop within the current secondary structure model (Fig. 2). The potential use of one or several of these consensus sites is suggested by our previous observations that 1) the apparent molecular mass of mOAT1 in mouse kidney membrane fraction was ~90 kDa (after treatment with endoglycosidase H, the molecular mass was reduced to ~55 kDa, the size of the unmodified protein (6)); 2) the microsomal dependent increase in the apparent molecular mass of mOAT1 products obtained by in vitro translation is completely reversed by treatment with endoglycosidase H (3); and 3) the treatment of mOAT1-expressing COS-7 cells with tunicamycin resulted in an almost complete loss of mOAT1-mediated organic anion transport. Immunofluorescence provided the evidence that most of the protein remained in the intracellular compartment.

**Fig. 7. Functional analysis of the effect of multiple disruptions of putative glycosylation sites.** Mutant transporters were generated by simultaneously replacing all the asparagines (N) with glutamine (Q) in mOAT1 (a) and hOAT1 (b). The mutants were expressed in HeLa cells followed by measuring [14C]PAH uptake (20 μM). The data are presented as the percentage of the control. Values are the mean ± S.E. (n = 3).

**Fig. 8. Functional analysis of unglycosylated mOAT1.** a, efflux of [14C]PAH. Cells expressing wild type mOAT1 and its quadruple mutant were preloaded with [14C]PAH (100 μM) for 1 h followed by an exposure to PBS (solid bar) or PBS containing α-ketoglutarate (shaded bar). b, inhibition by probenecid. mOAT1 and its quadruple mutant were expressed in HeLa cells, followed by measuring [14C]PAH uptake in the absence (solid bar) or presence (shaded bar) of 1 mM probenecid.
in tunicamycin-treated cells (3). In total, these results indicate that mOAT1 is a glycoprotein, and glycosylation plays an important role for mOAT1 function.

In the present study we have used site-directed mutagenesis to provide molecular insight into the glycosylation of mOAT1 and the functional role of glycosylation. Because hOAT1, the human counterpart of mOAT1, is the ultimate target for improving human therapy, the glycosylation of hOAT1 was also investigated.

Based on the secondary structure model, both mOAT1 and hOAT1 contain five asparagines in the first extracellular loop, which are predicted to be the potential sites for glycosylation. To determine which of these sites is utilized for glycosylation, we generated mutants by replacing asparagine with glutamine singly or in combination. Western blot analysis of these mutants revealed that only four of the five potential sites were normally used for glycosylation for both mOAT1 and hOAT1. Glycosylation at these sites confirms the predicted membrane topology of OAT; the large hydrophilic loop between transmembrane domains 1 and 2 faces extracellular fluid.

Asp-113 in hOAT1 is not normally glycosylated (Fig. 3b). However, when other glycosylation sites are removed, Asp-113 becomes accessible for glycosylation (Fig. 5b). By steric hindrance, the bulky carbohydrate moieties at other sites may contribute to the absence of glycosylation at Asp-113 in wild type hOAT1. Alternatively, folding of the extracellular loop near Asp-113 may make this asparagine an intrinsically poor substrate for the oligosaccharyl transferase in the endoplasmic reticulum.

Functional analysis of the effect of a single disruption of the glycosylation sites revealed that disrupting Asp-39 in both mOAT1 and hOAT1 resulted in an almost complete loss of transport activity without affecting the cell surface expression of these transporters. Interestingly, Asp-39 in mOAT1 is not used for glycosylation, whereas Asp-39 in hOAT1 is glycosylated. This finding suggests that the loss of function is not because of deglycosylation of Asp-39 per se but is rather because of the change of an amino acid in an important position in the protein. Asp-39 in mOAT1 is located in the same position as Asp-39 in hOAT1, two amino acid residues from the mouth of the first transmembrane domain. It is therefore likely that the first transmembrane domain may form part of the translocation pathway for the substrates. Single replacement of asparagines at other sites had no effect on transport activity for either mOAT1 or hOAT1, suggesting that glycosylation at individual sites is not essential for OAT function. In contrast, when all the glycosylation sites were simultaneously removed, the membrane abundance of unglycosylated mOAT1 and hOAT1 was dramatically reduced. Immunofluorescence demonstrated that most of the unglycosylated mOAT1 and hOAT1 remained in the intracellular compartment. These results suggest that glycosylation plays an essential role for the proper targeting of these transporters onto the plasma membrane. Of course, it is important to acknowledge that the loss of the surface expression observed in these mutants may also have been because of the changes in amino acid sequences and not necessarily because of the deglycosylation per se. However, our previous results (3) in COS-7 cells treated with tunicamycin, a condition in which the asparagines in the transporter were not changed, would strongly argue that deglycosylation is sufficient to impair the proper trafficking of the transporters onto the plasma membrane.

In conclusion, the present study provided molecular evidence that 1) Asp-39 in both mOAT1 and hOAT1 plays an important role in the substrate binding/recognition of these transporters, 2) glycosylation at individual sites is not required for the transport function, and 3) glycosylation is essential for the targeting of the transporter to the plasma membrane. This study is the first identification and characterization of glycosylation in OAT1 and may provide important insights into the structure-function relationships of the organic ion transporter family.

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