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Conserved Tryptophan Residues within Putative Transmembrane Domain 6 Affect Transport Function of Organic Anion Transporting Polypeptide 1B1

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Running title: *Roles of conserved tryptophan residues in TM6 of OATP1B1*

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Abbreviations: E-3-S, estrone-3-sulfate; NHS-SS-biotin, Sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate; OATP, organic anion transporting polypeptide; PBS, phosphate-buffered saline; TM, transmembrane domain.

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Abstract

The organic anion-transporting polypeptides (OATPs, gene symbol *SLCO*) are a family of transporters that play important roles in the absorption, distribution, metabolism and excretion of various drugs. Although substrate specificity of transporter proteins is under extensive study, the underlying mechanisms for substrate binding and/or recognition remain largely unknown. Transmembrane domain 6 (TM6) is a relatively conserved region within OATP family members and several amino acid residues on its extracellular half are part of the OATP family signature sequence D-X-RW-(I,V)-GAWWX-G-(F,L)-L. In the present study, two adjacent tryptophan residues (Trp258 and Trp259) within TM6 were identified as critical amino acids for the transport function of OATP1B1. Kinetic studies showed that substitution of Trp258 with alanine resulted in monophasic kinetics for estrone-3-sulfate uptake, with a significantly higher Km value (K_m=12.0±2.8μM) compared to the high affinity component of wild-type OATP1B1 (Km=0.38±0.06μM). On the other hand, W259A retained the biphasic characteristic of the transporter. Km values of the high and low affinity components for estrone-3-sulfate of W259A are 1.93±0.76μM and 30.8±4.4μM, respectively. Further studies revealed that W258A retained transport function of another prototypic substrate taurocholate; while W259A displayed a dramatically reduced uptake of the substrate and exhibited an eight-fold increase in the Km value compared with that of the wild-type and W258A. Our results suggested that Trp258 and Trp259 may play different roles for the uptake of different substrates by OATP1B1.

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Introduction

The organic anion-transporting polypeptides (OATPs, gene symbol *SLCO*) are a family of transporters that mediate sodium-independent transport of wide spectrum of structurally independent compounds (Hagenbuch and Gui, 2008). Because of their broad substrate specificity and distribution in several important tissues involved in the absorption, distribution, metabolism and excretion (ADME) of drugs, altered transport kinetics of OATPs may contribute to the inter-individual variability in drug responses (König, 2011). So far there are 12 members of the human OATP family: OATP1A2, 1B1, 1B3, 1B7, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1 and 6A1 (Hagenbuch and Meier, 2003; Mikkaichi et al., 2004; König et al., 2006; Nakanishi and Tamai, 2012). Some OATPs are expressed ubiquitously; while others, such as OATP1B1 and OATP1B3, are predominantly found in certain organs or tissues. OATP1B1 is mainly located at the basolateral membrane of human hepatocytes and plays an essential role in drug clearance from the body (Kalliokoski and Niemi, 2009). This transporter protein has been found to mediate the uptake of a variety of different drugs from blood into hepatocytes (König, 2011).

Although substrate specificity of transporter proteins is under extensive study, the underlying mechanisms for substrate binding and/or recognition remain largely unknown because crystal structures of mammalian drug transporters have not yet been solved (Miyagawa et al., 2009). Transmembrane domains (TMs) are essential structural features of membrane proteins and have been proposed to be critically involved in proper functions of other transporters such as organic anion transporters (Hong et al, 2004; Hong et al., 2010). It has been shown that TM 8 and 9 in

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OATP1B1 are critical for its substrate recognition (Miyagawa et al., 2009). Several important amino acids were also identified in TM10 of OATP1B1 (Gui and Hagenbuch, 2009). Previous study in our lab showed that four amino acids within TM2 of OATP1B1 are essential for its uptake of the prototypic substrate estrone-3-sulfate (E-3-S). Asp70 and Phe73 may interact with the substrate; while Glu74 and Gly76 are important in maintaining the proper structure of the transporter protein (Li et al., 2012). According to the computer-based hydropathy analysis, OATP1B1 as well as other OATP members are predicted to share a similar transmembrane domain organization of 12 transmembrane domains and a large extracellular loop 5 with conserved cysteine residues (Hagenbuch and Meier, 2003). There is an OATP family signature with the sequence D-X-RW-(I,V)-GAWWX-G-(F,L)-L located at the border of extracellular loop 3 and TM 6 (Hagenbuch and Meier, 2003), which are well conserved among human, rat and mouse OATPs/Oatps. However, the underlying importance of this highly conserved domain within OATPs remains unclear. In a computer-generated model of OATP1B3, an OATP family member that has high homology compared with OATP1B1, it was proposed that TM1, 2, 4 and 5 of the N-terminal half and helices 7, 8, 10 and 11 of the C-terminal half of the uptake transporter face the pore that interacts with the substrate, while TM 3, 6, 9 and 12 are largely embedded in the bilayer (Meier-Abt et al., 2005). In our previous study, a model of OATP1B1was generated using *Escherichia Coli* glycerol-3-phosphate transporter (PDB 1pw4) as the template (Li et al., 2012). Similar with that of OATP1B3, our model implied that TM6 is mostly embedded within the lipid layer. However, part of the extracellular portion seems to participate in the formation of

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the substrate interaction pore (Fig. 1).

In the present study, we performed alanine-scanning and site-directed mutagenesis of all the amino acid residues within the putative TM6 of OATP1B1. The substitution of two conserved tryptophan residues (Trp258 and Trp259) with alanine greatly reduced E-3-S uptake by the transporter protein. Further studies were conducted to investigate the roles of these two amino acids in proper function of OATP1B1.

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Materials and Methods

Materials - [^{3}H]estrone-3-sulfate (E-3-S) and [^{3}H]taurocholic acid were purchased from PerkinElmer Life Sciences (Waltham, MA). Sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate (NHS-SS-biotin) and streptavidin-agarose beads were from Thermo Scientific (Rockford, IL). All other reagents were purchased from Sigma except where otherwise stated.

Site-directed Mutagenesis - Mutant transporters were generated using QuickChange Lightning Site-Directed Mutagenesis Kit from Agilent (Santa Clara, CA). pReceiver M07 vector containing the *SLCO1B1* cDNA and 3-HA tags at the C-terminus was obtained from Genecopoeia (Rockville, MD) and used as the template for mutagenesis. All mutant sequences were confirmed by the dideoxy chain termination method.

Cell culture and transfection of plasmid constructs into cells - HEK293 cells (ATCC, Manassas, VA) were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). Confluent cells in 48-well or 6-well plates were transfected with DNA plasmid using LipofectAMINE 2000 reagent (Invitrogen) following manufacturer's instructions. Transfected cells were incubated for 48 hrs at 37 °C and then used for transport assay and cell surface biotinylation.

Uptake assay —Cells in 48-well plates were used for transport measurement as previously described (Li et al., 2012) with minor modification. Briefly, cells were incubated with uptake solution containing [^{3}H]E-3-S or [^{3}H] taurocholic acid at 37°C for 2 min (1min for kinetic

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analysis) and uptake was stopped by addition of ice-cold phosphate-buffered saline (PBS) solution. Cells were then washed with cold PBS and solubilized in 0.2 M NaOH. The radioactivity of the cell lysate was measured with a liquid scintillation counter Triathler-Hidex (Hidex, Finland). The uptake count was standardized by the amount of protein in each well.

Cell Surface Biotinylation and Western blot - Cell surface expression of OATP1B1 and its mutants was examined with the membrane-impermeable biotinylation reagent NHS-SS-biotin using a method described previously (Li et al., 2012).

Statistical analysis - Data statistical analysis was carried out using one-way analysis of variance. Differences between means are regarded as significant if $p<0.05$.

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Results

Alanine-scanning of amino acids within TM6 - The contribution of each amino acid in the putative TM6 to the transport function of OATP1B1 was probed by systematically mutating each residue into alanine. A combination of four different transmembrane helices prediction servers was used to locate the position of TM6 (Table1). According to the predictions, 24 amino acids from position 257 to 280 of OATP1B1 were selected for alanine-scanning analysis (Fig.2). These amino acids were chosen because they are predicted to be part of TM6 according to at least two topology prediction methods. Uptake function of the mutants was first analyzed using 100nM of E-3-S as the substrate. As shown in Figure 3, sixteen mutants showed higher than 60% of the uptake by wild-type OATP1B1, suggesting that they retained most of the transport function. Six mutants, i.e. F262A, V264A, L267A, S269A, F276A and F278A, demonstrated around 60-70% reduction of the transport activity. Mutants W258A and W259A showed an even greater reduced uptake function (more than 90% reduction) and presented a similar transport capability of E-3-S as that of the mock control (data not shown), suggesting these two amino acids may play a more critical role for transport function than other amino acids within TM6 of OATP1B1.

Protein expression of mutants with reduced transport activity – Since OATP1B1 is a membrane protein, whether it is correctly targeted to the plasma membrane could greatly affect its proper function. Therefore, we first investigated whether the loss of transport function is due to reduced surface expression of the mutant protein. Our results showed that both W258A and W259A were abundantly expressed on the cell surface (Fig. 4A), indicating

that the alteration of transport activity in W258A and W259A was not caused by the change of protein expression but rather the change of their interaction with the substrate. On the other hand, F262A, V264A, L267A, S269A, F276A and F278A all had a dramatically reduced level of the transporter protein (Fig. 4B). Therefore, the reduced uptake function of these mutants was mainly due to decreased protein expression.

The role of Trp258 and Trp259 on OATP1B1 transport activity – To investigate whether the structural characteristics of Trp258 and Trp259 are essential for transport function of OATP1B1, we substituted these two tryptophan residues with phenylalanine, another amino acid that contains the aromatic ring. As shown in Figure 5, such a replacement fully recovered the activity of the transporter, suggesting that the benzene ring structure is crucial for these two positions. Since OATP1B1 has been proposed to contain two binding components for E-3-S (Noé et al., 2007; Gui and Hagenbuch, 2009; Tamai et al., 2001), we next investigated whether alanine substitution at Trp258 and Trp259 had effects on the transport of high concentrations of E-3-S (50μM). As demonstrated in Figure 6, W258A still showed a significantly reduced transport function of E-3-S, while W259A had an uptake function comparable to that of wild-type OATP1B1. We then further compared the kinetic parameters of W258A and W259A with wild-type OATP1B1. The Eadie–Hofstee plot showed biphasic transport kinetics of E-3-S in wild-type OATP1B1 and W259A. However, W258A only showed a single binding component for this substrate (Fig.7). Km value of W258A was significantly higher than the high affinity component but markedly lower than the low affinity component of those of the wild-type

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OATP1B1. On the other hand, W259A, which retained the biphasic kinetics, had a high affinity Km value much greater than that of the wild-type. The Km value for the low affinity component and Vmax of W259A, however, were unchanged (Table2).

Effects of mutations on uptake of another OATP prototypic substrate taurocholate- Since W258A and W259A seemed to affect E-3-S uptake in different manners, we wanted to see if such mutations also affect the transport of taurocholate, a bile acid often used as model substrate for the study of OATP-mediated transport (Noé et al., 2007; Huber et al., 2007). As shown in Figure 8A, uptake of taurocholate in W258A was comparable to that of wild-type OATP1B1; while W259A showed a significantly decreased transport function of the substrate. We also found that taurocholate exerted a significant suppression effect on uptake of both low (50nM) and high (50μM) concentrations of E-3-S in W258A. On the other hand, a greater inhibitory effect on uptake of high concentration of E-3-S was shown in both W259A and wild-type OATP1B1 (Table 3). Moreover, taurocholate inhibition of E-3-S uptake by W259A was to a lesser extent as compared with the wild-type. Further analysis demonstrated that taurocholate Km and Vmax values of W258A were comparable to those of wild-type; while W259A exhibited an eight-fold increase in the Km value (Fig. 8 & Table 2).

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Discussion

Amino acids within TMs have been shown to play important roles in substrate binding, maintenance of protein stability and correct folding of proteins (Hong et al., 2007; Hong et al., 2010; Gui and Hagenbuch, 2009; Miyagawa et al., 2009; Li et al., 2012). Studies on single nucleotide polymorphism (SNP) also pointed out that mutants located within TMs often result in functional changes (Kalliokoski and Niemi, 2009). In the present study, we used site-directed mutagenesis to study the involvement of amino acid residues within the putative TM6 of OATP1B1 in substrate transport. Among the 24 amino acids analyzed, F262A, V264A, L267A, S269A, F276A and F278A showed a more than 60% reduction of transport activity. Further studies found out that these mutants had much reduced transporter protein expression compared with the wild-type. If protein expression is considered in the comparison of the transport activity, E-3-S uptake by these mutants was more than 60% of that by wild-type OATP1B1 (data not shown). Therefore, the reduced uptake function of these mutants may be mainly accounted for by decreased transporter protein level. W258A and W259A, on the other hand, showed a greater reduction (>90%) in transport function. In fact, no significant difference was observed when uptake of E-3-S by these two mutants was compared to that of the mock control. Therefore, Trp258 and Trp259 may play more critical roles for OATP1B1 function. Cell surface biotinylation and western blot analysis demonstrated that expression of these two mutants on the plasma membrane was comparable to that of wild-type OATP1B1, suggesting the alteration of transport function was not due to changes of protein expression. Trp258 and Trp259 are part of

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the OATP superfamily signature sequence and located in the extracellular half of TM6. Interestingly, mutation of other conserved amino acids of this region that are possibly involved in the formation of TM6, i.e. Ala257 and Leu263 still retained a significant uptake function; while F262A showed a much reduced expression level, indicating that phenylalanine at this position may be important for the correct processing of the transporter protein. Substitution of Trp258 and Trp259 with aromatic amino acid phenylalanine fully recovered the transport function, which suggested that the aromatic ring structure is essential in these two positions. We also investigated the effect of alanine substitution on these two positions for the low affinity component of E-3-S transport. The uptake of 50μM E-3-S by W259A was comparable to that of the wild-type; while W258A showed significant reduction of transport activity. These results implied that tryptophan at these two locations, though adjacent to each other, play different roles in maintaining the proper function of OATP1B1. Although the uptake of E-3-S by wild-type OATP1B1 displayed biphasic saturation kinetics with two distinct affinity components (K_m=0.38±0.06 and 36.1±12.1μM in our current study), W258A only showed one component with the K_m value of 12.0±2.8μM, suggesting that there may be a structural alteration in the mutant. Such a change was also observed in a previous study on TM10 of OATP1B1. Simultaneous mutation of four residues (Leu545, Phe546, Leu550, and Ser554) in TM10 resulted in significantly reduced OATP1B1-mediated transport and a 10-fold decreased affinity for E-3-S with respect to the high-affinity component of wild-type OATP1B1 (Gui and Hagenbuch, 2009). On the other hand, W259A retained both binding components with a much higher Km for the high affinity

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component (K_m=1.93±0.76μM) and a similar low-affinity Km (K_m=30.8±4.4μM) compared with those of the wild-type OATP1B1. When we studied the responses of these two mutants to taurocholate, another prototypic substrate for OATPs, it was found that taurocholate had similar inhibitory effects on uptake of both high and low concentrations of E-3-S in W258A, while a more significant inhibition on transport of 50μM E-3-S was observed in wild-type OATP1B1 and W259A (Table 3). These results also suggested that alanine substitution of Trp258 may convert two binding sites within OATP1B1 into one site. Kinetic analysis demonstrated that both Km and Vmax values of W258A for taurocholate were similar to those of wild-type OATP1B1, which indicated that the mutation of Trp258 affected transport of E-3-S but not taurocholate. Replacement of Trp259 with alanine, however, resulted in a dramatically decreased transport function of taurocholate. The Km of this mutant for taurocholate was increased around eight-fold as compared with that of the wild-type OATP1B1, implying a much reduced affinity for the substrate.

Structural models for OATP family members OATP1B3 and OATP2B1 were generated based on the known structures of major facilitator superfamily transport proteins glycerol-3-phosphate transporter (PDB1pw4) and lactose permease (PDB 1pv6) (Meier-Abt et al., 2005). In our previous study of transmembrane domain 2, we also used PDB1pw4 as a template for the homology modeling of OATP1B1 (Li et al., 2012). According to these models, TM6 is mostly embedded in the membrane bilayer and thus may not directly interact with the substrates (Meier-Abt et al., 2005; Li et al., 2012). However, in our OATP1B1 model, a portion of the TM6

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extracellular side is facing the pore in which the interaction with substrates occurs (Fig. 1). Consistent with such a model, the two conserved tryptophan residues identified in our current study, which are positioned at the border of extracellular loop 3 and TM6 and thought to be a part of the 'mouth' region of the substrate interaction pore, were found to be important for substrate transport. The mutant W258A may go through a conformation change and result in altered protein-substrate interaction. On the other hand, Vmax values for both high and low affinity components of W259A were similar with those of wild-type OATP1B1, and the lower Km was elevated in this mutant, suggesting that Trp259 may directly interact with the substrates.

In conclusion, our present study identified two critical tryptophan residues in the putative transmembrane domain 6 of OATP1B1. These aromatic amino acids may play an important role in maintaining the proper structure of the transporter protein through a π-π stacking (Ruddat et al., 2004) or directly interact with the substrates. Substitution of Trp258 with alanine resulted in one substrate binding site that altered its capability to interact with E-3-S but retained the ability to transport taurocholate. On the other hand, mutation of Trp259 retained two interaction components with E-3-S but showed a greatly reduced transport function of taurocholate. The results indicated that though these two tryptophan residues are located adjacent to each other in the sequence of OATP1B1, they may play different roles in the transport function of various substrates.

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Participated in research design: M. Hong.

Conducted experiments: J. Huang, Li, W. Hong, Zhan, Yu.

Performed data analysis: H. Huang and M. Hong.

Wrote or contributed to the writing of the manuscript: M. Hong

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Footnotes

J.H. and N.L. contributed equally to this work.

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Figure legends

Figure 1. Location of putative TM6 within structure of OATP1B1. Viewed from **A**. the extracellular side. **B.** the intracellular side. The *E.coli* glycerol-3-phosphate transporter (PDB 1pw4) was used as the template for homology modeling of OATP1B1. The structure of OATP1B1 was modeled with the web-based protein structure prediction service Phyre2 (http://www.imperial.ac.uk/phyre/). Location of TM6 is indicated with arrows.

Figure 2. TM6 sequence alignment of OATP family members. Full length sequences of 11 OATP family members were aligned with ClustalW. Only partial sequences were shown here. Corresponding sequences of TM6 were in bold. Sequence of OATP superfamily signature was underlined in OATP1B1.

Figure 3. Estron-3-sulfate uptake of OATP1B1 transmembrane domain 6 mutants. Transport of 100nM estrone-3-sulfate was measured at 37°C for 2 min in HEK293 cells expressing OATP1B1 and its alanine-substituted mutants. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from cells expressing wild-type OATP1B1 and mutants. Transport activity was expressed as a percentage of the uptake measured in wild-type OATP1B1 (hOATP1B1WT). The results represent data from three experiments, with triplicate measurements for each mutant. The results shown are means ± S.E. (n = 3).

Figure 4. Plasma membrane protein expression of mutants with reduced transport activity**. A.** Cell surface expression of W258A and W259A. Upper panel, representative blot of OATP1B1 and mutants W258A and W259A. Cells were biotinylated with NHS-SS-biotin, and the

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biotin-labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by western blotting with anti-HA antibody. Same blot was probed with integrin antibody as surface protein loading control. Lower panel, relative protein expression level of W258A and W259A. **B.** Protein expression F262A, V264A, L267A, S269A, F276A and F278A. The intensity of each protein band was quantified with Image J software and expressed as a percentage to the wild-type. Each column shows the mean of three experiments and the error bars show the range of observations. The results shown are means ± S.E. (n = 3).

Figure 5. Estron-3-sulfate uptake of Trp258 and Trp259 mutants. Uptake of 100nM E-3-S was measured at 37°C for 2 min in HEK293 cells expressing OATP1B1 wild-type, W258A, W258F as well as W259A and W259F. Net uptake was obtained by subtracting the uptake of cells transfected with empty vector from cells expressing wild-type OATP1B1 and mutants. The results represent data from three experiments, with triplicate measurements for each mutant. The results shown are means ± S.E. (n = 3). Different letters indicate values that are significantly different (p< 0.05).

Figure 6. Uptake of high concentration of estrone-3-sulfate by Trp-258 and Trp-259 mutants. Uptake for 50μM E-3-S was measured at 37°C for 2 min in HEK293 cells expressing OATP1B1 wild-type, W258A and W259A. The results represent data from three experiments, with triplicate measurements for each mutant. The results shown are means ± S.E. (n = 3). Asterisks indicate values significantly different (p< 0.05) from that of wild-type.

Figure 7. Eadie-Hofstee plots of estrone-3-sulfate uptake by OATP1B1 and its mutants. **A**.

OATP1B1WT, **B**.W258A, **C**.W259A. Uptake of E-3-S was measured at concentrations range from 0.05 to 50μM at 37°C for 1 min. The results represent data from three experiments, with triplicate measurements for each mutant. The results shown are means ± S.E. (n = 3).

Figure 8. Uptake of taurocholate by Trp-258 and Trp-259 mutants. **A.** Uptake of taurocholate. Uptake of 20μM taurocholate was measured at 37°C for 2 min in HEK293 cells expressing OATP1B1 wild-type, W258A and W259A. **B**. Eadie-Hofstee plots of taurocholate uptake by OATP1B1 and **C.** W258A, **D.** W259A. Uptake of taurocholate was measured at concentrations range from 1 to 50μM for wild-type OATP1B1 and W258A, and from 10 to 200μM for W259A at 37°C for 1 min. The results represent data from three experiments, with triplicate measurements for each mutant. The results shown are means ± S.E. (n = 3).

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Table 1 Position of TM6 according to different transmembrane domain prediction servers

Transmembrane helices prediction servers	Predicted position of TM6
TopPred 0.01	
Goldman Engelman Steitz scale	262-282
Kyte Doolittle scale	259-279
TMHMM server V2.0	257-279
TMpred	258-280
HMMTOP	257-279

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Table 2 Kinetic parameters of OATP1B1 wild-type and its mutants

	Km (uM)	Vmax (pmol/mg protein/min)
E-3-S		
OATP1B1	0.38±0.06	40.2±12.4
	36.1±12.1	484±157
W258A	12.0*±2.8	167*±46
W259A	1.93*±0.76	42.1±18.4
	30.8±4.4	426±132
Taurocholate		
OATP1B1	21.3±3.5	139±19
W258A	17.2±1.2	144±22
W259A	161*±1	688*±63

Uptake of E-3-S was measured at concentrations from 0.05 to 50μM, while uptake for taurocholate was measured at concentrations from 1 to 50μM for wild-type OATP1B1 and W258A, and from 10 to 200μM for W259A at a 1-min interval. Transport kinetic values were calculated using the Eadie-Hofstee transformation. The results shown are means ± S.E. (n = 3). Asterisks indicate values significantly different (p< 0.05) from that of wild-type control.

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Table 3 Inhibitory effect of taurocholate on transport of estrone-3-sulfate by OATP1B1 wild-type and its mutants

	50nM E-3-S	50μM E-3-S
	(% of uptake function for E-3-S in the absence of taurocholate)	
OATP1B1	81.4±2.1	23.1*±9.5
W258A	34.4±2.5	46.6±6.6
W259A	84.8±3.9	59.6*±3.1

Uptake of 50nM and 50μM of E-3-S was measured in OATP1B1 wild-type and mutants in the presence of 10μM taurocholate and compared with the uptake of those without the addition of taurocholate. The results shown are means ± S.E. (n = 3). Asterisks indicate inhibitory effect is significantly different (p< 0.05) between two E-3-S concentrations.

Figure 1

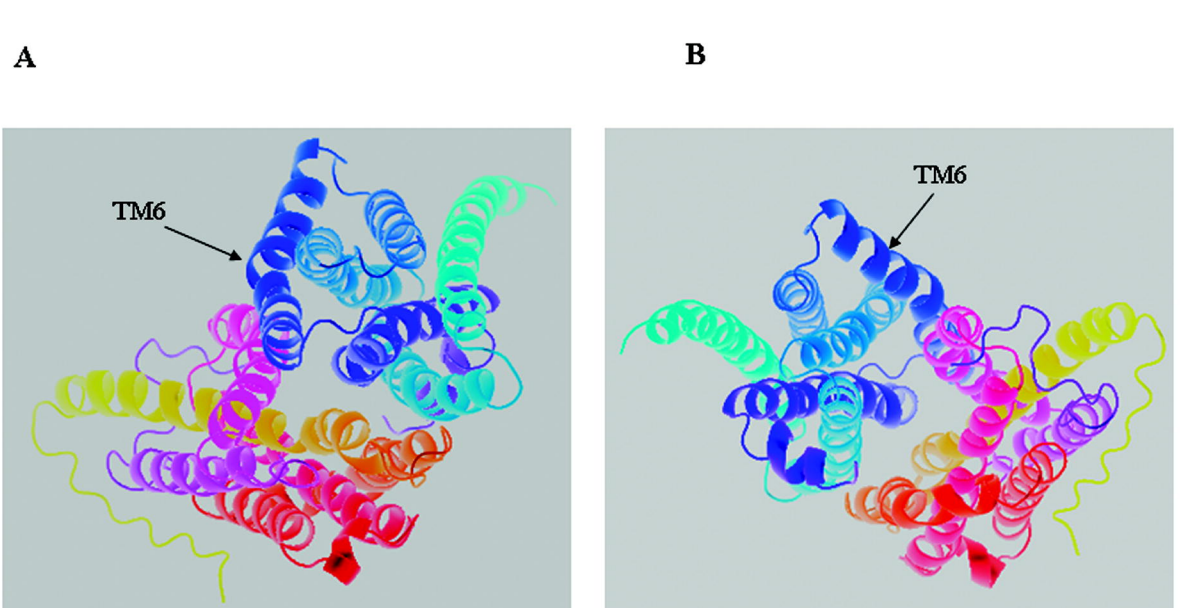


Figure 2

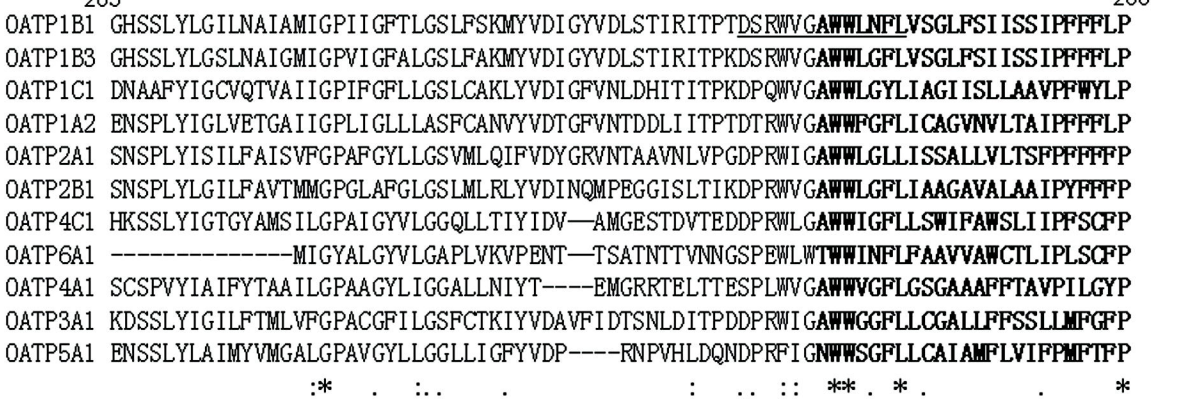

203
280
OATP1B1 GHSSLYLGILNAIAMIGPIIGFTLGSLFSKMYVDIGYVDLSTIRITPTDSRWVGAWWLNFLVSGLFSIISSIPFFFLP
OATP1B3 GHSSLYLGSLNAIGMIGPVIGFALGSLFAKMYVDIGYVDLSTIRITPKDSRWVGAWWLGFLVSGLFSIISSIPFFFLP
OATP1C1 DNAAFYIGCVQTVAIIGPIFGFLLGSLCAKLYVDIGFVNLDHITITPKDPQWVGAWWLGYLIAGIISLLAAVPFWYLP
OATP1A2 ENSPLYIGLVETGAIIGPLIGLLLASFCANVYVDTGFVNTDDLIITPTDTRWVGAWWFGFLICAGVNVLTAIPFFFLP
OATP2A1 SNSPLYISILFAISVFGPAFGYLLGSVMLQIFVDYGRVNTAAVNLVPGDPRWIGAWWLGLLISSALLVLTSFPFFFFP
OATP2B1 SNSPLYLGILFAVTMMGPGLAFGLGSLMLRLYVDINQMPEGGISLTIKDPRWVGAWWLGFLIAAGAVALAAIPYFFFP
OATP4C1 HKSSLYIGTGYAMSILGPAIGYVLGGQLLTIYIDV—AMGESTDVTEDDPRWLGAWWIGFLLSWIFAWSLIIPFSCFP
OATP6A1 ----------MIGYALGYVLGAPLVKVPENT—TSATNTTVNNGSPEWLWTWWINFLFAAVVAWCTLIPLSCFP
OATP4A1 SCSPVYIAIFYTAAILGPAAGYLIGGALLNIYT----EMGRRTELTTESPLWVGAWWVGFLGSGAAAFFTAVPILGYP
OATP3A1 KDSSLYIGILFTMLVFGPACGFILGSFCTKIYVDAVFIDTSNLDITPDDPRWIGAWWGGFLLCGALLFFSSLLMFGFP
OATP5A1 ENSSLYLAIMYVMGALGPAVGYLLGGLLIGFYVDP----RNPVHLDQNDPRFIGNWWSGFLLCAIAMFLVIFPMFTFP

Figure 3

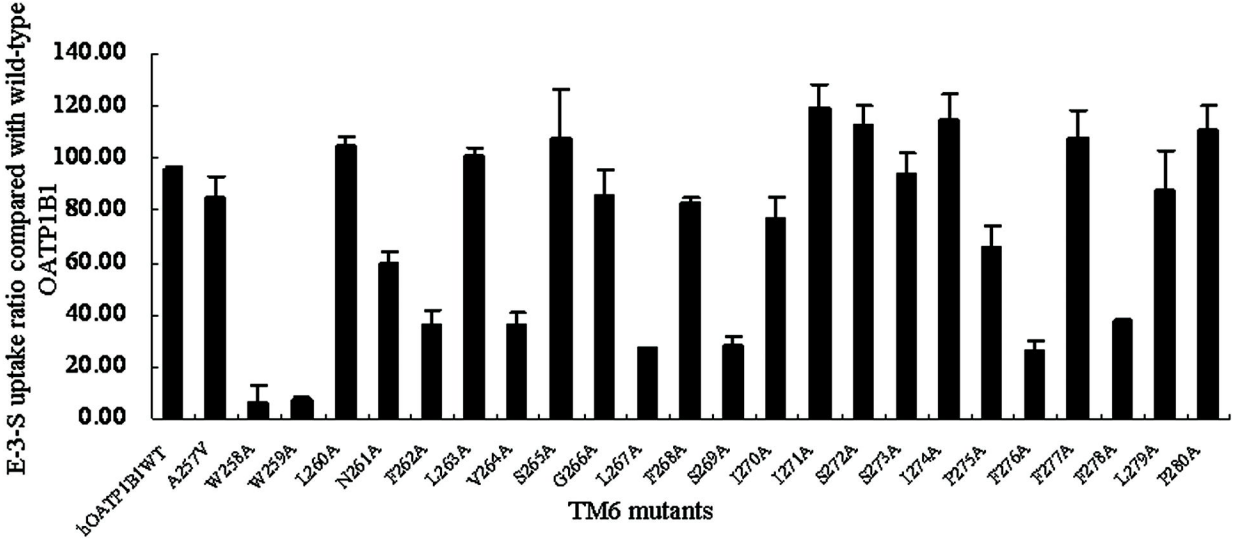

E-3-S uptake ratio compared with wild-type OATP1B1
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
hOATP1B1WT
A257V
W258A
W259A
L260A
N261A
F262A
L263A
V264A
S265A
G266A
L267A
F268A
S269A
I270A
I271A
S272A
S273A
I274A
P275A
F276A
F277A
F278A
L279A
F280A
TM6 mutants

Figure 4

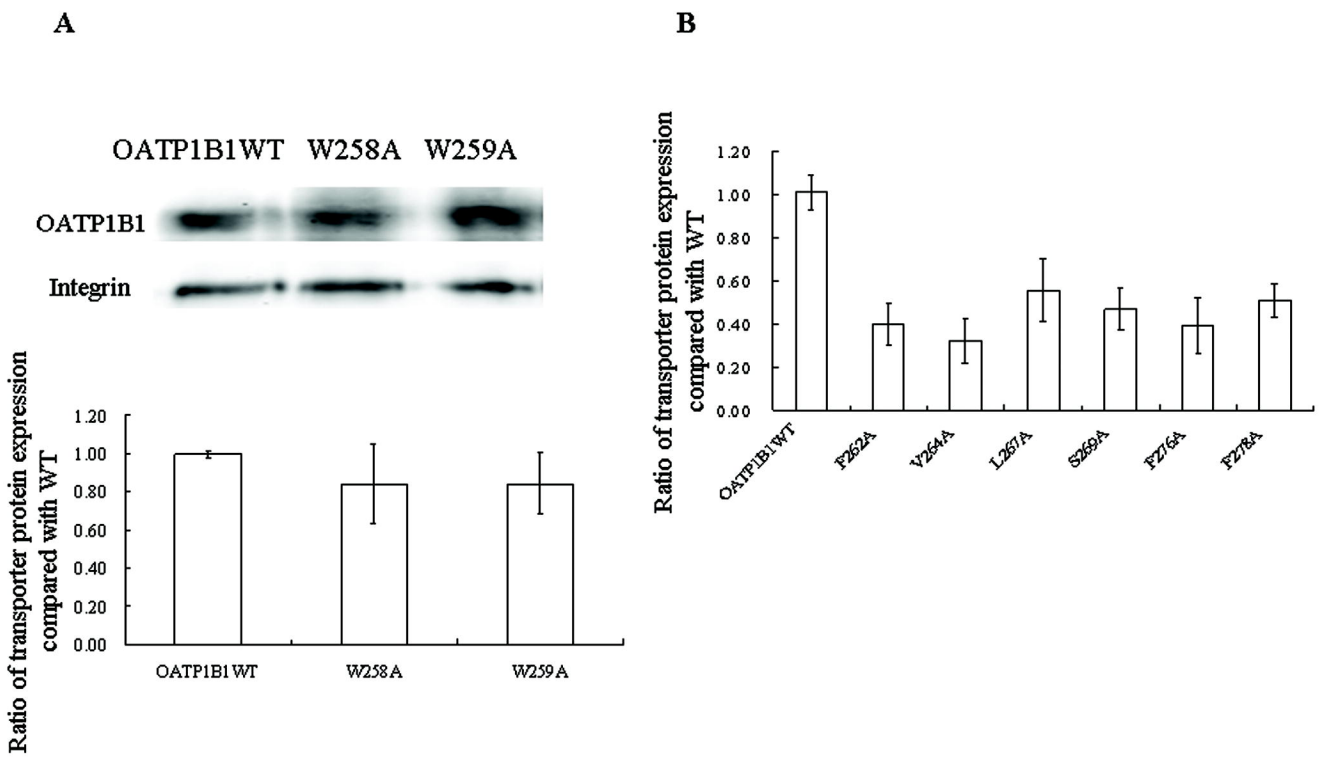

A
OATP1B1WT W258A W259A
OATP1B1
Integrin
Ratio of transporter protein expression compared with WT
1.20
1.00
0.80
0.60
0.40
0.20
0.00
OATP1B1WT
W258A
W259A
B
Ratio of transporter protein expression compared with WT
1.20
1.00
0.80
0.60
0.40
0.20
0.00
OATP1B1WT
F262A
V264A
L267A
S269A
F276A
F278A

Figure 5

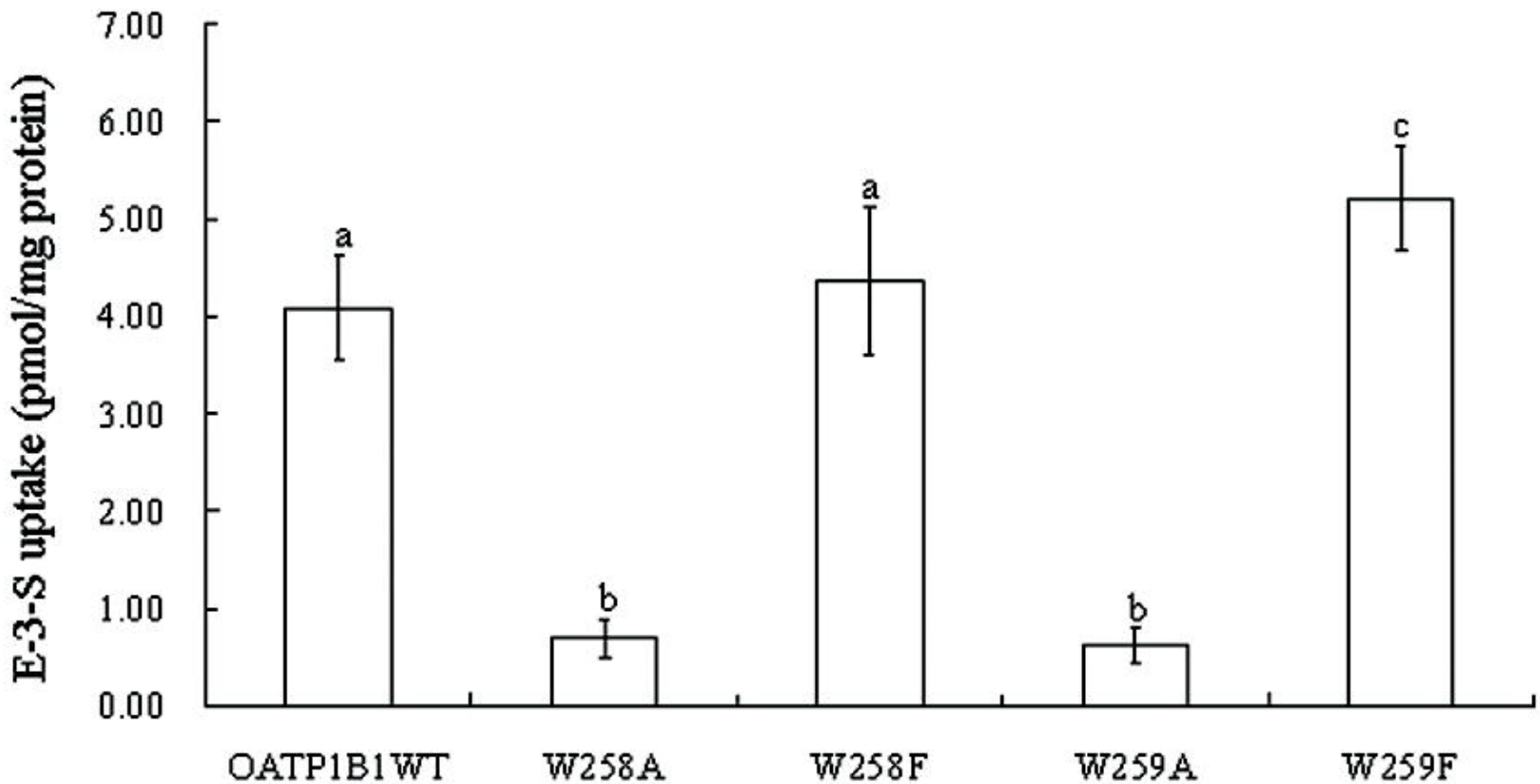

E-3-S uptake (pmol/mg protein)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
a
b
c
OATP1B1WT
W258A
W258F
W259A
W259F

Figure 6

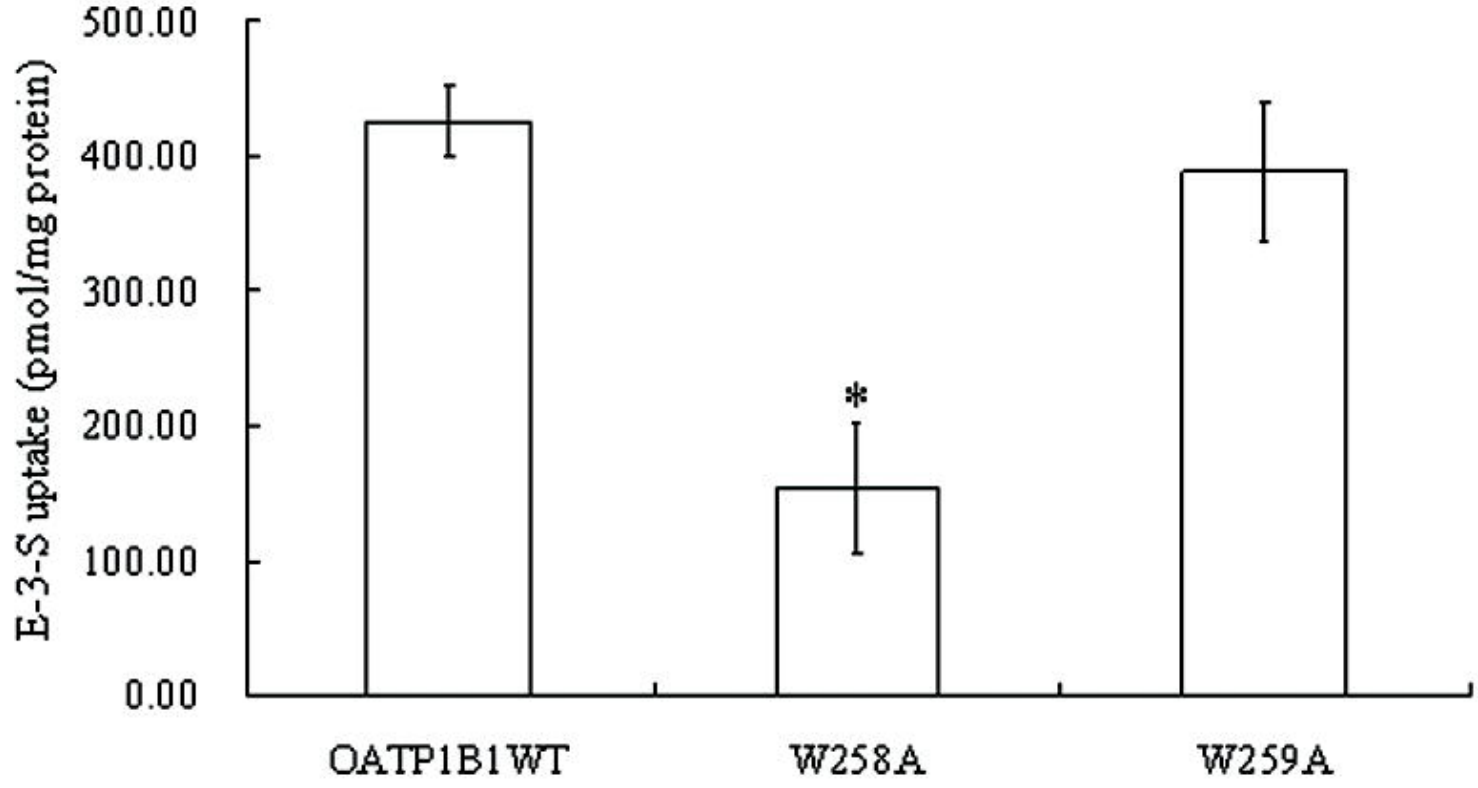

E-3-S uptake (pmol/mg protein)
500.00
400.00
300.00
200.00
100.00
0.00
*
OATP1B1WT
W258A
W259A

Figure 7

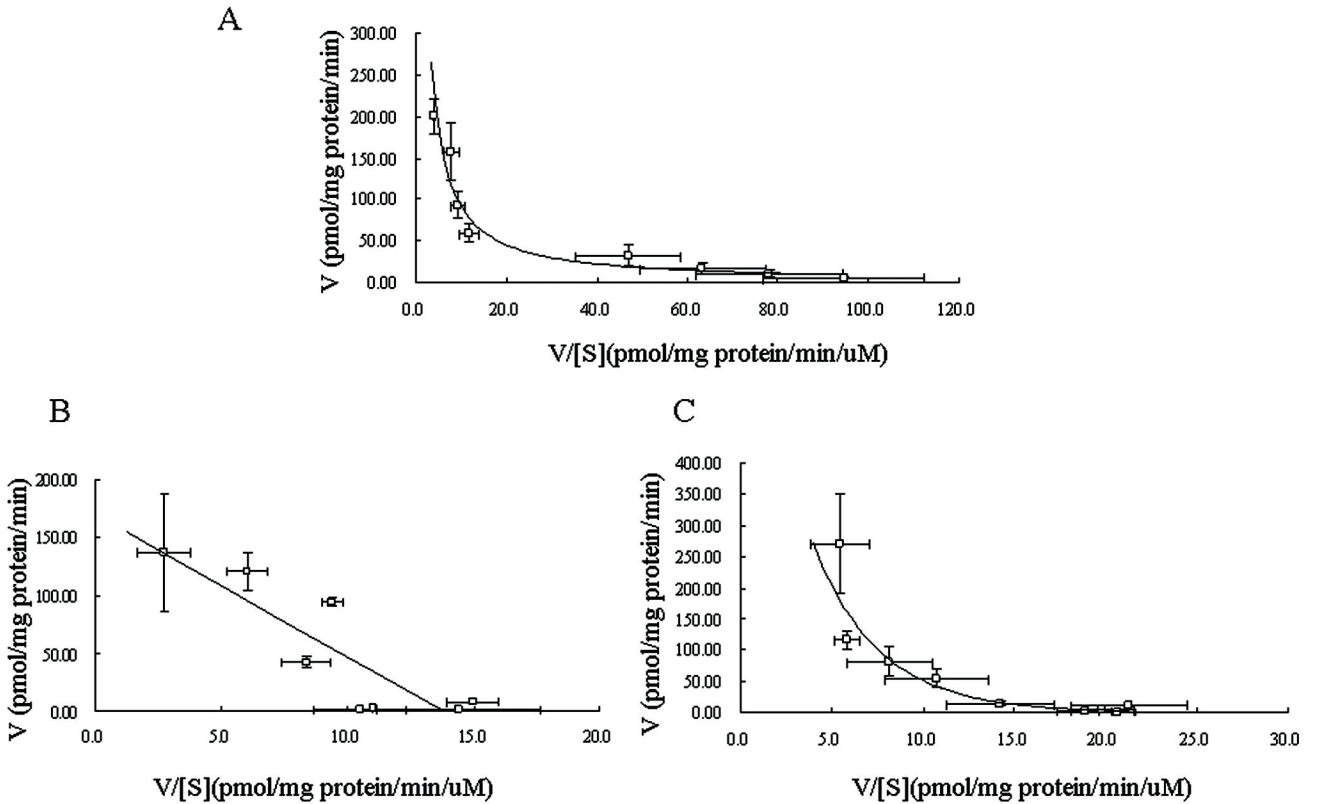
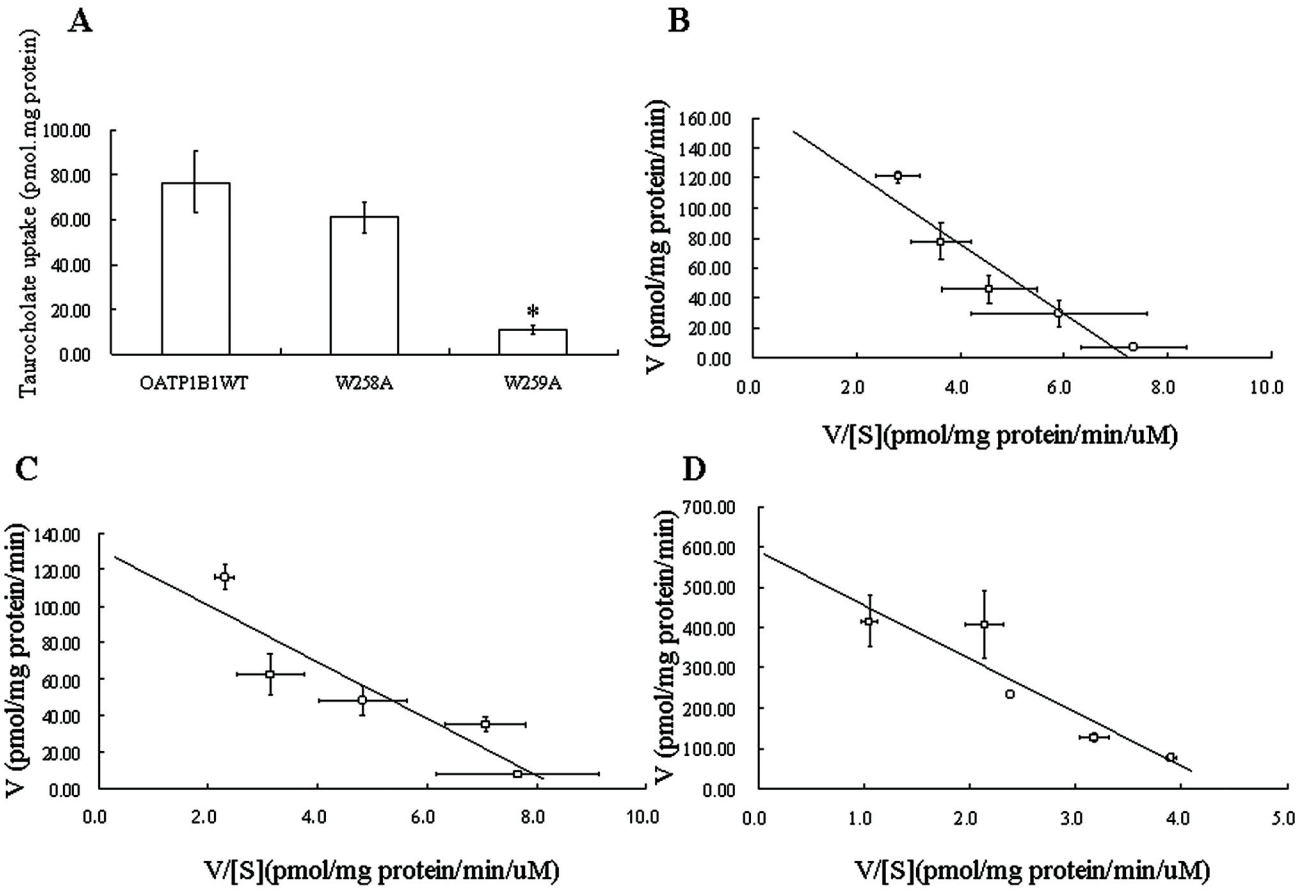

A
V (pmol/mg protein/min)
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.0
20.0
40.0
60.0
80.0
100.0
120.0
V/[S](pmol/mg protein/min/uM)
B
V (pmol/mg protein/min)
200.00
150.00
100.00
50.00
0.00
0.0
5.0
10.0
15.0
20.0
V/[S](pmol/mg protein/min/uM)
C
V (pmol/mg protein/min)
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0
V/[S](pmol/mg protein/min/uM)

Figure 8


A
Taurocholate uptake (pmol.mg protein)
100.00
80.00
60.00
40.00
20.00
0.00
*
OATP1B1WT
W258A
W259A
B
V (pmol/mg protein/min)
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0.0
2.0
4.0
6.0
8.0
10.0
V/[S](pmol/mg protein/min/uM)
C
V (pmol/mg protein/min)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0.0
2.0
4.0
6.0
8.0
10.0
V/[S](pmol/mg protein/min/uM)
D
V (pmol/mg protein/min)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
0.0
1.0
2.0
3.0
4.0
5.0
V/[S](pmol/mg protein/min/uM)