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RESEARCH PAPER

Functional characterization of nonsynonymous single nucleotide polymorphisms in the human organic anion transporter 4 (hOAT4)

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Background and purpose: The human organic anion transporter (hOAT) family of transmembrane carrier proteins mediate the cellular flux of anionic substances, including certain hormones and anti-cancer drugs. hOAT4 is highly expressed at the apical membrane of the renal tubular cell and facilitates drug re-absorption in the kidney. In the present study, the impact of 10 nonsynonymous single nucleotide polymorphisms (SNPs) of hOAT4 on transport function in COS-7 cells was characterized.
Experimental approach: Transport uptake assay was used to assess the function of the variant transporters. Cell surface biotinylation and western blot analysis were used to investigate the expression characteristics of the transporter proteins. Comparative modelling was used to interpret the influence of nonsynonymous changes in terms of hOAT4 structure.
Key results: Four naturally occurring hOAT4 variants (L29P, R48Y, V155G and T392I) exhibited a significant loss of function. Substitution of leucine-29, which is a conserved residue in OATs, with a proline residue, impaired the synthesis or the apparent stability of the transporter and membrane insertion was disrupted in the R48Y variant. In the case of the V155G and T392I variants, impaired function was due to decreased affinity of the transporter for oestrone sulphate and impaired transporter-substrate turnover respectively. The T392I variant was inhibited more extensively than the wild-type transporter by the cationic substrate tetraethyl ammonium.
Conclusions and implications: Several naturally occurring SNPs encode variant hOAT4s that may impair the renal tubular re-absorption of important drug substrates.
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Keywords: organic anion transporter; oestrone sulphate transport; single nucleotide polymorphisms; genetic variants

Abbreviations: GlpT, glycerol 3-phosphate transporter; hOAT, human organic anion transporter; SLC, solute carrier; SNP, single nucleotide polymorphism; TEA, tetraethylammonium

Introduction

Organic anion transporters (OATs) are a family of important solute carrier (SLC) proteins that mediate the flux of organic anions into cells (You, 2002). Apart from their roles in the transport of physiologically important anions, OATs also transport a range of chemical toxins and drugs, such as anti-cancer drugs, antibiotics and ACE inhibitors (Zhou and You, 2007). Thus, OATs play a major role in the cellular uptake of numerous drugs, which influences drug efficacy and the duration of drug action (Srimaroeng *et al.*, 2008).

From computer modelling and hydropathy analysis, OATs are predicted to share common structural features, including 12 putative trans-membrane domains and multiple consensus sites for glycosylation and phosphorylation (Zhou and You, 2007). The critical roles of protein N-glycosylation in the maintenance of OAT function and expression have been established (Tanaka *et al.*, 2004; Zhou *et al.*, 2005). Important studies of the structure-function relationships of OATs have also been conducted and have identified amino acid residues that influence transporter function and stability (Feng *et al.*, 2001; 2002; Zhou *et al.*, 2004a,b; Perry *et al.*, 2006). In addition, information on the regulatory control of OAT expression by hormones or protein kinases has emerged (You *et al.*, 2000; Sauvant *et al.*, 2003; Hesse *et al.*, 2004; Zhou *et al.*, 2007; Zhang *et al.*, 2008; Hocherl *et al.*, 2009).

Human OAT4 (SLC22A11) is highly expressed at the basolateral membrane of placental syncytiotrophoblast cells and at the apical membrane of renal proximal tubular cells (Cha *et al.*, 2000; Ugele *et al.*, 2003). Thus, OAT4 is an organic

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anion/dicarboxylate exchanger with a major role in the tubular reabsorption of organic anions from urine (Ekaratanawong *et al*., 2004). Polymorphisms in the genes encoding OAT1 and OAT3 and their consequences for transporter function have been reported previously (Nishizato *et al*., 2003; Bleasby *et al*., 2005; Erdman *et al*., 2006; Ogasawara *et al*., 2008). However, there is little information at present on the impact of pharmacogenetic variation in the *SLC22A11* gene on transporter function. Oestrone sulphate is a typical hOAT4 substrate which undergoes desulphation to the active hormone within cells following transporter-mediated uptake (Tilson-Mallett *et al*., 1983; Santner *et al*., 1986). Thus, functional information on the impact of SNPs in the hOAT4 gene could be relevant for oestrogen-dependent human diseases such as osteoporosis (Lee *et al*., 2008) and pharmacokinetic performance of important drugs like torsemide (Vormfelde *et al*., 2006). In the present study, information on the non-synonymous SNPs that have been reported in the *SLC22A11* gene was used to evaluate the impact of genetic polymorphism on the kinetics and membrane expression of variant hOAT4 transporters.

Methods

Generation of hOAT4 cDNA and its polymorphic variants

The cDNA for the *SLC22A11* gene that encodes hOAT4 (NCBI protein access number: NP_060954) was amplified from Quick-Clone cDNA and confirmed by sequencing. The primers (5′-GCAGCTAGCTCCAAACAGCAGTTAGGTCAGC-3′, sense) and 5′- CAGGGGTACCTGACTAAAGGGGCTCCA TGC-3′, antisense) corresponded to the published sequences of the *SLC22A11* gene (NM_018484). The cDNA was then subcloned into the PCI vector through its *NheI* and *KpnI* sites. Specific nucleotide changes were generated using Pfu DNA polymerase following the manufacturer's instructions. The sequences of the oligonucleotides used in the mutagenesis procedure are shown in Table 1. All sequences were confirmed by the dideoxy chain termination method (Supamac, Camperdown, NSW, Australia).

Expression in COS-7 cells

COS-7 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Cells were transfected with plasmid DNA using Lipofectamine 2000 Reagent following the manufacturer's instructions. Twenty-four hours after transfection of the cells, substrate transport activities were measured.

Transport uptake assay

Uptake of [^{3}H]-oestrone sulphate was initiated in phosphate-buffered saline (PBS), pH 7, containing 5 mM glucose and 100 nM [^{3}H]-oestrone sulphate and was terminated at varying times by rapidly washing the cells in ice-cold PBS. The cells were then dissolved in 0.2 M NaOH, neutralized with 0.2 M HCl, and aliquoted for liquid scintillation counting. Uptake count was standardized by the amount of protein in each well. Data are presented as mean ± SE (n = 3).

Cell-surface biotinylation

Cell-surface expression levels of hOAT4 and its variants were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin. The transporters were expressed in COS-7 cells in six-well plates using Lipofectamine 2000, as previously described. After 24 h, the medium was removed, and the cells were washed successively with 3 mL aliquots of ice-cold PBS (pH 8.0). Cells were incubated on ice with 1 mL of freshly prepared NHS-SS-biotin (0.5 mg·mL^{-1} in PBS) for 30 min with gentle shaking. After biotinylation, cells were washed with 3 mL of PBS containing 100 mM glycine and then incubated on ice for 20 min to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then treated with 400 µL of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and containing the protease inhibitors phenylmethylsulphonyl fluoride, 200 µg·mL^{-1}, and leupeptin, 3 µg·mL^{-1}, pH 7.4) for 30 min. Unlysed cells were removed by centrifugation at 13 200 rpm at 4°C. Streptavidin-agarose beads (50 µL) were then added to the supernatant to isolate cell membrane protein.

Table 1 Oligonucleotide sequences used for site-directed mutagenesis of hOAT4

Nucleic acid change	*Mutants*	*Forward primers (5′-3′)*	*Reverse primers (5′-3′)*	*Allele frequency*
G37A	V13M[a]	CAA GCC GGA GGC ATG GGC CTC TTC C	GGA AGA GGC CCA TGC CTC CGG CTT G	0.005
T86C	L29P[b]	CATCCTCCCCTGCCCCATGATACCTTCC	GGAAGGTATCATGGGGCAGGGGAGGATG	ND*
A91G	I31V[b]	CCC TGC CTC ATG GTA CCT TCC CAG ATG C	GCA TCT GGG AAG GTA CCA TGA GGC AGG G	ND*
C142T	R48Y[a]	CGC CAT CCC AGG CCA CTA CTG CTG GAC ACA CAT G	CAT GTG TGT CCA GCA GTA GTG GCC TGG GAT GGC G	0.005[1]/0.008[2]
C185G	T62R[a]	GCT CTG CGG TTT CCA GAA ACA TGA CCC CC	GGG GGT CAT GTT TCT GGA AAC CGC AGA GC	0.005
T464G	V155G[b]	CGG GAT CT GGG GGG CTC CTT TAT CTG	CAG ATA AAG GAG CCC CCC AGG ATC CCG	0.01
G463A	V155M[a]	CGG GAT CCT GAT GGG CTC CTT TAT CTG	CAG ATA AAG GAG CCC ATC AGG ATC CCG	0.01
C732T	A244V[a]	GCA GGC CAG GCG GTG CTG GGC GGC C	GGC CGC CCA GCA CCG CCT GGC CTG C	0.005
G1015A	T339M[a]	GAC CTG TTC TGC ATG CCC GTG CTC CG	CGG AGC ACG GGC ATG CAG AAC AGG TC	0.01
C1175T	T3921[a]	GGC CGG GCC ACC ATT GCC CTC TTG C	GCA AGA GGG CAA TGG TGG CCC GGC C	0.005

[a]Xu *et al.* (2005).
[b]NCBI SNP database: http//www.ncbi.nlm.nih.gov/SNP/
*Not determined.

Electrophoresis and immunoblotting
Cell membrane protein samples were loaded onto 7.5% polyacrylamide minigels and electrophoresed using a mini cell. Proteins were transferred to PVDF membranes in an electroelution cell and blocked for 1 h with 5% nonfat dry milk in PBS-Tween (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 100 mM NaCl, and 0.05% Tween 20, pH 7.5), washed, and then incubated overnight at 4°C with anti-hOAT4 antibody (1 $\mu g \cdot mL^{-1}$). The membranes were washed, incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1 : 5000), and signals were detected using a SuperSignal West Dura extended duration kit.

Comparative modelling
The glycerol 3-phosphate transporter (GlpT) from *Escherichia coli* (PDB ID 1pw4) was used as the template for comparative modelling (Huang *et al.*, 2003). The sequence alignment for GlpT, hOAT1 and hOAT4 was performed using CLUSTALW (Thompson *et al.*, 1994) as implemented at EBI (Perry *et al.*, 2006). In the alignment used for modelling, the sequence identity was 13% in the regions of structural similarity; the modelling was undertaken following consideration of similarities between folds (Perry *et al.*, 2006). Because the loops in the GlpT structure were shorter than those in OAT4, those between helices 1 and 2, and helices 6 and 7, respectively (specifically, residues Asn63 to Ser138 and Glu307 to Arg343, respectively) were removed to facilitate the analysis. The sequence and template were used as inputs for the comparative protein modelling software MODELLER (Sali, 1995), using the graphical interface Discovery Studio (v1.7, Accelrys, San Diego, CA, USA). Modelling was then performed at higher optimization settings and loop refinement. After the modelling, Cys49-Thr62, Gln139-Gly140 and Arg281-Lys306 were also deleted as they did not form part of the secondary structure on either side of the putative transporter loops. The model was validated using MOLPROBITY (Davis *et al.*, 2007).

Statistics
Student's *t*-test was used to test the significance of differences between data sets. Differences in transport function and inhibition of hOAT4 and its variants were detected by analysis of variance and Fisher's protected least significant difference testing.

Materials
[^{3}H]-oestrone sulphate (57.3 $Ci \cdot mmol^{-1}$) and [^{14}C]-tetraethylammonium (TEA; 3.5 $mCi \cdot mmol^{-1}$) were purchased from PerkinElmer (Melbourne, VIC, Australia). Culture media were obtained from Thermo Scientific (Lidcombe, NSW, Australia). All other chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Quick-Clone cDNA was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA, USA); PCI vector and Pfu DNA polymerase were from Promega (Alexandria, NSW, Australia); Lipofectamine 2000 Reagent was from Invitrogen (Mount Waverley, VIC, Australia) and NHS-SS-biotin from Quantum Scientific (Lane Cove West, NSW, Australia). The mini cell and the electroelution cell were obtained from Bio-Rad (Gladesville, NSW, Australia); the streptavidin-agarose beads and SuperSignal West Dura extended duration kit were from Quantum Scientific; the anti-hOAT4 antibody was from Jomar Bioscience Pty Ltd (Kensington, South Australia).

Results

Transport of [^{3}H]-oestrone sulphate by hOAT4 variants
Ten nonsynonymous SNPs within the *SLC22A11* gene that encode variant hOAT4 transporters have been reported previously (http://www.ncbi.nlm.nih.gov/projects/SNP/) (Xu *et al.*, 2005). Allele frequencies are reportedly in the range 0.005–0.01, with the exception of the T86C and A91G variants, for which no information is currently available. The corresponding SNPs were incorporated into the wild-type *SLC22A11* gene by site-directed mutagenesis in order to undertake the present study.

To explore the impact of SNPs on the variant transporters, the uptake of [^{3}H] oestrone sulphate was measured in transfected COS-7 cells. As shown in Figure 1, compared with wild-type OAT4, transport function was essentially lost in the case of the L29P and R48Y transporter variants, significantly decreased in the case of the V155G and T392I variants and retained by the other six variants.

Immunoblot analysis of the membrane and total cellular expression of hOAT4 and its L29P, R48Y, V155G and T392I variants
Biotinylation and immunoblot analysis was undertaken to evaluate the expression of hOAT4 and its variant transporters at the plasma membrane of COS-7 cells. As shown in Figure 2A, expression of the R48Y and V155G variants at the cell surface was decreased, while that of the L29P and T392I variants was only ~10% of wild-type or lower. In control experiments, the specificity of biotinylation was assessed by reprobing western immunoblotting membranes with an anti-actin antibody. The absence of a signal indicates that biotinylation was specific for proteins expressed at the cell membrane. The molecular masses of the variant hOAT4 proteins expressed at the cell surface were ~86kD in each case, which is consistent with previous findings (Zhou *et al.*, 2004a,b; 2005).

Immunoblot analysis of total cellular expression of the hOAT4 variants was also undertaken. In the cases of the R48Y, V155G and T392I variant transporters, the signal at ~86 kD was markedly decreased and was completely undetectable in the case of the L29P variant (Figure 2B). Interestingly, a signal near ~60kD was detected in cells that expressed the R48Y hOAT4 variant. Although the nature of this protein was not investigated, it may represent the partially glycosylated transporter (Zhou *et al.*, 2005).

Assessment of transporter characteristics of hOAT4 and its V155G and T392I variants
Apart from reduced expression of transporter protein at the plasma membrane, decreased transporter function could also

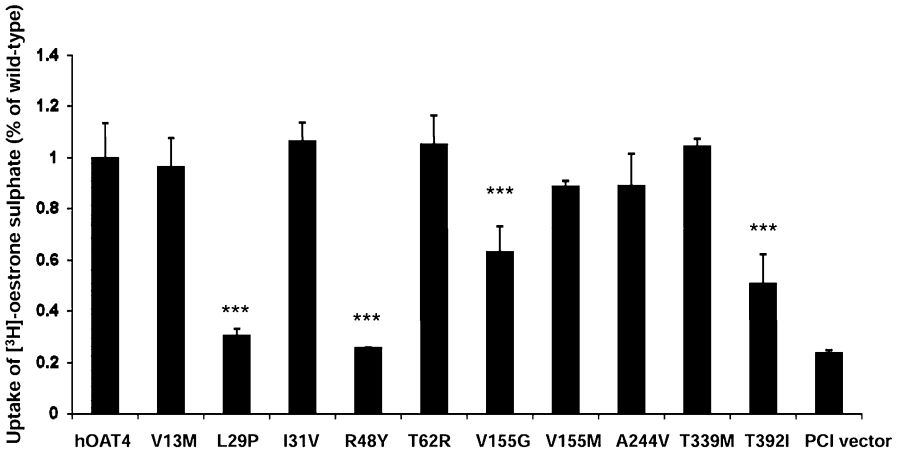


Figure 1 Uptake of [^{3}H]-oestrone sulphate by cells expressing wild-type hOAT4 and single nucleotide polymorphisms. The transport of 100 nM [^{3}H]-oestrone sulphate in COS-7 cells transfected with wild-type and mutant variants of the *SLC22A11* gene. Values are mean ± SE (n = 3). ***Different from wild-type hOAT4: $P < 0.001$.

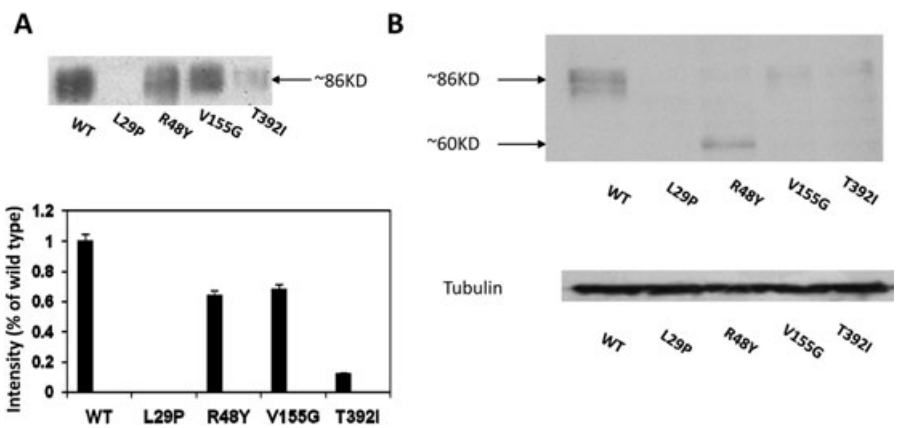
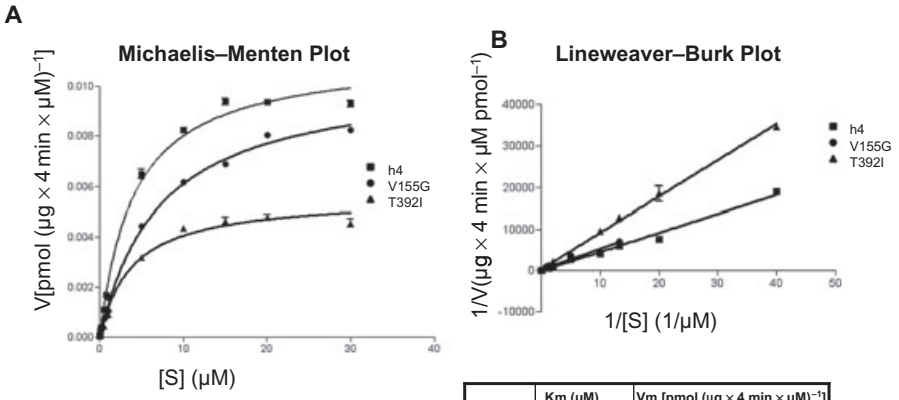


Figure 2 Cell surface expression of hOAT4 and single nucleotide polymorphisms. (A) Western blot analysis of cell surface expression of wild-type hOAT4 and its variant transporters. Top panel: cells were biotinylated, and the labelled cell surface proteins were precipitated with streptavidin beads and separated by gel electrophoresis, followed by Western blotting with anti-hOAT4 antibody. Bottom panel: the transporter expression after densitometry. (B) Western blot analysis of total cell expression of hOAT4 and its variants in cells. Top panel: cells were lysed, and proteins were separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody. Bottom panel: after being stripped, the blot was reprobed with anti-tubulin antibody.

be due either to altered substrate binding affinity or turnover. To assess the mechanisms underlying the impaired transport function of the V155G and T392I variants of hOAT4, we conducted kinetic analyses in cells that had been transiently transfected with the corresponding *SLC22A11* gene variants. As shown in Figure 3, an increase in the Michaelis constant for [^{3}H]-oestrone sulphate was noted with the V155G variant transporter (K_m 7.17 ± 0.4 μM compared with 4.06 ± 0.3 μM for wild-type hOAT4), but not with the T392I variant (K_m 3.79 ± 0.4 μM). However, the latter substitution decreased V_{max} (T392I: 0.0056 ± 0.00015 pmol·μg^{-1} × 4 min × μM) compared with wild-type (0.011 ± 0.00022 pmol·μg^{-1} × 4 min × μM), whereas the apparent V_{max} for the V155G hOAT4 variant was similar to wild type (0.0010 ± 0.00019 pmol·μg^{-1} × 4 min × μM). Thus, the loss of function in the case of the V155G variant may be due to decreased substrate binding affinity, while the impaired function of the T392I variant was due primarily to decreased substrate turnover.

hOAT4 may also efflux organic anion substrates (Ekaratanawong *et al*., 2004). To test whether the hOAT4 variants retain this characteristic, cells that expressed wild-type hOAT4 or the V155G and T392I variants were pre-loaded with 500 nM [^{3}H]-oestrone sulphate for 1 h and then incubated with oestrone sulphate or the alternative substrate β-oestradiol-3-sulphate (Ekaratanawong *et al*., 2004; Zhou *et al*., 2005). The efflux of [^{3}H]-oestrone sulphate from cells that expressed the wild-type hOAT4 was 64% and 57% in the presence of oestrone sulphate and β-oestradiol-3-sulphate, respectively, compared with control; similar rates were observed for both hOAT4 variants (data not shown). The present findings are similar to an earlier report that anion exchange capacity was retained in loss-of-transport function hOAT1 mutants (Hong *et al*., 2004).

The inhibition of wild-type hOAT4 and its V155G and T392I variants by a range of ionic substrates was assessed. As shown in Figure 4, [^{3}H]-oestrone sulphate uptake by wild-type hOAT4 was modulated by β-oestrone 3-sulphate, β-oestrone 3,17-disulphate and diclofenac (all 1 μM) whereas oestradiol, p-aminohippurate, captopril and diflunisal, as well as the cationic agent TEA (all 1 μM), had no effect on oestrone



	Km (µM)	Vm [pmol (µg × 4 min × µM)$^{-1}$]
hOAT4	4.06±0.3	0.011 ± 0.00022
V155G	7.17±0.4	0.0010±0.00019
T392I	3.79±0.4	0.0056 ± 0.00015

Figure 3 Kinetic analysis of oestrone sulphate transport mediated by hOAT4 and its variants. Kinetic characteristics were determined at substrate concentrations ranging from 0.025 to 30 µM (4-min uptake) using cells expressing hOAT4 wild-type, V155G or T392I. Transporter kinetic parameters were calculated using GraphPad Prism 4. Values are mean ± SE ($n = 3$). (A) The direct Michaelis–Menten plot is shown and (B) Lineweaver–Burk plot. The K_m and V_{max} values of hOAT4 and single nucleotide polymorphisms are summarized in the table.

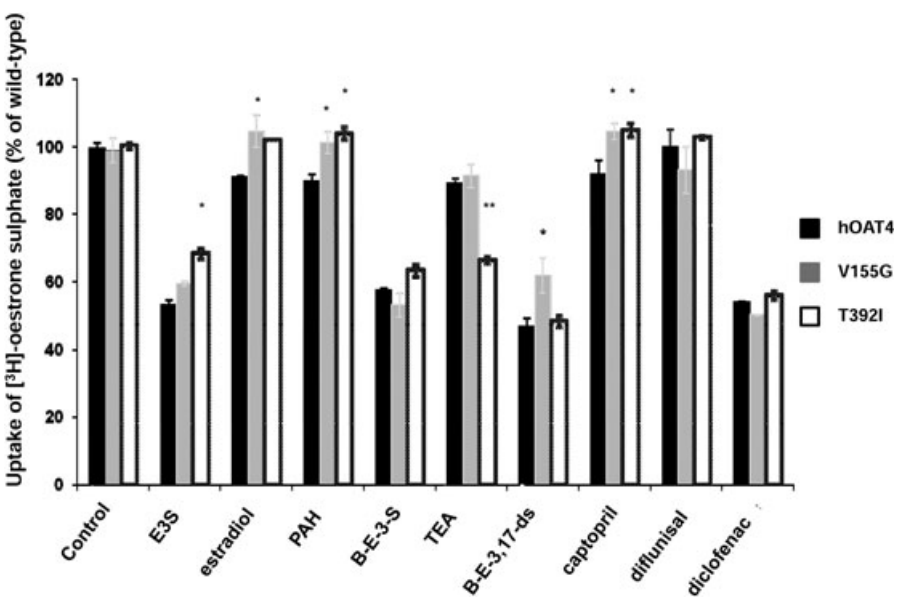


Figure 4 Inhibition of [^{3}H]-oestrone sulphate (100 nM) transporter in the absence (control) and presence of various unlabelled compounds (1 µM). The data are presented as a percentage of control uptake. PAH: p–aminohippurate; B-E-3-S: β-oestrone-3-sulphate; TEA: tetraethylammonium; B-E-3,17-ds: β-oestrone-3,17-disulphate. Values are mean ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$, different from observed inhibition of the wild-type transporter.

sulphate influx. Essentially, similar findings were made with the V155G and T392I variant hOAT4 transporters, although the extent of the responses to certain ionic substances was altered. Thus, relatively small changes in the inhibition of transport by oestrone 3-sulphate, β-oestrone 3-sulphate, PAH, β-oestrone 3,17-disulphate and captopril occurred with the variant hOAT4s. Interestingly, however, the T392I variant was somewhat more susceptible to inhibition by TEA, which is a typical cationic substance and which has been reported previously not to be a hOAT4 substrate. The possibility that the greater susceptibility of the T392I variant transporter to inhibition by TEA may reflect a change in transporter substrate specificity was assessed directly. However, the transport of [^{14}C]-TEA in cells that over-expressed hOAT4 and its T392I variant was extremely low (not shown).

Comparative modelling of the structure of hOAT4

No crystal structure of a protein that has a structural relationship with hOAT4 is currently available in the Worldwide Protein Databank (Berman *et al.*, 2000). The glycerol 3-phosphate transporter (GlpT) from *E. coli* (PDB ID 1pw4; Huang *et al.*, 2003), which shares 13% sequence relatedness across the aligned regions, was therefore selected as the best

available template. According to the protein sequence alignment of GlpT, hOAT1 and hOAT4 (Figure 5) and the proposed model of hOAT1 (Perry *et al*., 2006), a predicted topological structure of hOAT4 was obtained (Figure 6). Model quality by Molprobity is summarized as follows: rotamer outliers 3.6%, Ramachandran outliers 1.1%, Ramachandran favoured 92%, 10 Cβ deviations >0.25 Å, residues with bad bonds 0.0% and residues with bad angles 1.3% (Davis *et al*., 2007); this level of quality is suitable for the present purpose. The template structure of *E. coli* GlpT used in comparative modelling was determined at 3.3 Å resolution. As shown in the present model, T392 resides on trans-membrane domain 8 of OAT4 whereas V155 is buried in trans-membrane domain 2 (Figure 6).

Discussion

OATs are important membrane transporters, which influence the absorption, distribution and elimination of anionic endogenous and exogenous chemicals and metabolites. OATs are highly expressed in human kidney and are also found in a range of other tissues. hOAT4 is abundantly expressed in human placenta as well as in the kidney. Several nonsynonymous SNPs in the *SLC22A11* gene that encodes hOAT4 have been reported recently and are also listed in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) (Xu *et al*., 2005). In the present study, the impact of these SNPs in *SLC22A11* on the function of the hOAT4 variant transporters were characterized.

Transport function in the L29P, R48Y, V155G and T392I variants of hOAT4 was decreased. From biotinylation and immunoblot analysis, membrane expression was somewhat impaired in the cases of the R48Y and V155G variants and particularly so with the L29P and T392I variants. hOAT4 immunoreactive protein was not detected in the case of the L29P variant; it is feasible that the relatively inflexible proline residue may have influenced protein synthesis or stability. From the amino acid alignment of the sequences of hOAT1, hOAT3, hOAT4 and rOAT3 shown in Figure 5, leucine-29 is a conserved amino acid within the OAT family, as is glutamate-278. Indeed, transporter function of E278K was 13.7 ± 1.9% of wild-type hOAT4 function when expressed in COS-7 cells (data not shown), which is consistent with previous findings (Lee *et al*., 2008); this variant has been associated with the incidence of osteoporosis in Korean women.

Impaired cellular expression of the R48Y variant was observed. Expression of the mature (glycosylated) form of the protein (~86 kD) was decreased, while expression of a ~60 kD form of protein was increased; this is likely to be the non-glycosylated immature form of the protein. Previous reports have highlighted the importance of asparagine glycosylation for correct membrane insertion of hOAT4 (Zhou *et al*., 2005). Thus, it is feasible that R48 is important for N-glycosylation of hOAT4 in the Golgi apparatus prior to its translocation to the cell surface. Replacement of the basic arginine-48 with the larger aromatic tyrosine residue may also disrupt substrate binding (Zhou *et al*., 2004b).

In the case of the hOAT4 V155G and T392I variants, the membrane and total cellular expression of the transporters were both decreased, which accounts in part for the loss of function. From kinetic analysis, the V155G variant also exhibited an increased K_m, consistent with a decrease in the affinity of the transporter for its substrate, while the V_{max} was essentially unchanged. In contrast, the T392I variant exhibited an unchanged K_m but a decreased V_{max}, which is consistent with decreased turnover of the transporter substrate complex. The transporter turnover rate is a combination of rate constants, including those describing a conformational switch in the transporter and several association/dissociation steps in substrate cycling, which are also influenced by the total number of surface transporter molecules (Eraly, 2008).

In cells that over-expressed the T392I variant, the cationic substrate TEA was found to inhibit oestrone sulphate uptake to an extent greater than that with the wild-type or V155G variant hOAT4s. Although the variant remained an anion transporter, it emerges that residue 392, or residues in the vicinity, may be important for interaction with substrates and inhibitors. The substitution of the polar threonine by the non-polar isoleucine at residue 392 occurs in transmembrane domain 8 of OAT4 (Figure 6). From the present kinetic analysis, the substrate affinity was unaltered in the T392I variant but the V_{max} was decreased. In OAT4, threonine-392 is adjacent to an arginine at residue 389; this corresponds to the lysine/arginine residue that is common to several OATs (Figure 5). Indeed, in rat OAT3, the corresponding lysine-370 was one of a pair of basic residues that were found to be essential for anion transport (Feng *et al*., 2001). From these alignments and the present model of OAT4 leucine-29, glutamate-278, arginine-389 and threonine-392 are all oriented intracellularly. The model also suggests that arginine-389 and threonine-392, which are co-located on transmembrane domain 8 (Figure 6A), interact directly in the native transporter. In contrast, no direct interactions between arginine-389 and alternate amino acid residues on the nearby transmembrane domains 5, 9 and 10 appear likely. Thus, it is feasible that the replacement of threonine-392 by isoleucine disrupts local hydrogen bonding with arginine-389, and impairs transporter function. As to the present model, valine-155 resides within transmembrane domain 2, which could be part of the substrate binding pocket, because the variant V155G showed impaired substrate-transporter binding affinity.

In summary, in the present study, the impact of nonsynonymous SNPs in the *SLC22A11* gene on hOAT4 function has been characterized. A major loss of hOAT4 function occurred with four of these variants, apparently by different mechanisms. The R48Y substitution may impair the N-glycosylation of hOAT4, which is important for correct membrane localization, whereas the T392I substitution may impair the selectivity of the transporter for anionic substrates. The present findings now provide a firmer basis for pharmacogenomic studies of the impact of OAT variation on the disposition and elimination of drugs that are substrates for hOAT4, which could significantly contribute to the overall therapeutic performance of these drugs, including their pharmacokinetic profiles and various adverse events. The present findings may also contribute to the achievement of the optimum therapy with hOAT4 drug substrates, and could assist in the prediction of adverse effects due to co-administered agents that are also transported by hOAT4 in patients carrying certain naturally occurring SNPs.

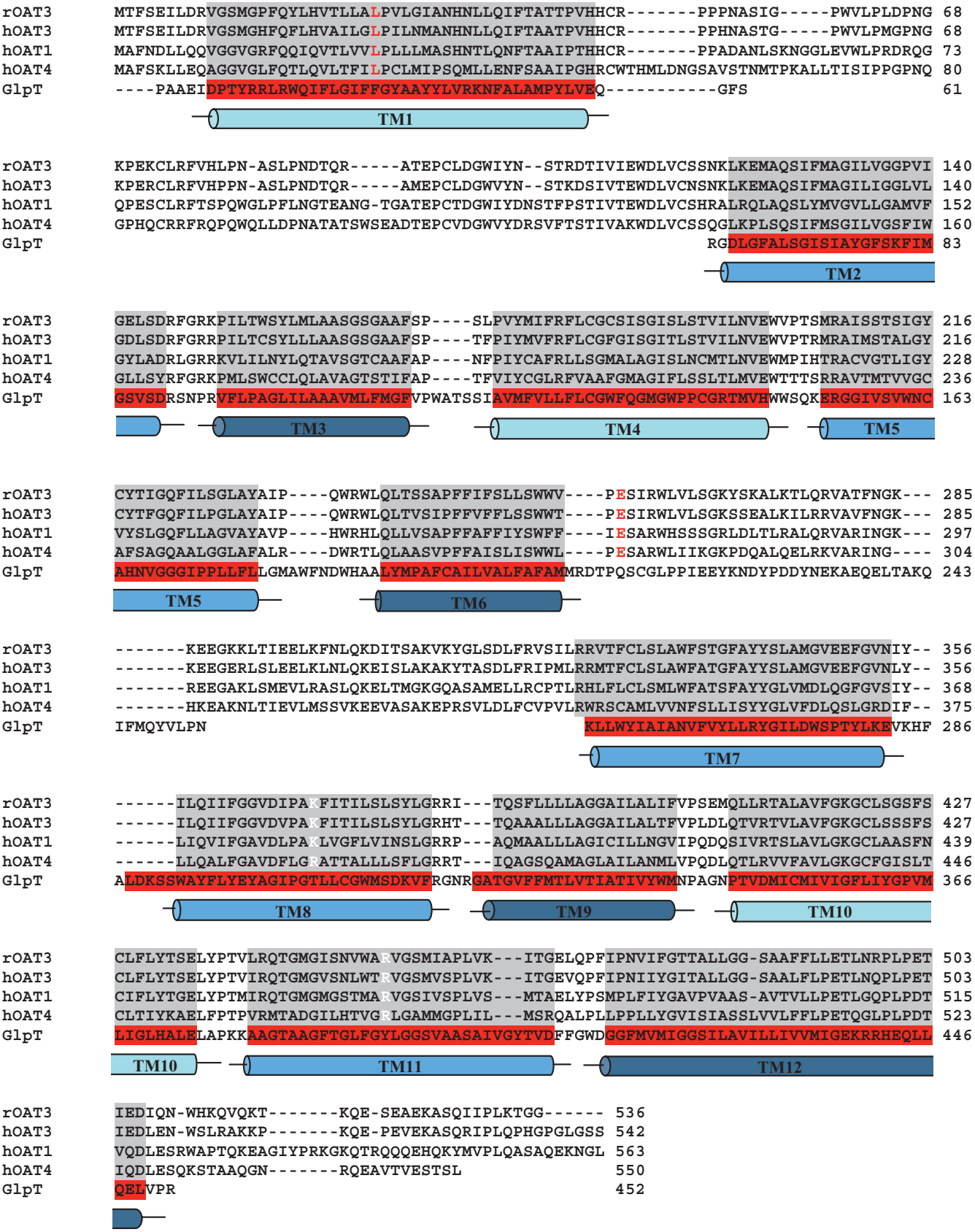


Figure 5 Amino acid alignment of hOAT1 (AAD55356), hOAT3 (NP_004245), hOAT4 (NP_060954), rOAT3 (NP_112622) and GlpT (PDB ID 1pw4). The alignment was performed by CLUSTALW2 (Larkin *et al.*, 2007) as implemented at EBI (Lopez and Lloyd, 1997). Shaded letters represent the predicted helices from the comparative modelling template, *Escherichia coli* glycerol 3-phosphate transporter.

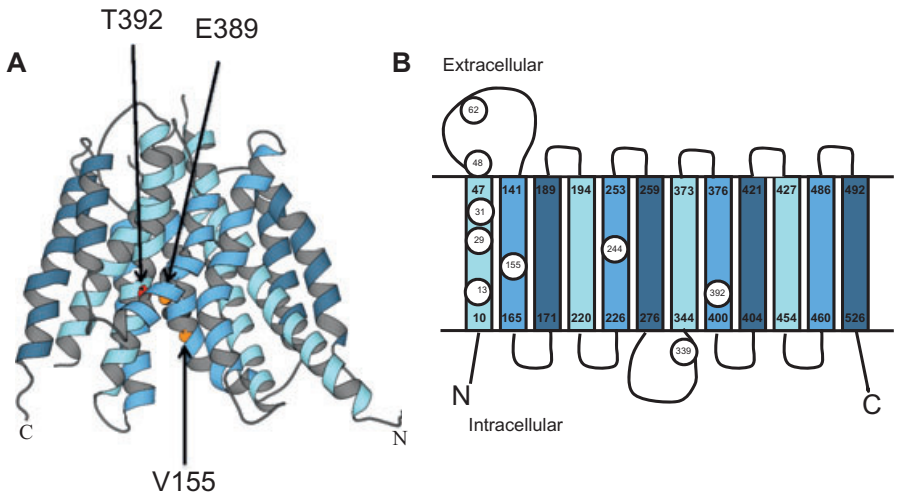


Figure 6 Predicted structural model of hOAT4. (A) The three-dimensional model of hOAT4 based on the structure of GlpT using MODELLER and the figure was drawn by Molescript (Kraulis, 1991). (B) The two-dimensional depiction of the structure showing the corresponding helices. Rectangular bars indicate the transmembrane helices; numbers indicate the amino acids at the start or end of each transmembrane helix; and circled numbers show the location of amino acid residues encoded by the nonsynonymous single nucleotide polymorphisms evaluated in the present study.

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Conflicts of interest

None declared.

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