Biochemical Pharmacology 82 (2011) 1692–1699

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Functional analysis of pharmacogenetic variants of human organic cation/carnitine transporter 2 (hOCTN2) identified in Singaporean populations

Dorothy Su Lin Toh [a,b], Michael Murray [a], Kuan Pern Tan [c], Vishwaroop Mulay [a], Thomas Grewal [a], Edmund Jon Deoon Lee [b], Fanfan Zhou [a,*]

[a] Faculty of Pharmacy, University of Sydney, NSW 2006, Australia
[b] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[c] Bioinformatics Institute, Agency for Science, Technology and Research (A*STAR), Singapore

ARTICLE INFO

Article history:
Received 17 June 2011
Accepted 5 August 2011
Available online 16 August 2011

Keywords:
Human organic cation/carnitine transporter 2
SLC22A5
Single nucleotide polymorphisms
Transporter function
L-Carnitine

ABSTRACT

The human organic cation/carnitine transporter-2 (hOCTN2; *SLC22A5*) mediates the cellular influx of organic cations such as carnitine, which is essential for fatty acid oxidation. Primary carnitine deficiency has been associated with a wide range of hOCTN2 gene mutations. Six novel nonsynonymous single nucleotide polymorphisms in the hOCTN2 gene were identified recently in Chinese and Indian populations of Singapore. The present study evaluated the impact of these polymorphisms on hOCTN2 function and expression in HEK-293 cells. Transport function was markedly impaired in variants that encoded amino acid substitutions D122Y (<20% of wild-type control) and K302E (~45% of wild-type) in the large extracellular loop and large intracellular loop of hOCTN2, respectively. The function of the other four variants was unimpaired (E109K, V175M, K191N and A214V). From biotinylation and immunofluorescence experiments, the expression of the D122Y and K302E-hOCTN2 variants at the plasma membrane of HEK-293 cells was decreased relative to the wild-type hOCTN2 but total cellular expression was unchanged. Transporter kinetic studies indicated a decrease in the V_{max} for L-carnitine influx by K302E-hOCTN2 to 49% of wild-type control, while K_m remained unchanged; kinetic evaluation of D122Y-hOCTN2 was not possible due to its low transport function. The K302E-hOCTN2 variant was also more susceptible than the wild-type transporter to inhibition by the drugs cimetidine, pyrilamine and verapamil. These findings indicate that impaired plasma membrane targeting of the D122Y and K302E-hOCTN2 variants that occur in Singaporean populations contributes to decreased carnitine influx.

1. Introduction

The SLC22A genes are members of the major facilitator superfamily that comprises 18 families of transporters from bacteria, plants, animals and humans [1]. These proteins mediate the transport of organic cations, zwitterions, and organic anions across cell membranes. *SLC22A5*, the gene encoding for the human organic cation/carnitine transporter 2 (hOCTN2), consists of 10 exons and is located in the cytokine cluster region on chromosome 5q31 [2,3]. hOCTN2 is a multispecific, bidirectional, and pH-dependent transporter for organic cations that consists of 557 amino acids and has a predicted molecular mass of 63 kDa. hOCTN2 is expressed in numerous tissues, including kidney, heart, and liver [4].

Carnitine is essential for the β-oxidation of fatty acids and energy production in skeletal muscle. Carnitine deficiency can lead to fasting-induced hypoketotic hypoglycaemia, hepatic encephalopathy and cardiac myopathy [5,6]. hOCTN2 mediates the cellular influx of the prototypic substrate L-carnitine in a sodium-dependent manner and defects in the *SLC22A5* gene underlie carnitine deficiency in man [7–10]. As is the case with other SLC transporters, hOCNT2 can also transport a number of xenobiotics, such as tetraethylammonium (TEA), verapamil, pyrilamine and the β-lactam antibiotics [11].

Like other members of the SLC superfamily, hOCTN2 has twelve predicted transmembrane domains (TMDs) with a large extracellular loop between TMDs 1 and 2 and a large intracellular loop located between TMDs 6 and 7 [2,7]. Such evidence for SLC transporter structure has been provided by hydropathy analysis, comparative modeling and the use of domain-specific antibodies [12]. As summarized recently, a number of single nucleotide polymorphisms (SNPs) have been identified in the *SLC22A5* gene [13]. Information on the functional consequences of some of these variants is available [14–17]. Recently we identified six non-synonymous variants in the

Abbreviations: GlpT, glycerol 3-phosphate transporter; hOCTN2, human organic cation/carnitine transporter 2; OPM, Orientations of Proteins in Membranes; PBS, phosphate-buffered saline; PDB, protein data bank; SNPs, single nucleotide polymorphisms; TEA, tetraethylammonium; TMD, transmembrane domain.

* Corresponding author. Tel.: +61 2 9036 3015; fax: +61 2 9036 3244.
E-mail address: fanfan.zhou@sydney.edu.au (F. Zhou).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.08.008

SLC22A5 gene that encoded variant hOCTN2 transporters in the Chinese (V175M, K191N and K302E) and Indian (E109Q, D122Y and A214V) populations of Singapore [18]. The D122Y and A214V variants were also identified subsequently in a cohort of infants who exhibited abnormal carnitine screening results and their relatives [13]. However, to date there is no information on the impact of these recently identified variants on hOCTN2 function.

The present study assessed the functional significance of each of the nonsynonymous polymorphisms that were identified in Singaporean populations on hOCTN2-dependent transport of L-carnitine. The principal finding to emerge was that the transport function was markedly impaired in D122Y and K302E-hOCTN2 but not in the other variants. From biotinylation and immunofluorescence experiments, the underlying mechanism was impaired maturation of the D122Y- and K302E-hOCTN2 variants to the plasma membrane.

2. Materials and methods

2.1. Materials

[^{14}C]-L-carnitine (56 mCi/mmol) and [^{14}C]-tetraethylammonium (TEA; 3.5 mCi/mmol) were purchased from BioScientific Pty. Ltd., Gymea, NSW, Australia. Culture media was obtained from Thermo Scientific (Lidcombe, NSW, Australia). Unless stated otherwise all other chemicals and biochemicals were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia).

2.2. Construction of SLC22A5 gene variants

A plasmid containing the full-length cDNA of human *SLC22A5* (reference sequence: NM_003060) was obtained from Gene-Ethics (Asia) Pty Ltd., Singapore. Specific nucleotide changes were generated by site-directed mutagenesis essentially as described previously [19], using Pfu DNA polymerase (Promega, Singapore) with the reference clone as template. The sequences of the oligonucleotides used in mutagenesis are shown in Table 1. The sequences of all variant *SLC22A5* cDNAs were confirmed by the dideoxy chain termination method (Ramaciotti Centre, University of New South Wales, Kensington, NSW, Australia).

2.3. Expression of hOCTN2 variants in HEK-293 cells

Human embryonic kidney (HEK)-293 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with plasmid DNA using Lipofectamine 2000 Reagent (Invitrogen, Mount Waverley, VIC, Australia) following the manufacturer's instructions. Twenty-four hours after transfection, substrate transport activities were measured.

2.4. Transport studies

Uptake of [^{14}C]-L-carnitine (5 μM) and [^{14}C]-TEA (100 μM) was initiated at room temperature in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3; *N*-methyl-D-glucamine chloride replaced NaCl in the sodium free condition) and was terminated at intervals by rapidly washing the cells in ice-cold PBS. The cells were then solubilized in 0.2 M NaOH, neutralized with 0.2 M HCl, and aliquoted for liquid scintillation counting. Uptake was standardized to the amount of protein in each well. Data are presented as mean ± SE (n = 3). Kinetic studies with L-carnitine were performed by varying the concentration of unlabelled substrate added to the uptake buffer.

2.5. Cell-surface biotinylation of hOCTN2 variants

Cell-surface expression of hOCTN2 and its variants was determined using the membrane impermeable biotinylation reagent NHS-SS-biotin (Quantum Scientific, Lane Cove West, NSW, Australia). Transporter cDNAs were expressed in HEK-293 cells in six-well plates using Lipofectamine 2000, as previously described [19]. After 24 h, the medium was removed and the cells were washed with ice-cold PBS (pH 8.0; 3 mL). Cells were incubated on ice with 1 mL of freshly prepared NHS-SS-biotin (0.5 mg in PBS) for 30 min with gentle shaking. After biotinylation, cells were washed with PBS containing 100 mM glycine (3 mL) and then incubated on ice for 20 min to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then treated for 30 min with lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, that contained the protease inhibitors phenylmethylsulphonyl fluoride, 200 mg mL^{-1}, and leupeptin, 3 mg mL^{-1}, pH 7.4; 400 mL). Unlysed cells were removed by centrifugation at 14,000 × g at 4 °C. Streptavidin-agarose beads (50 μL; Quantum Scientific, Lane Cove West, NSW, Australia) were then added to the supernatant to isolate biotinylated cell membrane proteins.

2.6. Electrophoresis and immunoblotting

Protein samples were loaded onto 7.5% polyacrylamide minigels and electrophoresed using a mini cell (Bio-Rad, Gladesville, NSW, Australia). Proteins were transferred to polyvinylidene fluoride membranes in an electroelution cell (Bio-Rad) 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-hOCTN2 antibody (1 μg mL^{-1}; Sapphire Biosciences, Cat. No: Abcam-ab79964, Redfern, NSW, Australia). The membranes were washed, incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Sapphire Biosciences, Cat. No: sc-2004), and signals were detected using a SuperSignal West Dura extended duration kit (Thermo Scientific, Lidcombe, NSW, Australia).

2.7. Immunofluorescence of transfected cells

Cells expressing hOCTN2 variants and pDsRed2-ER or pDsRed-Monomer-Golgi (Scientifix, Cheltenham Victoria, Australia) were washed three times in PBS, fixed for 20 min at room temperature in 4% paraformaldehyde in PBS, and then rewashed in PBS. The fixed

Table 1
Primer sequences for mutagenesis.

Variants	Forward primer (5′–3′)	Reverse primer (5′–3′)
E109K	ACCTGGGGCAGCTGCAGCAGGAGAGCTGTCTGGATG	GACAGCTCTCCTGCTGCAGCTGCCCCAGGTCCACGTCGCGC
D122Y	CTGGGAGTTCAGTCAGTACGTCTACCTGTCCACCATTGTG	GGACAGGTAGACGTACTGACTGAACTCCCAGCCATCCAGACAGC
V175M	AAGAATGTGCTGTTCATGACCATGGGCATGCAGACAGGCTTC	GCATGCCCATGGTCATGAACAGCACATTCTTCCGGCCAAACCT
K191N	AGATCTTCTCGAATAATTTTGAGATGTTTGTCGTGCT	CAAACATCTCAAAATTATTCGAGAAGATCTGCAGGAAGCTG
A214V	TCCAACTATGTGGCAGTATTTGTCCTGGGGACAGAAAT	CCCCAGGACAAATACTGCCACATAGTTGGAGATCTGG
K302E	CCGCAAGGCTGCCGAAGCCAATGGGATTGTTGTGCCTT	AATCCCATTGGCTTCGGCAGCCTTGCGGATGATCAC

cells were permeabilized with 0.1% Triton X-100 for 10 min, incubated for 30 min at room temperature in PBS containing 5% goat serum and then incubated for 2 h in the same medium containing anti-OCTN2 antibody (1:100). The cells were washed, and bound primary antibody was detected using Alexa Fluor® 488 conjugate goat anti-rabbit IgG (1:1000 dilution; 1 h; Invitrogen, Mount Waverley, VIC, Australia; Cat. No: A11012). Treated cells were washed thoroughly, and the cover glasses were mounted in VECTASHIELD Mounting Medium (Abacus ALS, East Brisbane QLD, Australia). Samples were visualized with a Leica DMI3000 B epi fluorescence microscope (Leica Microsystems, North Ryde, NSW, Australia).

2.8. Comparative modeling

No close homologs (sequence identity > 30%) of hOCTN2 were found in the Protein Data Bank (PDB) and hence only coarse-grained modeling was undertaken. A BLAST search of the non-redundant protein database confirmed that OCTN2 belongs to the Major Facilitator Superfamily. Of four non-redundant structures (PDB: 1PW4, 2CFP, 2GFP, 3O7Q) of the superfamily that were deposited in the Orientations of Proteins in Membranes (OPM) database, the *Escherichia coli* Glycerol-3-Phosphate Transporter (GlpT; PDB: 1PW4) was used as the template for comparative modeling in consideration of sequence similarity. Topologies of OCTN2 and GlpT were estimated by the TransMembrane prediction using Hidden Markov Models approach [20].

From alignments with other SLC transporters, hOCTN2 is predicted to form 12 TMD α-helixes and to possess several extracellular and intracellular domains. Amino acid residues 44–142 in hOCTN2 are predicted to form a large extracellular domain. In this study the transmembrane, intracellular and extracellular regions of hOCTN2 and GlpT were aligned separately using CLUSTALW. The large extracellular domain was modeled separately using three other templates (PDB: 2VQ3, 2H0A and 1NDH) on the basis of local sequence similarity. The individual models were then reassembled to produce the final alignment. In 3D model building, additional α-helix constraints were applied to predicted TMD of hOCTN2.

2.9. Statistics

The Student's *t*-test was used to test for differences between two data sets. Differences in transport function and inhibition of hOCTN2 and its multiple variants were detected by one-way analysis of variance and Dunnett's Testing.

3. Results

3.1. Transport of [^{14}C]-L-carnitine and [^{14}C]-TEA by hOCTN2 variants

To explore the functional impact of the variant hOCTN2 transporters, the uptake of [^{14}C]-L-carnitine and [^{14}C]-TEA was measured in transfected HEK-293 cells. As shown in Fig. 1, relative to wild-type hOCTN2, L-carnitine and TEA transport was extremely low in the case of the D122Y transporter variant (<10% of wild-type) and significantly decreased in the case of the K302E variant (<50% of wild-type). As expected, carnitine transport by the hOCTN2 variants in sodium-free media was not different from vector alone, whereas TEA influx was quite similar in sodium-containing and sodium-free media. Transport function remained essentially intact in the case of the other four hOCTN2 variants (E109K, V175M, K191N and A214V).

3.2. Immunoblot analysis of the membrane and total cellular expression of hOCTN2 and its D122Y and K302E variants

Biotinylation and immunoblot analyses were undertaken in transfected HEK-293 cells to evaluate the plasma membrane expression of hOCTN2 and the variant transporters in which function was most impaired. Expression of the D122Y- and K302E-hOCTN2 variants was decreased markedly to ~10% and ~40% of wild type, respectively (Fig. 2A). The molecular masses of the variant hOCTN2 proteins expressed at the cell surface were ~78 kDa in each case, which is consistent with both the anticipated sizes of the proteins and with the antibody manufacturer's specifications. In control experiments, the specificity of biotinylation was confirmed by reprobing the membranes used for hOCTN2 immunoblotting with an anti-actin antibody. Total cellular expression of hOCTN2 and its variants was also assessed. In the cases of the D122Y and K302E variant transporters, total protein expression was not significantly different from wild-type hOCTN2 (Fig. 2B). This strongly suggests that incorporation of the variant transporters within the plasma membrane is selectively impaired (Fig. 2C). Immunocolocalization experiments confirmed that expression of both D122Y- and K302E-hOCTN2 at the plasma membrane was decreased and that there was significant retention of the transporters in both the Golgi apparatus and the endoplasmic reticulum (Fig. 3).

3.3. Functional characterization of hOCTN2 and its K302E variant

Apart from impaired expression of transporter protein at the cell membrane, decreased transport function could also be due to

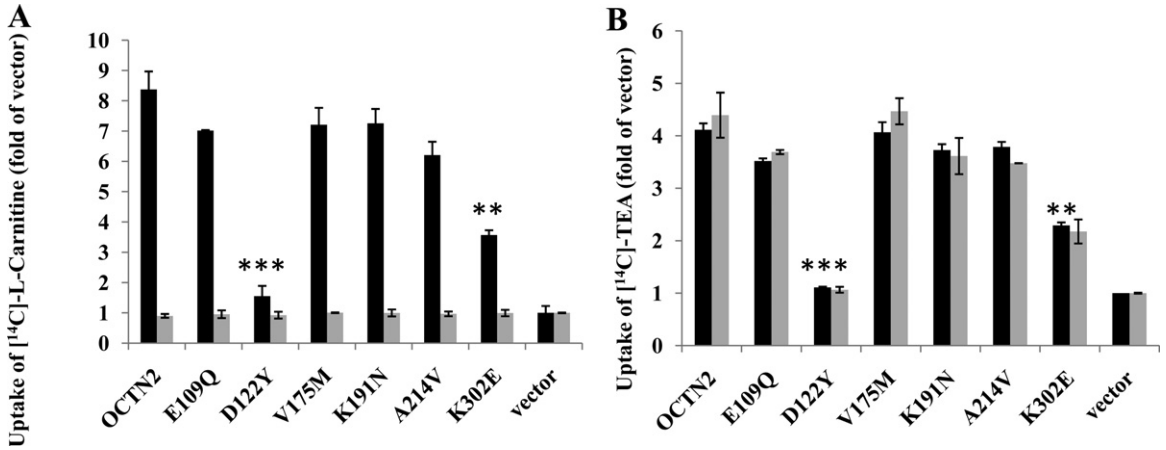


Fig. 1. Uptake of [^{14}C]-L-carnitine and [^{14}C]-TEA by HEK293 cells expressing wild type hOCTN2 and its variants in the presence and absence of sodium. Transport of 5 μM [^{14}C]-L-carnitine (A) and 100 μM [^{14}C]-TEA (B) in HEK293 cells transfected with wild type and mutagenized variants of the *SLC22A5* gene. Uptake was conducted for 4 min in the presence of sodium (black bars) and in the absence of sodium (grey bars). Values are mean ± SE ($n = 3$) Different from wild-type hOCTN2, $^{**}p < 0.01$; $^{***}p < 0.001$.

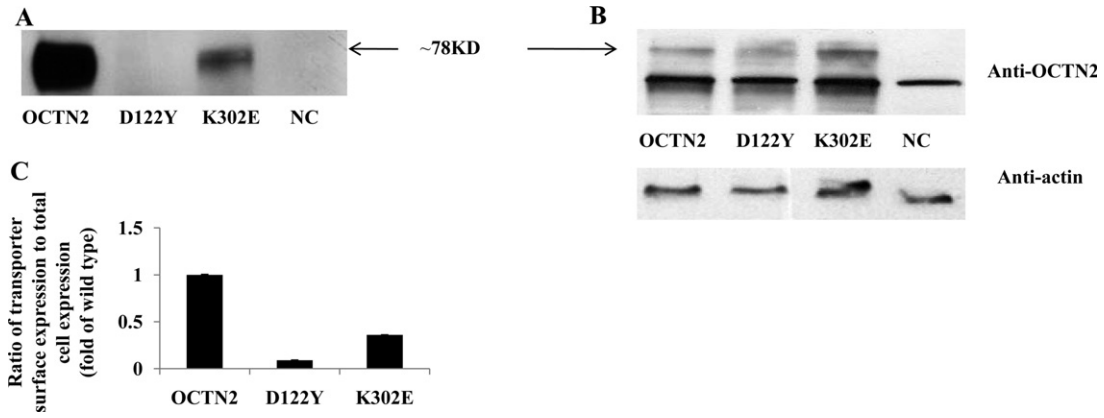


Fig. 2. Expression of hOCTN2 and its variants in HEK-293 cells. (A) Western blot analysis of cell surface expression of wild-type hOCTN2 and its variant transporters. Cells were biotinylated, and the labelled cell surface proteins were precipitated with streptavidin beads and separated by gel electrophoresis, followed by Western blotting with anti-hOCTN2 antibody. (B) Western blot analysis of total cell expression of hOCTN2 and its variants in cells. Top panel: hOCTN2 expression in total cell lysates. Bottom panel: after being stripped, the blot was reprobed with anti-actin antibody. (C) Ratio of transporter surface expression to its total expression after densitometry. NC: negative control.

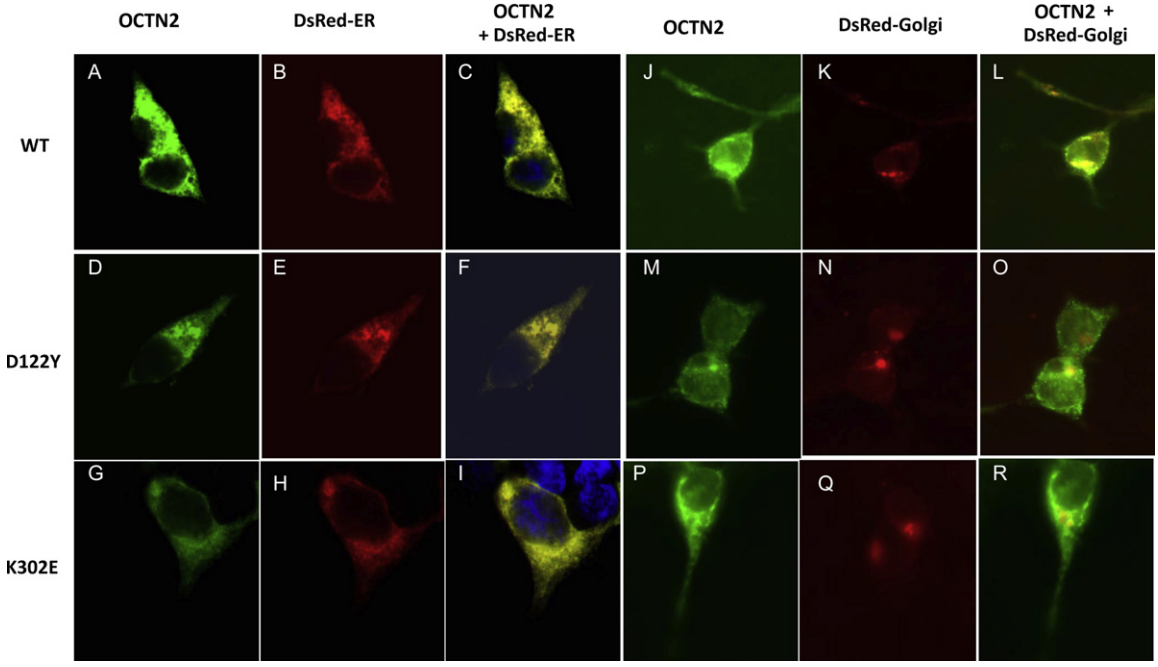
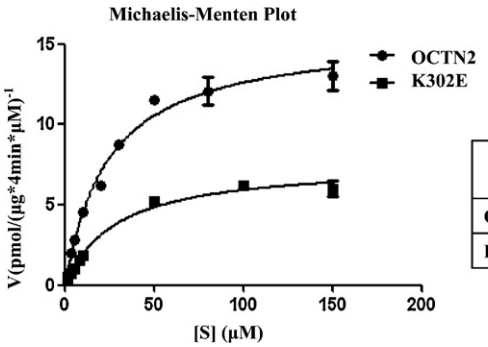


Fig. 3. Immunocolocalization analysis of hOCTN2 and its variants with endoplasmic reticulum (ER) and Golgi apparatus markers. HEK 293 cells were cotransfected with hOCTN2 or its variants together with DsRed-ER or DsRed-Golgi expression plasmids. The cells were stained with anti-hOCTN2 antibody and Alexa Fluor® 488 conjugate goat anti-rabbit IgG. Panels A, D, G, J, M and P show the specific immunostaining of hOCTN2, which appears as bright green fluorescence; panels B, E, and H indicate the ER in bright red fluorescence, panels K, N and Q show the Golgi apparatus in bright red fluorescence; panels C, F and I are the merged images of transporter and ER; panels L, O and R show the merged images of transporter and Golgi apparatus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



	Km (µM)	Vm (pmol *(µg*4min*µM)$^{-1}$)
OCTN2	24±3	15.6 ± 0.6
K302E	28±3	7.6 ± 0.3

Fig. 4. Michaelis–Menten plot showing kinetic analysis of carnitine transport mediated by hOCTN2 and the K302E-variant. Transport kinetic parameters were calculated using GraphPad Prism 5. Values are mean ± SE (n = 3).

altered affinity for substrate or to altered substrate turnover. To assess these mechanisms, we conducted kinetic analyses in cells that had been transiently transfected with the wild-type hOCTN2 and the K302E-hOCTN2 variant. As shown in Fig. 4, the K_m for L-carnitine transport by the variant was slightly, but not significantly, increased relative to wild-type (28 ± 3 μM vs. 24 ± 3 μM for wild-type hOCTN2). However, the V_{max} was markedly impaired to ~50% of control (K302E-hOCTN2: 7.6 ± 0.3 pmol *(μg*4 min*μM)$^{-1}$ compared with wild-type 15.6 ± 0.6 pmol *(μg*4 min*μM)$^{-1}$). This suggests that decreased transporter activity in cells that contained K302E-hOCTN2 was primarily due to decreased substrate turnover. The corresponding analysis with the D122Y-hOCTN2 variant was not undertaken because of low and variable rates of L-carnitine uptake at low substrate concentrations.

The inhibition of wild-type hOCTN2 and the K302E variant by a range of cationic substrates was assessed. As shown in Fig. 5, uptake of 1 μM [^{14}C]-L-carnitine by wild-type hOCTN2 was modulated by carnitine, TEA, cimetidine, pyrilamine and verapamil (1 mM) whereas 1 mM ergothioneine, guanidine, 1-methyl-4-phenylpyridinium (MMP+), spermine and p-aminohippurate did not affect L-carnitine uptake. Similar findings were seen with the K302E-hOCTN2 variant but the extent of inhibition of the variant transporter by cimetidine, pyrilamine and verapamil was increased.

3.4. Comparative modeling of the structure of hOCTN2

The sequence alignment of GlpT and hOCTN2 is shown in Fig. 6. Alignments of the predicted extracellular domain of hOCTN2 (residues 44–142) with its partial templates 2VQ3 (A191–L209), 2H0A (L266–I293) and 1NDH (P15–L52) are shown in Fig. 7A. Of 99 residues in the extracellular loop, 33 are aligned with residues from the other templates. Structural modeling was also undertaken to locate the amino acid residues that are subject to polymorphisms. The 3D model of hOCTN2 from which the extracellular domain is excluded shows the location of the residues V174, K191, A214 and K302 (Fig. 7B) and the extracellular domain model shows the location of the residues E109 and D122 (Fig. 7C). Together these models indicate that residues V174 and A214 are located within α-helixes that comprise TMDs, E109 and D122 are in the large extracellular loop and K191 and K302 are situated in intracellular loops. From Protein Structural Analysis the model has a *Z*-score of −1.72, which is consistent with a high quality model that is appropriate for the present analysis [21].

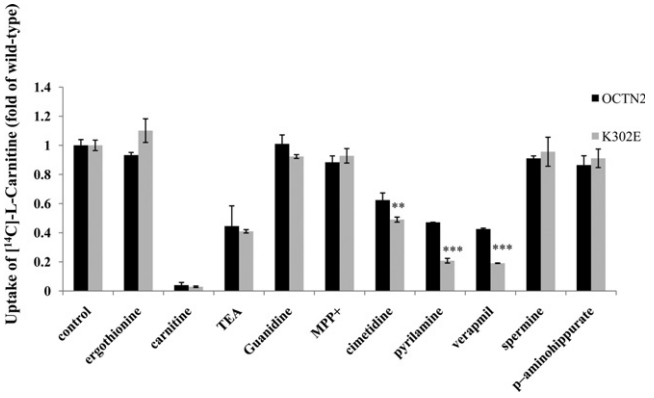


Fig. 5. Inhibition of [^{14}C]-L-carnitine transport. [^{14}C]-L-carnitine transport (1 μM) was measured in the absence (control) and presence of various unlabelled compounds (1 mM). The data are presented as a percentage of control uptake. MPP$^+$: 1-methyl-4-phenylpyridinium; TEA: tetraethylammonium. Values are mean ± SE ($n = 3$). Different from observed inhibition of the wild-type transporter, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation due to naturally occurring mutations in the *SLC22A5* gene that encode functionally defective hOCTN2 transporter variants. An increasing number of mutations in the *SLC22A5* gene have been reported but there is no clear relationship between genotype and clinical phenotype, especially among asymptomatic individuals [13,22]. The present study evaluated the functional significance of six hOCTN2 variants that were identified in the Chinese and Indian populations of Singapore [18]. Compared to the wild-type hOCTN2 L-carnitine uptake in transfected HEK-293 cells was significantly decreased with two of the six novel variants. Consistent with altered function, the expression of the D122Y- and K302E-hOCTN2 variant transporters at the cell membrane was significantly impaired even though total cellular expression was unchanged from wild-type. The present data also suggest that the variant transporter proteins were retained to a greater extent than the wild-type protein in the endoplasmic reticulum and Golgi apparatus.

The variant D122Y-hOCTN2 was detected in the Indian subset of the Singaporean population at a frequency of 0.01 [18]. More recently the D122Y-hOCTN2 variant was also detected in a 19 year old heterozygous female who presented clinically with cardiomyopathy and myopathy but no information on serum carnitine concentrations was available [13]. In the D122Y-hOCTN2 variant an evolutionarily conserved aspartic acid residue in the large extracellular loop is replaced by the uncharged, aromatic amino acid tyrosine. The Grantham value of 160 indicates profound chemical dissimilarity between these alternate amino acids [23].The large extracellular loop in SLC transporters is important for correct trafficking to the plasma membrane. In hOCTN2 this loop is 99 residues in length and contains four arginine residues that are putative *N*-glycosylation sites (N57, N64, N91 and N133). With the exception of N133, mutagenesis of these residues abolished carnitine transport, caused cytoplasmic transporter retention and prevented correct maturation of the transporter to the cell surface [24]. Few of the naturally occurring mutations in hOCTN2 in patients with primary carnitine deficiency encode amino acid substitutions in the extracellular loop. However, the OCTN2VT splice variant of OCTN2 encodes a 24-amino acid insertion between Glu-131 and Trp-132 [24]. This variant was non-functional and was retained within the cytoplasm. In the present study the D122Y-hOCTN2 variant that had minimal function was also retained intracellularly. In contrast, alternate variant E109K that was also identified in the Indian population of Singapore retained full function. These findings indicate that not all variants in the large extracellular loop alter membrane trafficking and transporter function.

Transport function was also decreased in the K302E-hOCTN2 variant. Replacement of the basic lysine residue by the acidic glutamate corresponds to a Grantham value of 56, which is consistent with an amino acid replacement of intermediate chemical difference [23]. Wang et al. identified the A301D-hOCTN2 variant in a homozygous patient with acute metabolic decompensation. *In vitro* analysis in transfected CHO cells found that this variant was essentially devoid of substrate transport activity [25]. In the present study biotinylation and immunofluorescence revealed that maturation of the K302E-hOCTN2 variant to the plasma membrane was impaired. In clear contrast, however, despite being adjacent to the K302 residue, the A301D-hOCTN2 variant matured normally to the plasma membrane even though the cytoplasmic distribution appeared to be more diffuse than with the wild-type transporter [6]. Both A301 and K302 are located in the largest of the intracellular loops of hOCTN2, which strongly suggests that specific amino acid residues within this domain may

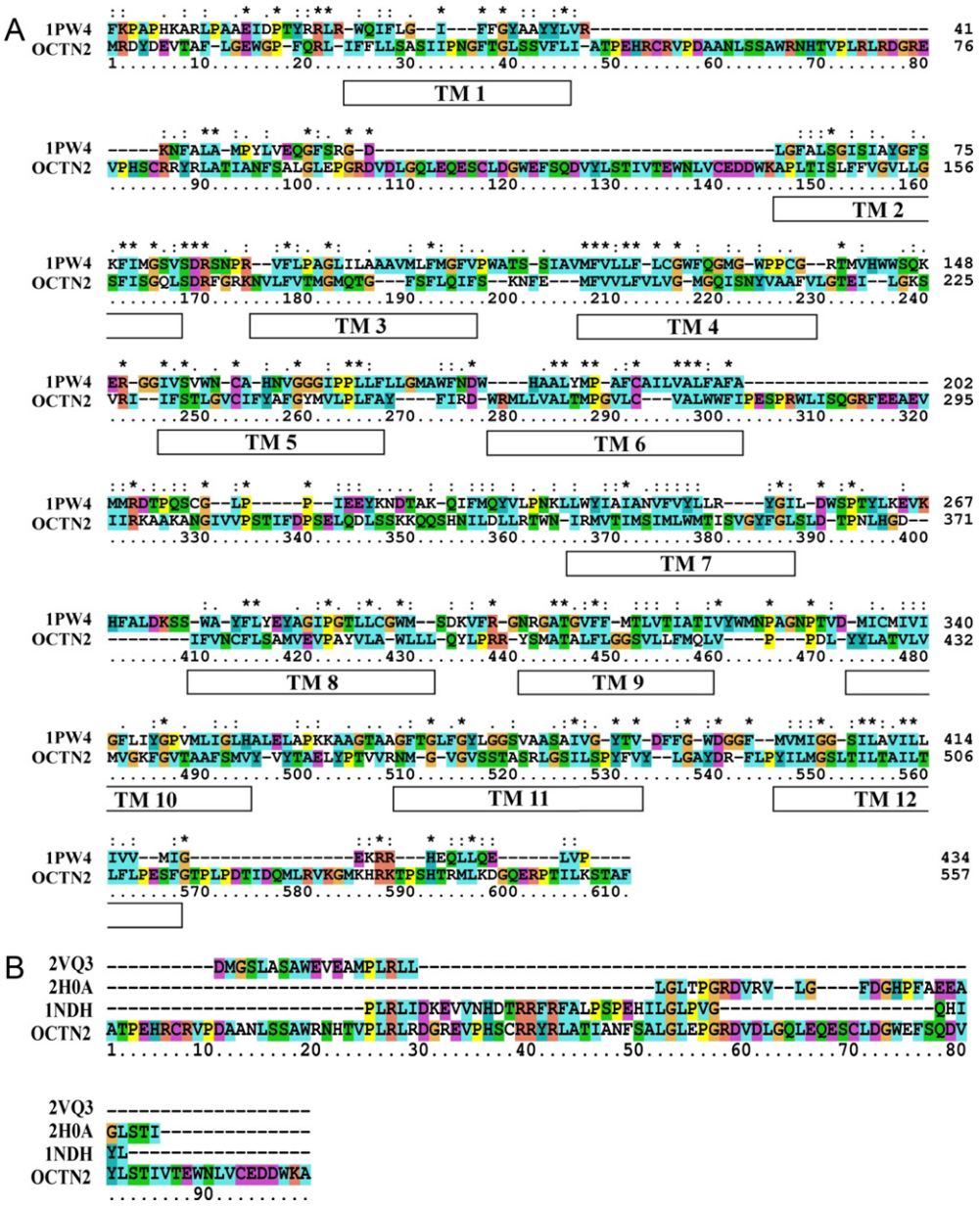


Fig. 6. Sequence alignments of hOCTN2. (A) Sequence alignment of hOCTN2 and *Escherichia coli* glycerol 3-phosphate transporter GlpT (PDB: 1PW4). The alignment was performed in a piecewise manner. Predicted transmembrane regions, intracellular regions and extracellular regions from OCTN2 and GlpT were matched and aligned separately, after which these fragments were assembled to produce the final alignment. (B) Sequence alignment between OCTN2 putative first extracellular domain (A44–A142) and structural templates PDB: 2VQ3 (A191–L209), PDB: 2H0A (L266–I293), PDB: 1NDH (P15–L52).

be particularly important in optimal plasma membrane maturation and targeting.

Replacement of the basic lysine at 302 with the negatively charged glutamate would be expected to favor increased inhibition by cationic drugs. This is supported by the observations that pyrilamine and verapamil and, to a lesser extent cimetidine, were somewhat more effective inhibitors of L-carnitine transport by K302E-hOCTN2 than the wild-type transporter. We have reported similar differences in the action of inhibitors with naturally occurring variants of the human organic anion transporter 4 [19]. Thus, pharmacokinetic interactions elicited by some cationic drugs may be more pronounced in individuals who carry certain nonsynonymous polymorphisms in SLC transporters that alter critical amino acid residues.

Carnitine and TEA transport by hOCTN2 are functionally quite different [16,17,26]. Carnitine transport is sodium dependent, while TEA is transported in a sodium-independent manner [7,8,17]. Thus, hOCTN2 transporter is a Na^+-dependent, high affinity symporter for L-carnitine but is a polyspecific and Na^+-independent cation antiporter [7–11]. Such substrate-dependent differences in transport mechanisms are consistent with differences in the specificity of binding or recognition by hOCTN2 for substrates and co-transported ions. Previous studies have suggested that different amino acid residues are important in TEA transport (residues 1–122 and 240–449) and carnitine transport (residues 123–239 and 450 to the carboxy terminus) [16]. The amino acid substitution in the D122Y-hOCTN2 variant is adjacent to the residues that influence the transport of both substrates, which likely accounts for the present observations. The observation that the decline in carnitine transport is more pronounced than the decrease in TEA transport in the case of the K302E-hOCTN2 was somewhat unexpected. However, it is possible that

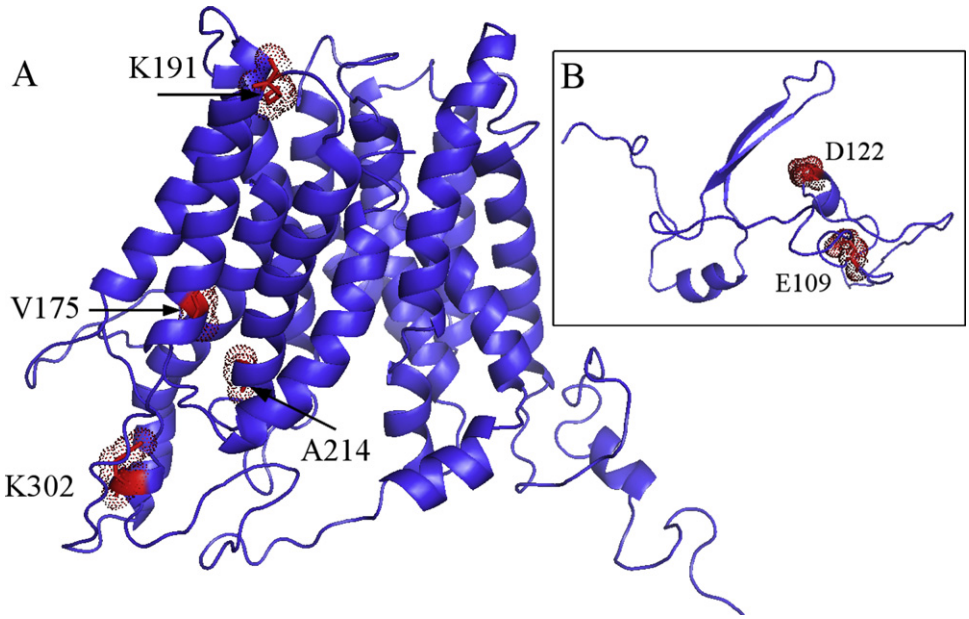


Fig. 7. Homology three-dimensional models of hOCTN2. The position of amino acid substitutions evaluated in this study (E109, D122, V175, K191, A214, K302) are shown in dotted regions. (A) Homology model of OCTN2 using GlpT (PDB: 1PW4) as structural template. The putative first extracellular domain (A44–A142) is not shown. (B) Homology model of the putative OCTN2 extracellular domain (A44–A142) using multiple structural templates (PDB: 1NDH, 2H0A, 2VQ3).

this may be due to the observed impairment of protein membrane targeting in conjunction with effects on substrate transport.

The remaining four variants that were detected in Singaporean populations did not affect hOCTN2 function significantly. These were E109K, located in the large extracellular loop, V175M in TMD3, K191N in a smaller extracellular loop and A214V in the intracellular loop located between TMD4 and TMD5. The A214V variant has also been detected more recently in three individuals who exhibited abnormal serum carnitine concentrations [13]. Each of these subjects was heterozygous for the A214V variant, so it is possible that they carried additional SLC22A5 mutations that may have impacted on L-carnitine uptake into cells.

In summary, the impact of 6 novel nonsynonymous SNPs in the *SLC22A5* gene on hOCTN2 function has been characterized. The present findings may assist in the development of personalised therapy with hOCTN2 substrates, including early supplementation with L-carnitine. They could also assist in the prediction of adverse effects due to co-administered agents that are also transported by hOCTN2 in patients carrying certain naturally occurring mutations.

Funding

This work was supported in part by The Pharmacy Research Trust of NSW.

Acknowledgements

The authors are grateful to Dr. Cynthia Sung for her assistance with the statistical aspects of this study.

References

[1] Pao SS, Paulsen IT, Saier Jr MH. Major facilitator superfamily. Microbiol Mol Biol Rev 1998;62(1):1–34.
[2] Wu X, Prasad PD, Leibach FH, Ganapathy V. cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. Biochem Biophys Res Commun 1998;246(3):589–95.
[3] Shoji Y, Koizumi A, Kayo T, Ohata T, Takahashi T, Harada K, et al. Evidence for linkage of human primary systemic carnitine deficiency with D5S436: a novel gene locus on chromosome 5q. Am J Hum Genet 1998;63(1):101–8.
[4] Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res 2007;24(7):1227–51.
[5] Longo N, Amat di San Filippo C, Pasquali M. Disorders of carnitine transport and the carnitine cycle. Am J Med Genet C Semin Med Genet 2006;142C(2):77–85.
[6] Amat di San Filippo C, Pasquali M, Longo N. Pharmacological rescue of carnitine transport in primary carnitine deficiency. Hum Mutat 2006;27(6):513–23.
[7] Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A, Shimane M, et al. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. J Biol Chem 1998;273(32):20378–82.
[8] Wu X, Huang W, Prasad PD, Seth P, Rajan DP, Leibach FH, et al. Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. J Pharmacol Exp Ther 1999;290(3):1482–92.
[9] Ohashi R, Tamai I, Nezu Ji J, Nikaido H, Hashimoto N, Oku A, et al. Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2. Mol Pharmacol 2001;59(2):358–66.
[10] Lahjouji K, Mitchell GA, Qureshi IA. Carnitine transport by organic cation transporters and systemic carnitine deficiency. Mol Genet Metab 2001;73(4):287–97.
[11] Ohashi R, Tamai I, Yabuuchi H, Nezu JI, Oku A, Sai Y, et al. Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. J Pharmacol Exp Ther 1999;291(2):778–84.
[12] Meyer-Wentrup F, Karbach U, Gorboulev V, Arndt P, Koepsell H. Membrane localization of the electrogenic cation transporter rOCT1 in rat liver. Biochem Biophys Res Commun 1998;248(3):673–8.
[13] Li FY, El-Hattab AW, Bawle EV, Boles RG, Schmitt ES, Scaglia F, et al. Molecular spectrum of SLC22A5 (OCTN2) gene mutations detected in 143 subjects evaluated for systemic carnitine deficiency. Hum Mutat 2010;31(8):E1632–51.
[14] Urban TJ, Gallagher RC, Brown C, Castro RA, Lagpacan LL, Brett CM, et al. Functional genetic diversity in the high-affinity carnitine transporter OCTN2 (SLC22A5). Mol Pharmacol 2006;70(5):1602–11.
[15] Tahara H, Yee SW, Urban TJ, Hesselson S, Castro RA, Kawamoto M, et al. Functional genetic variation in the basal promoter of the organic cation/carnitine transporters OCTN1 (SLC22A4) and OCTN2 (SLC22A5). J Pharmacol Exp Ther 2009;329(1):262–71.
[16] Seth P, Wu X, Huang W, Leibach FH, Ganapathy V. Mutations in novel organic cation transporter (OCTN2), an organic cation/carnitine transporter, with differential effects on the organic cation transport function and the carnitine transport function. J Biol Chem 1999;274(47):33388–92.
[17] Ohashi R, Tamai I, Inano A, Katsura M, Sai Y, Nezu J, et al. Studies on functional sites of organic cation/carnitine transporter OCTN2 (SLC22A5) using a Ser467Cys mutant protein. J Pharmacol Exp Ther 2002;302(3):1286–94.
[18] Toh DS, Yee JY, Koo SH, Murray M, Lee EJ. Genetic variations of the SLC22A5 gene in the Chinese and Indian populations of Singapore. Drug Metab Pharmacokinet 2010;25(1):112–9.
[19] Zhou F, Zhu L, Cui PH, Church WB, Murray M. Functional characterization of nonsynonymous single nucleotide polymorphisms in the human organic anion transporter 4 (hOAT4). Br J Pharmacol 2010;159(2):419–27.

[20] Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 1998;6:175–82.
[21] Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 2007;35(Web Server issue):W407–10.
[22] Wang Y, Taroni F, Garavaglia B, Longo N. Functional analysis of mutations in the OCTN2 transporter causing primary carnitine deficiency: lack of genotype–phenotype correlation. Hum Mutat 2000;16(5):401–7.
[23] Grantham R. Amino acid difference formula to help explain protein evolution. Science 1974;185(4154):862–4.
[24] Maekawa S, Mori D, Nishiya T, Takikawa O, Horinouchi T, Nishimoto A, et al. OCTN2VT, a splice variant of OCTN2, does not transport carnitine because of the retention in the endoplasmic reticulum caused by insertion of 24 amino acids in the first extracellular loop of OCTN2. Biochim Biophys Acta 2007;1773(6):1000–6.
[25] Filippo CA, Ardon O, Longo N. Glycosylation of the OCTN2 carnitine transporter: study of natural mutations identified in patients with primary carnitine deficiency. Biochim Biophys Acta 2011;1812(3):312–20.
[26] Koizumi A, Nozaki J, Ohura T, Kayo T, Wada Y, Nezu J, et al. Genetic epidemiology of the carnitine transporter OCTN2 gene in a Japanese population and phenotypic characterization in Japanese pedigrees with primary systemic carnitine deficiency. Hum Mol Genet 1999;8(12):2247–54.