This article has not been copyedited and formatted. The final version may differ from this version.
DMD Fast Forward. Published on February 27, 2009 as doi:10.1124/dmd.108.023762

DMD #23762

Functional characterization of the human organic cation transporter 2 variant p.270Ala>Ser

Oliver Zolk, Thomas F. Solbach, Jörg König, Martin F. Fromm

Institute of Experimental and Clinical Pharmacology and Toxicology

University of Erlangen-Nuremberg

Fahrstr. 17, 91054 Erlangen, Germany (OZ, TFS, JK, MFF)

Running title: Functional characterization of OCT2 p.270Ala>Ser

Correspondence to:

Oliver Zolk, MD

Institute of Experimental and Clinical Pharmacology and Toxicology

University of Erlangen-Nuremberg

Fahrstr. 17

91054 Erlangen, Germany

Phone: +49-9131-8522783

Fax: +49-9131-8522773

Zolk@pharmakologie.med.uni-erlangen.de

Number of text pages: 22

Number of tables: 2

Number of figures: 5

Number of references: 25

Number of words in the Abstract: 242

Number of words in the Introduction: 401

Number of words in the Discussion: 989

Nonstandard abbreviations:

OCT2, organic cation transporter 2; HEK, human embryonic kidney. MPP^+, 1-methyl-4-phenylpyridinium.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The organic cation transporter 2 (OCT2, *SLC22A2*) plays an important role for renal drug elimination. Recent clinical studies indicate an impact of the frequent non-synonymous c.808G>T (p.270Ala>Ser) polymorphism on renal clearance of metformin and the extent of the metformin-cimetidine interaction. The role of this polymorphism for renal disposition of endogenous compounds and drugs other than metformin has not been investigated. In addition, it is unclear whether the observed genotype-dependence of an OCT2-mediated drug-drug interaction might occur also with other OCT inhibitors. To address these issues we generated HEK cells stably expressing wildtype OCT2 or the p.270Ala>Ser variant. No differences in protein expression levels and membrane incorporation pattern were observed between the two cell lines. The p.270Ala>Ser variant significantly impaired uptake kinetics of MPP^+, dopamine, norepinephrine, and propranolol. V_{max} values were significantly reduced for uptake of all four compounds mediated by the p.270Ala>Ser variant compared to wildtype OCT2. In addition, a significant difference in the affinity to wildtype and mutant OCT2 was observed for dopamine (K_m dopamine: 932±77 vs 1285±132 µM). Moreover, out of a set of 27 compounds p.270Ala>Ser OCT2 was significantly less sensitive to inhibition by cimetidine, flurazepam, metformin, mexiletine, propranolol, and verapamil than wildtype OCT2 (e.g. for propranolol: IC_{50} wildtype vs p.270Ala>Ser 189 vs. 895 µM, P<0.001). Our results indicate that the common OCT2 c.808G>T SNP significantly alters uptake of endogenous compounds and drugs. Moreover, for selected compounds the extent of OCT2-mediated drug interactions could depend on OCT2 c.808G>T genotype.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Renal secretion is an important pathway for the elimination of many clinically used drugs. Transporters in the renal tubule epithelium mediate drug secretion into urine and thus play a critical role in detoxification. Approximately 40% of all drugs on the market are organic cations (Kim and Shim, 2006). Various clinically important cationic therapeutics (e.g., metformin), endogenous compounds (e.g., dopamine), and toxic substances (e.g., 1-methyl-4-phenylpyridinium [MPP^+]) enter the tubule epithelial cell via organic cation transporters (OCTs) (Schömig et al., 2006; Wright, 2005; Koepsell et al., 2007). OCTs belong to the solute carrier *SLC22A* gene family. Three human OCTs have been cloned: OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), and OCT3 (*SLC22A3*). In humans, in particular OCT2 is important for the renal elimination of organic cations (Motohashi et al., 2002; Shikata et al., 2007; Song et al., 2008a; Wang et al., 2008). *In vitro* and *in vivo* data indicate that inhibition of OCT function by concomitantly administered drugs is a newly recognized mechanism of drug-drug interactions (Somogyi et al., 1987; Bachmakov et al., 2008).

Several variants in the *SLC22A2* gene have been identified (Leabman et al., 2002; Fukushima-Uesaka et al., 2004; Takane et al., 2008; Wang et al., 2008; Ogasawara et al., 2008). Among all OCT2 protein sequence variants identified so far, the p.270Ala>Ser (c.808G>T) OCT2 variant is the most common one. The allele frequencies of the c.808G>T SNP in different populations are 7-16% (Leabman et al., 2002). Some (Kang et al., 2007; Song et al., 2008a; Song et al., 2008b), but not all *in vitro* studies (Leabman et al., 2002) indicate altered uptake activity of the p.270Ala>Ser OCT2 variant compared to wildtype OCT2. Recent studies in healthy volunteers reported a significantly reduced renal clearance of metformin in subjects with the 808TT genotype compared to the GT and GG groups (Wang et al., 2008; Song et al., 2008a). Moreover, the extent of inhibition of metformin tubular secretion by cimetidine was significantly lower in healthy volunteers with the 808TT compared to the GT and GG groups (Wang et al., 2008).

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

The present *in vitro* study was designed to extend the previous *in vitro* and *in vivo* findings by addressing the following two issues: (1) Investigation of OCT2-mediated transport of additional endogenous compounds (dopamine, norepinephrine) and drugs (metformin, propranolol) by p.270Ala>Ser OCT2 variant compared to wildtype OCT2. (2) Characterization of the impact of the p.270Ala>Ser OCT2 variant on the inhibition of uptake by a set of 27 drugs.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Drugs. Carvedilol was purchased from Chemos (Regenstauf, Germany), desloratadine was from Toronto Research Chemicals (North York, ON, Canada), flurazepam was from Lipomed (Bad Säckingen, Germany), sibutramine, and tamoxifen were from BioTrend (Cologne, Germany). All other drugs were purchased from Sigma (Taufkirchen, Germany).

Cell culture and transfection. HEK293 cells were stably transfected with human *SLC22A2* cDNA cloned from human kidney using the mammalian expression vector pcDNA3.1/Hygro(-) (Invitrogen, Carlsbad, CA). The c.808G>T mutation was introduced into the vector pcDNA3.1/Hygro(-)-OCT2 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's protocol. Full length sequencing confirmed identity with the reference sequence and presence of the c.808G>T mutation after mutagenesis. Transfectants expressing recombinant wildtype or p.270Ala>Ser OCT2 were selected with hygromycin and the transfectants were screened for OCT2 expression by real-time RT-PCR (forward primer 5'-CAA TGG CCT ATG AGA TAG TCT-3', reverse primer 5'-GCA GCA ACG GTC TCT CTT CTT-3') and immunoblot analyses (see below). The clones with the highest wildtype and p.270Ala>Ser OCT2 expression, respectively, were chosen for further studies. Cells transfected with the pcDNA3.1/Hygro(-) vector only were used as controls.

Transport assay in HEK-OCT2 cells. Transport assays were performed as described (Solbach et al., 2008; Zolk et al., 2008). Before uptake experiments were started, the cells were washed with prewarmed (37°C) uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). [^{3}H]MPP^+ (85 Ci/mmol, American Radiolabeled Chemicals, St Louis, MO) was dissolved in uptake buffer, and unlabeled MPP^+ was added to the required final concentration. For additional experiments [^{14}C]metformin (0.1 Ci/mmol, kindly provided by Merck KGaA Darmstadt, Germany), [^{3}H]dopamine (28 Ci/mmol, American Radiolabeled Chemicals),

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

[^{3}H]norepinephrine (49.5 Ci/mmol, American Radiolabeled Chemicals), and [^{3}H]propranolol (33 Ci/mmol, Amersham, UK) were used as substrates. To characterize clinically used drugs as inhibitors, 27 compounds were added in increasing concentrations (up to 1,000 µM). Data on inhibition of wildtype OCT2-mediated MPP^+ transport have been in part previously published {Zolk, 2008 1399 /id}. These potential inhibitors were selected based on previous publications suggesting them as substrates/inhibitors of OCT2, and/or on the presence of physicochemical characteristics generally associated with OCT2 substrates/inhibitors, such as net positive charge at the amine nitrogen at physiological pH and molecular weight <500 mol. Of note, these compounds were selected to cover a broad range of structural diversity.
The cells were incubated with the test solution at 37°C. Uptake was stopped after 2 min by washing the cells three times with ice-cold uptake buffer. Cells were then lysed with 5 mM Tris buffer (pH 7.4) containing 0.1% Triton X-100. The intracellular accumulation of radioactivity was determined by liquid scintillation counting (PerkinElmer, Rodgau-Jügesheim, Germany) and protein concentrations of the lysates were measured with a bicinchonic acid assay (BCA Protein Assay Kit, Peribo Science, Bonn, Germany). The OCT2-mediated net uptake was obtained by subtracting the uptake in vector-transfected cells from that in OCT2 (reference or mutant) expressing cells.

Michaelis-Menten type nonlinear curve-fitting was carried out to obtain estimates of the maximal uptake rate, V_{max}, and the concentration at which half the maximal uptake occurs, K_m (GraphPad Software, CA, USA). The IC_{50} was estimated by a sigmoidal inhibition model with GraphPad Prism (GraphPad Software, CA, USA) and was fit to the equation $V = V_0/(1 + (I/IC_{50})n)$ by nonlinear regression. V is the uptake of MPP^+ in the presence of the inhibitor, V_0 is the uptake of MPP^+ in the absence of inhibitor, I is the inhibitor concentration and n is the slope. Data from nonlinear regression are presented as mean±SEM values. The uptake experiments were repeated at least six times, inhibition experiments at least three times.

To determine the type of inhibitory interaction of metformin with OCT2-mediated MPP^+ uptake, the uptake of [^{3}H]MPP^+ (10, 25, 50 µM) was determined in the presence of

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

increasing concentrations of metformin (39-5000 μM). Data were analyzed by the method of Dixon, in which the reciprocal velocity, 1/V, is plotted against the inhibitor concentration.

Western blot analysis. Human placenta and HEK293 cells (vector control cells or HEK-OCT2 cells) were lysed in 50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mol/l EDTA and Complete Protease Inhibitors (Roche Applied Science, Mannheim, Germany). Aliquots of denatured protein were subjected to 10% SDS-PAGE, and separated proteins were electrophoretically transferred onto nitrocellulose membranes. Blots were probed with a polyclonal OCT2 antibody, which was generated by immunizing rabbits with the peptides RQCRRYEVDWNQSTFD (amino acids 87-102 of human OCT2) and SLDTNRSRLPLGPCRD (amino acids 109-124) coupled to keyhole limpet hemocyanin. As a control for loading, blots were also probed with a GAPDH antibody (1:2,000, HyTest, Turku, Finland).

To compare levels of plasma membrane expression of wildtype and p.270Ala>Ser OCT2 protein, cell surface proteins were first labeled with sulfo-NHS-SS-biotin, a membrane-impermeable biotinylation reagent (Cell Surface Protein Isolation Kit, Pierce, Rockford, IL). The cells were subsequently lysed and then labeled proteins were isolated with immobilized NeutrAvidin Gel according to the manufacturer's instruction. The bound proteins were released by incubating the resin with SDS-PAGE sample buffer containing 50 mM DTT and expression of OCT2 was analyzed by Western blotting as described above.

Immunofluorescence. Cellular localization of OCT2 was analyzed by immunofluorescent staining and confocal microscopy with a Zeiss LSM 5 Pascal system using a Zeiss Axiovert microscope. Incubation with the OCT2 antibody (1:500) was followed by incubation with an Alexa Fluor 488 conjugated secondary antibody (1:10,000, Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with Hoechst 33342.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Homology modeling. Sequence-based structural relatives to human OCT2 were searched with the Protein Homology/Analogy Recognition Engine *Phyre*, version 0.2 (Bennett-Lovsey et al., 2008). The glycerol-3-phosphate transporter (GlpT) of *Escherichia coli* (Protein Data Bank code 1PW4) was returned as the highest score hit, followed by the multidrug transporter EmrD from *Escherichia coli* (code 2GFP), and the lactose permease from *Escherichia coli* (code 1PV7). Subsequent homology modeling of human OCT2 was based upon the crystal structure of GlpT. The human SLC22A2 gene was compared with the ortholog genes from *Mus musculus*, *Rattus norvegicus*, *Sus scrofa*, *Pan troglodytes*, *Oryctolagus cuniculus*, *Gallus gallus*, and *Canis lupus familiaris* to identify conserved regions. This multiple alignment was done with *ClustalW2*. The information was then used to optimize the binary alignment between human SLC22A2 and GlpT sequences with the *LALIGN* program. Finally, the 3-dimensional model was constructed with *SwissModel* and visualized with *DeepView*, version 3.7.

Statistical analysis. Differences between two groups were tested by the Student *t* test for unpaired data. Data are expressed as mean±SEM values. A value of $p<0.05$ was considered statistically significant.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Expression and subcellular localization of the p.270Ala>Ser variant. Stable transfection of the HEK293 cell line with *SLC22A2* cDNAs encoding either the wildtype human OCT2 or the p.270Ala>Ser mutant OCT2 yielded two clones with substantial OCT2 expression at comparable levels, as verified by real-time RT-PCR (expression of c.808G>T OCT2 mRNA normalized to β-actin, relative to wildtype OCT2 expression: 1.0±0.17, n.s.) and Western blotting of whole cell lysates (Fig. 1a) and cell surface protein extracts (Fig. 1b). Both, the wildtype and the mutant OCT2 protein were integrated into the plasma membrane of HEK-OCT2 cells (Fig. 1c) and were functionally active, as revealed by transport assays (Fig. 2). For example, at 10 µM MPP^+ accumulation was 36-fold higher in HEK cells expressing wildtype OCT2 compared to vector control cells.

Functional effects of the p.270Ala>Ser variant. Figure 2 shows the effects of increasing concentrations of [^{3}H]MPP^+ on the rate of OCT2-mediated MPP^+ uptake. The apparent K_m was 19.5±2.8 µM, and V_{max} was 579±17 pmol/mg/min. The p.270Ala>Ser variant showed a significantly reduced transport of MPP^+ (Figure 2, Table 1; V_{max} 414±18 pmol/mg/min, $p<0.0001$) compared to wildtype OCT2 despite unchanged K_m (20.9 µM). In addition, we also tested the impact of the mutant OCT2 protein on dopamine, norepinephrine, metformin, and propranolol uptake. V_{max} values for p.270Ala>Ser were significantly lower for dopamine, norepinephrine, and propranolol than those of the OCT2 reference. In particular, transport of propranolol was markedly impaired with 56% ($p<0.01$) reduction in V_{max}. Moreover, substrate-specific changes in the affinities to mutant OCT2 were observed for dopamine with an increase in K_m compared with the reference OCT2 (wildtype OCT2 vs mutant OCT2: 932±77 vs 1285±132 µM, $p<0.05$).

To clarify the type of interaction of metformin with OCT2-mediated transport of MPP^+, Dixon plot analyses were performed (Fig. 3). The lines drawn for each concentration of substrate intersect at a single point above the x axis, indicating competitive inhibition. K_i

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

values determined from the Dixon plots were 286 µM and 590 µM for wildtype and p.270Ala>Ser OCT2, respectively.

To determine whether the p.270Ala>Ser mutation altered interaction of OCT2 with other molecules, we carried out inhibition studies. We investigated the inhibition of [^{3}H]MPP$^+$ (10 µM) uptake by a set of 27 structurally unrelated (Tanimoto pairwise similarity index 0.25) cationic drugs. As summarized in Table 2 and Figure 4a, IC_{50} values for inhibition of MPP$^+$ uptake mediated by mutant OCT2 were significantly increased compared to wildtype OCT2 for cimetidine (422 vs. 126 µM, $p<0.01$), flurazepam (110 vs. 53 µM, $p<0.05$), metformin 936 vs. 397 µM, $p<0.001$), mexiletine (78 vs. 42 µM, $p<0.05$), propranolol (895 vs. 189 µM, $p<0.001$), and verapamil (120 vs. 70 µM, $p<0.01$), while the inhibition kinetics of all other compounds showed no significant differences.

Based on the observation that the extent of inhibition of metformin tubular secretion by cimetidine was significantly lower in subjects with the 808TT compared to the GT and GG genotype (Wang et al., 2008), we also addressed the question whether the p.270Ala>Ser variant was less sensitive to cimetidine inhibition than the wildtype OCT2 transporter. As shown in Fig. 4b, the IC_{50} value for inhibition of metformin uptake by mutant OCT2 was significantly higher compared to wildtype OCT2 (45 vs. 11 µM, $p<0.01$).

Location of the p.270Ala>Ser SNP and sequence alignment. The predicted location of the p270Ala>Ser variant in the secondary structure of OCT2 is shown in Fig. 5a/b. It occurs in the transmembrane domain 6 facing the extracellular membrane surface. Fig. 5c shows the amino acid sequence of human OCT2 at position 270 with flanking amino acids, which was aligned with the orthologs from chimpanzee, Bornean orangutan, mouse, rat, swine, dog, rabbit, cattle, and chicken. The small polar amino acid Thr is conserved among all species except for some species within the family of man-like primates, such as human and chimpanzee, where the Thr is replaced by the small nonpolar amino acid Ala at position 270.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The major findings of the present investigation were that the OCT2 c.808G>T (p.270Ala>Ser) SNP does not affect expression and membrane localization, but causes compound-specific alterations of transport function compared with wildtype OCT2. While the p.270Ala>Ser variant had only minor or no effects on the substrate affinity, V_{max} was decreased for most compounds investigated. However, the degree of reduction varied substantially (10%-56% reduction) between these compounds. Moreover, p.270Ala>Ser OCT2 was less sensitive to inhibition by cimetidine, flurazepam, metformin, mexiletine, propranolol, and verapamil than wildtype OCT2, whereas the remaining drugs tested in this study showed non-significantly different IC_{50} values for inhibition of MPP^+ uptake in cells expressing p.270Ala>Ser or wildtype OCT2. Altogether, the p.270Ala>Ser SNP markedly decreased maximum transport rate and impaired the inhibitory interaction with MPP^+ uptake by some but not all drugs. These findings highlight the need for testing several compounds when characterizing *in vitro* functional differences between a reference transporter and its protein sequence variant.

In the first *in vitro* study on the functional consequences of *SLC22A2/OCT2* polymorphisms, Leabman et al. (Leabman et al., 2002) reported non-significant differences for the affinity of the prototype substrate MPP^+ to wildtype and p.270Ala>Ser OCT2, which is in line with the present findings. In contrast, using the same substrate, Kang et al. (Kang et al., 2007) reported a higher K_m value for p.270Ala>Ser OCT2 compared to wildtype OCT2 (K_m 3.5 vs 7.0 µM), although they did not state whether this difference was statistically significant. In accordance with our results, Kang et al. observed lower V_{max} values for p.270Ala>Ser OCT2 compared to wildtype OCT2.

Several clinical observations suggest a functional role of the p.270Ala>Ser variant. For example, a lower prevalence of OCT2 p.270Ala>Ser was reported for patients with essential hypertension (Lazar et al., 2006). While the molecular mechanism of this clinical observation has not been elucidated so far, our *in vitro* data with dopamine and

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

norepinephrine indicate that the p.270Ala>Ser variant alters transport of endogenous compounds possibly resulting in alterations of e.g. blood pressure.

Drugs, such as metformin, are dependent on renal transporters for their ultimate urinary elimination. Interindividual variations in the renal secretion of metformin have been documented (Sirtori et al., 1978; Yin et al., 2006). Therefore, recent studies in healthy volunteers have investigated the effect of the c.808G>T genotype on renal elimination of metformin (Song et al., 2008a; Wang et al., 2008). Renal metformin clearance was significantly reduced resulting in higher peak plasma concentration (C_{max}) and area under the curve (AUC) in individuals with the c.808TT genotype (p.270Ser/Ser) compared to carriers of wildtype alleles. These studies highlight the potential importance of this polymorphism for drug elimination.

In an attempt to directly demonstrate the significance of the *SLC22A2* c.808G>T SNP for transport of metformin, *in vitro* studies were preformed. Using transiently transfected HEK cells, Song et al. (Song et al., 2008a) observed similar K_m values, but 41% lower V_{max} for metformin uptake mediated by p.270Ala>Ser OCT2 compared to wildtype OCT2. Similarly, we observed no significant differences for the affinity of metformin to wildtype and p.270Ala>Ser OCT2. However, using stably transfected HEK cells, we also did not find a significant difference in V_{max}. We cannot explain these discrepant findings definitely, however one reason might relate to the different models of transfection (transient vs. stable). It should be noted that in the present study particular attention was drawn to wildtype and variant OCT2 cell surface expression levels.

By focusing on drug-drug interactions, a recent clinical study (Wang et al., 2008) determined the effect of cimetidine coadministration (an OCT inhibitor) on genotype-dependent metformin renal clearance (CL_r). In subjects homozygous for the c.808G>T mutation only a small change of metformin CL_r (12.5%) was observed when cimetidine was coadministered compared with the much larger change in subjects with the GT or the GG genotype (26.0% and 42.4%). That finding clearly suggests that TT carriers (p.270Ser/Ser) are much less sensitive to the inhibition by cimetidine. This is in agreement with our finding

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

that metformin uptake was less sensitive to inhibition by cimetidine in cells expressing the p.270Ala>Ser variant compared to wildtype OCT2. Of note, our results indicate that some compounds may act as competitive inhibitors of OCT2, as exemplified for inhibition of OCT2-mediated MPP^+ uptake by metformin.

The homology model of the three-dimensional structure of OCT provides a means to interpret the effect of specific residues on transport activity (Zhang et al., 2005; Koepsell et al., 2007; Pelis et al., 2006). The template structure of GlpT was used to develop a structural model of human OCT2. Similar to the model constructed by Zhang et al. (Zhang et al., 2005) the amino acid Ala-270 was predicted to face the outer pore of the transporter. There are, however, distinct differences in the absolute position of the Ala-270 residue. In the present study the amino acid Ala-270 was predicted to lie within the transmembrane domain 6 directly facing the extracellular membrane surface whereas in the model developed by Zhang et al. Ala-270 was located in the short extracellular loop between transmembrane helix 5 and 6. Nevertheless, Ala-270 locates in a region of the OCT2 protein that is potentially important for substrate recognition. This raises the possibility that the p.270Ala>Ser SNP may impair substrate-transporter interactions. Interestingly, Ala-270 occurs only in some species from the Hominidae family, whereas in orthologs from other species Thr, a small polar amino acid like Ser, is conserved at this position. It is tempting to speculate that Thr in OCT2 orthologs affects OCT2 transport function similarly to the p.270Ala>Ser variant and contributes in part to the observed species differences for example between human and rabbit OCT2 (Suhre et al., 2005).

In summary, we demonstrate that the common SLC22A2 c.808G>T SNP (p.270Ala>Ser) significantly alters uptake of endogenous compounds and drugs. Genotype-dependent alterations in the affinity (K_m) appear to be substrate-dependent, whereas for most compounds investigated we found significantly reduced maximal transport rates (V_{max}). Moreover, for selected compounds the extent of OCT2-mediated drug interactions could depend on *SLC22A2* c.808G>T genotype.

References

Bachmakov I, Glaeser H, Fromm MF and König J (2008) Interaction of oral antidiabetic drugs with hepatic uptake transporters: focus on organic anion transporting polypeptides and organic cation transporter 1. *Diabetes* **57**:1463-1469.

Bennett-Lovsey RM, Herbert AD, Sternberg MJ and Kelley LA (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. *Proteins* **70**:611-625.

Fukushima-Uesaka H, Maekawa K, Ozawa S, Komamura K, Ueno K, Shibakawa M, Kamakura S, Kitakaze M, Tomoike H, Saito Y and Sawada J (2004) Fourteen novel single nucleotide polymorphisms in the SLC22A2 gene encoding human organic cation transporter (OCT2). *Drug Metab Pharmacokinet* **19**:239-244.

Kang HJ, Song IS, Shin HJ, Kim WY, Lee CH, Shim JC, Zhou HH, Lee SS and Shin JG (2007) Identification and functional characterization of genetic variants of human organic cation transporters in a Korean population. *Drug Metab Dispos* **35**:667-675.

Kim MK and Shim CK (2006) The transport of organic cations in the small intestine: current knowledge and emerging concepts. *Arch Pharm Res* **29**:605-616.

Koepsell H, Lips K and Volk C (2007) Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* **24**:1227-1251.

Lazar A, Zimmermann T, Koch W, Gründemann D, Schömig A, Kastrati A and Schömig E (2006) Lower prevalence of the OCT2 Ser270 allele in patients with essential hypertension. *Clin Exp Hypertens* **28**:645-653.

Leabman MK, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T, Clark AG, Herskowitz I and Giacomini KM (2002) Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered function. *Pharmacogenetics* **12**:395-405.

Motohashi H, Sakurai Y, Saito H, Masuda S, Urakami Y, Goto M, Fukatsu A, Ogawa O and Inui K (2002) Gene expression levels and immunolocalization of organic ion transporters in the human kidney. *J Am Soc Nephrol* **13**:866-874.

Ogasawara K, Terada T, Motohashi H, Asaka J, Aoki M, Katsura T, Kamba T, Ogawa O and Inui K (2008) Analysis of regulatory polymorphisms in organic ion transporter genes (SLC22A) in the kidney. *J Hum Genet* **53**:607-614.

Pelis RM, Zhang X, Dangprapai Y and Wright SH (2006) Cysteine accessibility in the hydrophilic cleft of human organic cation transporter 2. *J Biol Chem* **281**:35272-35280.

Schömig E, Lazar A and Gründemann D (2006) Extraneuronal monoamine transporter and organic cation transporters 1 and 2: a review of transport efficiency. *Handb Exp Pharmacol* **175**:151-180.

Shikata E, Yamamoto R, Takane H, Shigemasa C, Ikeda T, Otsubo K and Ieiri I (2007) Human organic cation transporter (OCT1 and OCT2) gene polymorphisms and therapeutic effects of metformin. *J Hum Genet* **52**:117-122.

Sirtori CR, Franceschini G, Galli-Kienle M, Cighetti G, Galli G, Bondioli A and Conti F (1978) Disposition of metformin (N,N-dimethylbiguanide) in man. *Clin Pharmacol Ther* **24**:683-693.

Solbach TF, Paulus B, Weyand M, Eschenhagen T, Zolk O and Fromm MF (2008) ATP-binding cassette transporters in human heart failure. *Naunyn Schmiedebergs Arch Pharmacol* **377**:231-243.

Somogyi A, Stockley C, Keal J, Rolan P and Bochner F (1987) Reduction of metformin renal tubular secretion by cimetidine in man. *Br J Clin Pharmacol* **23**:545-551.

Song I, Shin H, Shim E, Jung I, Kim W, Shon J and Shin J (2008a) Genetic Variants of the Organic Cation Transporter 2 Influence the Disposition of Metformin. *Clin Pharmacol Ther* **84**:559-562.

Song IS, Shin HJ and Shin JG (2008b) Genetic variants of organic cation transporter 2 (OCT2) significantly reduce metformin uptake in oocytes. *Xenobiotica* **38**:1252-1262.

Suhre WM, Ekins S, Chang C, Swaan PW and Wright SH (2005) Molecular determinants of substrate/inhibitor binding to the human and rabbit renal organic cation transporters hOCT2 and rbOCT2. *Mol Pharmacol* **67**:1067-1077.

Takane H, Shikata E, Otsubo K, Higuchi S and Ieiri I (2008) Polymorphism in human organic cation transporters and metformin action. *Pharmacogenomics* **9**:415-422.

Wang ZJ, Yin OQ, Tomlinson B and Chow MS (2008) OCT2 polymorphisms and in-vivo renal functional consequence: studies with metformin and cimetidine. *Pharmacogenet Genomics* **18**:637-645.

Wright SH (2005) Role of organic cation transporters in the renal handling of therapeutic agents and xenobiotics. *Toxicol Appl Pharmacol* **204**:309-319.

Yin OQ, Tomlinson B and Chow MS (2006) Variability in renal clearance of substrates for renal transporters in chinese subjects. *J Clin Pharmacol* **46**:157-163.

Zhang X, Shirahatti NV, Mahadevan D and Wright SH (2005) A conserved glutamate residue in transmembrane helix 10 influences substrate specificity of rabbit OCT2 (SLC22A2). *J Biol Chem* **280**:34813-34822.

Zolk O, Solbach TF, König J and Fromm MF (2009) Structural determinants of inhibitor interaction with the human organic cation transporter OCT2 (SLC22A2). *Naunyn Schmiedebergs Arch Pharmacol*. **379**:337-348.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Financial support

This work was supported by the Deutsche Forschungsgemeinschaft (Fr 1298/5-1).

Address reprint requests to:

Dr. Oliver Zolk

Institute of Experimental and Clinical Pharmacology and Toxicology

University of Erlangen-Nuremberg

Fahrstr. 17

91054 Erlangen, Germany

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Figure 1: Characterization of HEK293 cells overexpressing OCT2. (a) Western blot analysis of lysates from HEK293 cells stably transfected with the control vector, wildtype, or p.270Ala>Ser OCT2 cDNA. The bar graph shows the mean expression levels (± SEM) from n=4 experiments. (b) Representative OCT2 Western blot of cell surface proteins. Similar results were obtained in two independent experiments. (c) Immunofluorescence of wildtype or p.270Ala>Ser OCT2 in HEK-OCT2 cells. Nuclei were stained with Hoechst 33342. For control, cells transfected with the vector only are shown.

Figure 2: Kinetics of MPP^+, norepinephrine, dopamine, propranolol, or metformin transport by wildtype OCT2 (dots) and p.270Ala>Ser variant (squares). Data were fit to the Michaelis-Menten equation after subtracting background uptake in vector control cells. Data points represent the mean ± SEM obtained from 4-12 wells of 2-3 independent experiments.

Figure 3: Inhibition of OCT2-mediated uptake of $[^3H]MPP^+$ by metformin in cells stably expressing wildtype or p.270Ala>Ser OCT2. The reciprocal velocity is plotted against the inhibitor concentration. Data points represent the mean ± SEM of three determinations from one typical experiment.

Figure 4: Cimetidine, metformin, propranolol, and verapamil inhibition kinetics. HEK cells stably expressing wildtype (dots) and p.270Ala>Ser OCT2 (squares) were incubated with 10 µM $[^3H]MPP^+$ (a) or 50 µM $[^{14}C]$metformin (b) in the presence or absence of various concentrations of unlabeled drug. Results are expressed relative to the uptake in the absence of the drug. Data points represent the mean ± SEM of three determinations from one typical experiment.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5: Homology model of human OCT2 based upon the crystal structure of the glycerol 3-phosphate transporter of Escherichia coli, GlpT (Protein Data Bank code 1PW4). Marked are the transmembrane helix 6 (yellow) and Ala 270. (a) Side view of the model, with the cytoplasmic aspect of the protein directed toward the bottom. (b) View of the model from the extracellular aspect of the protein. (c) Amino acid sequence alignment. Shown is the region flanking amino acid 270 of human *SLC22A2* and the aligned sequences of known orthologs from other species.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Uptake of different OCT2 substrates in HEK cells stably expressing wildtype OCT2 or the OCT2 p.270Ala>Ser mutant protein. Each value represents the mean mean ± SEM of n determinations from 2-3 independent experiments.

	K_m (µM)		V_{max} (pmol/mg/min)	
	OCT2 wildtype	OCT2 p.270Ala>Ser	OCT2 wildtype	OCT2 p.270Ala>Ser
MPP^+ (n=6)	19.5 ± 2.8	20.9 ± 4.3	579 ± 17	414 ± 18***
Dopamine (n=8)	932 ± 77	1285 ± 132*	7559 ± 173	6820 ± 203*
Norepinephrine (n=12)	5452 ± 498	5282 ± 907	2135 ± 90	1671 ± 132*
Metformin (n=7)	3171 ± 401	3179 ± 390	10730 ± 582	10670 ± 561
Propranolol (n=6)	11.8 ± 3.7	5.8 ± 3.6	201 ± 24	89 ± 17**

***p<0.0001; **p<0.01; *p<0.05 vs OCT wildtype.

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.023762
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2: IC_{50} values for inhibition of MPP^+ (10 µM) uptake mediated by OCT2 p.270Ala>Ser mutant protein compared to wildtype OCT2. Each value represents the mean and the 95% confidence interval of 6-9 determinations from 2-3 independent experiments.

Drug	OCT wildtype		OCT2 p.270Ala>Ser		
	IC_{50} [µM]	95% CI	IC_{50} [µM]	95% CI	*p*
Amitriptyline	14	11 – 19	13	9 – 18	
Carvedilol	63	27 – 149	33	16 – 67	
Chloroquine	1087	604 – 1955	926	552 – 1553	
Chlorpromazine	14	11 – 18	19	13 – 26	
Cimetidine	126	91-176	422	316-564	0.0046
Clonidine	16	12 – 22	20	15 – 29	
Desloratadine	60	48 – 76	62	47 – 81	
Diphenhydramine	21	14 – 31	21	13 – 34	
Disopyramide	325	209 – 504	132	122 – 440	
Dopamine	3485	1683 – 7216	3325	2038 – 5425	
Doxepin	13	6 – 27	16	9 – 29	
Fenfluramine	10	5 – 19	16	7 – 33	
Flecainide	191	77 – 470	264	141 – 494	
Flurazepam	53	36 – 78	110	79 – 154	0.0167
Imipramine	6	4 – 8	9	6 – 13	
Ipratropium bromide	15	9 – 25	16	10 – 28	
Levomethadone	60	33 – 108	94	53 – 167	
Mefloquine	204	136 – 306	188	93 – 382	
Metformin	397	300 – 526	936	753 – 1163	0.0006
Mexiletine	42	32 –54	78	55 – 114	0.0198
Propafenone	25	16 – 39	17	11 – 27	
Propranolol	189	117 – 304	895	543 – 1473	0.0008
Quinidine	87	46 – 164	51	32 – 81	
Sibutramine	29	16 – 52	16	9 – 29	
Tamoxifen	87	37 – 205	92	26 – 331	
Trimethoprim	1327	725 – 2430	1001	698 – 1436	
Verapamil	70	60 – 82	120	87 – 165	0.0098

Fig. 1

DMD Fast Forward. Published on February 27, 2009 as DOI: 10.1124/dmd.108.0237
This article has not been copyedited and formatted. The final version may differ from this v

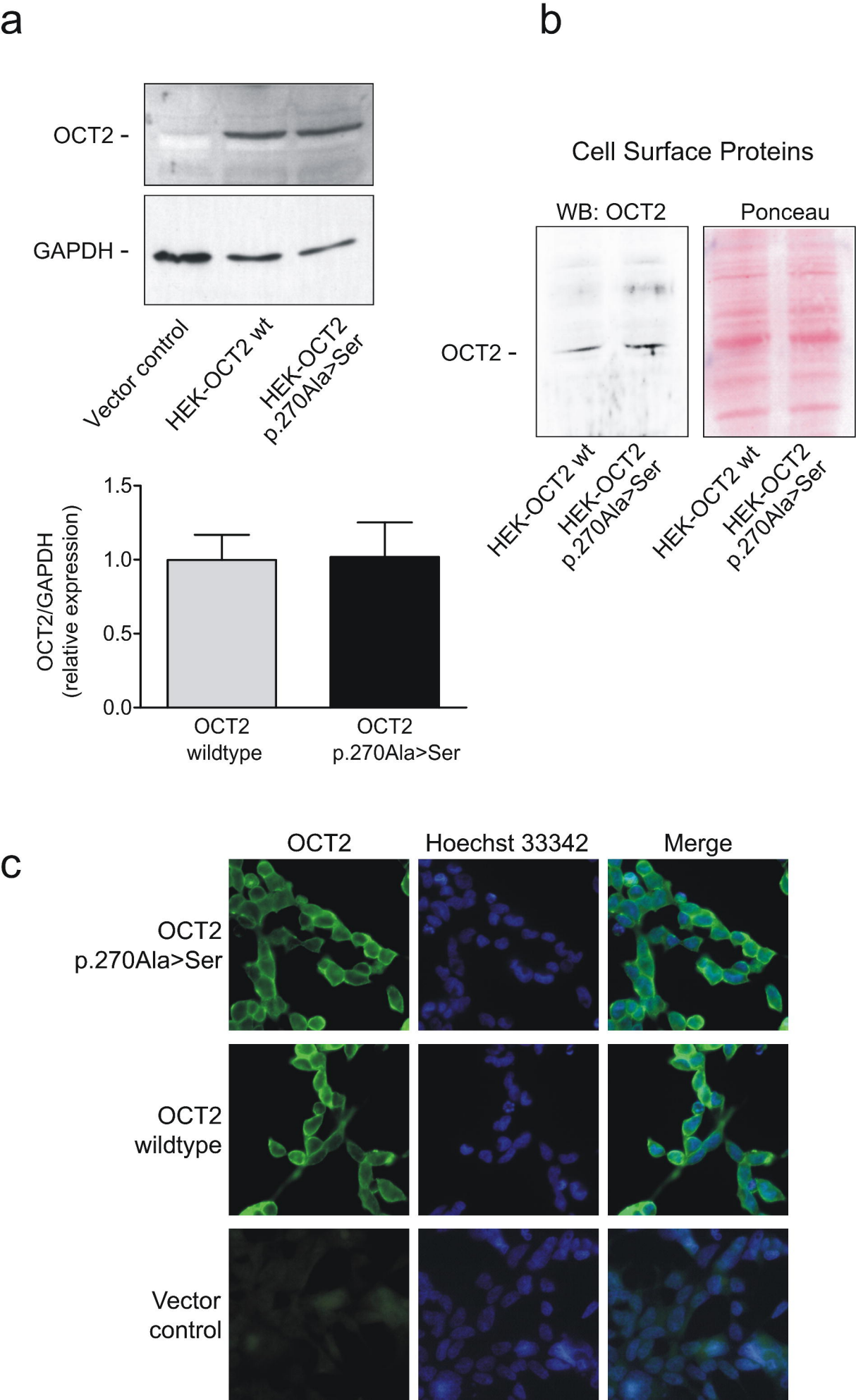


Fig. 2

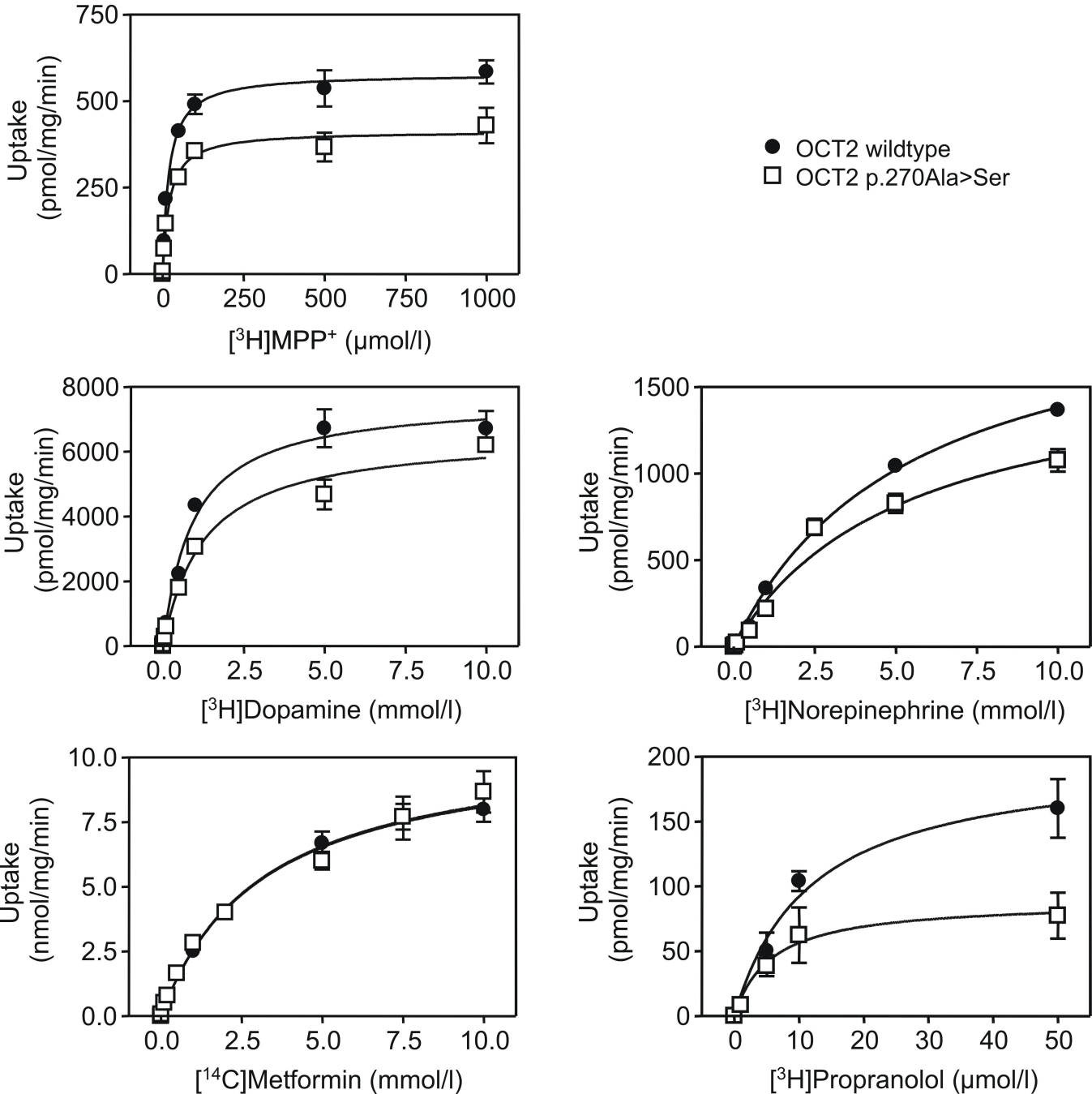
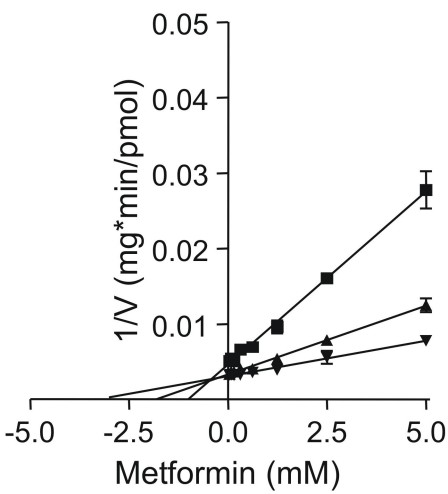

Uptake (pmol/mg/min)
750
500
250
0
0
250
500
750
1000
[3H]MPP+ (µmol/l)
OCT2 wildtype
OCT2 p.270Ala>Ser
Uptake (pmol/mg/min)
8000
6000
4000
2000
0
0.0
2.5
5.0
7.5
10.0
[3H]Dopamine (mmol/l)
Uptake (pmol/mg/min)
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
[3H]Norepinephrine (mmol/l)
Uptake (nmol/mg/min)
10.0
7.5
5.0
2.5
0.0
0.0
2.5
5.0
7.5
10.0
[14C]Metformin (mmol/l)
Uptake (pmol/mg/min)
200
150
100
50
0
0
10
20
30
40
50
[3H]Propranolol (µmol/l)

Fig. 3


0.05
0.04
0.03
0.02
0.01
1/V (mg*min/pmol)
-5.0
-2.5
0.0
2.5
5.0
Metformin (mM)
MPP+
10 µM
25 µM
50 µM

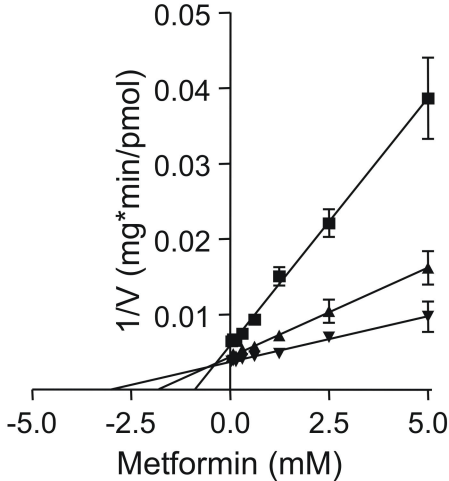
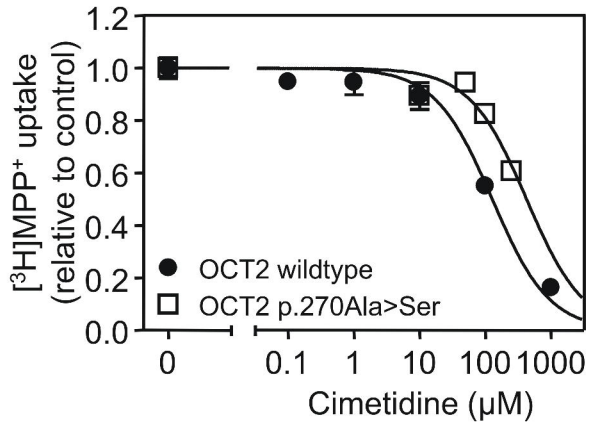
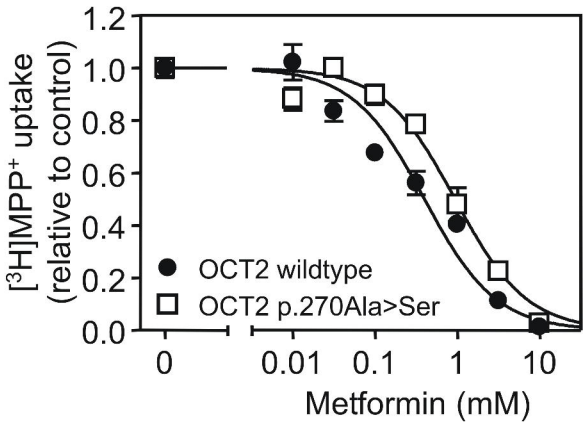
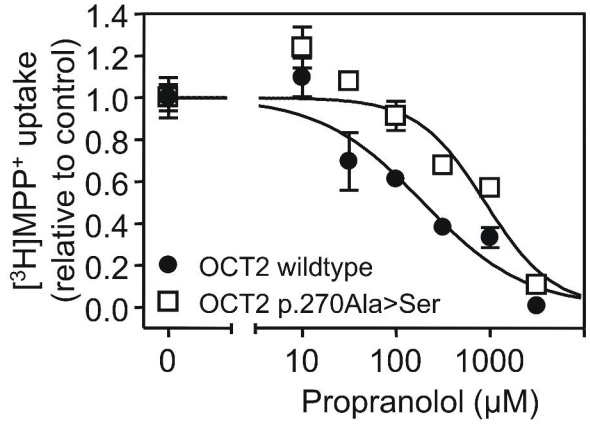

0.05
0.04
0.03
0.02
0.01
1/V (mg*min/pmol)
-5.0
-2.5
0.0
2.5
5.0
Metformin (mM)
MPP+
10 µM
25 µM
50 µM

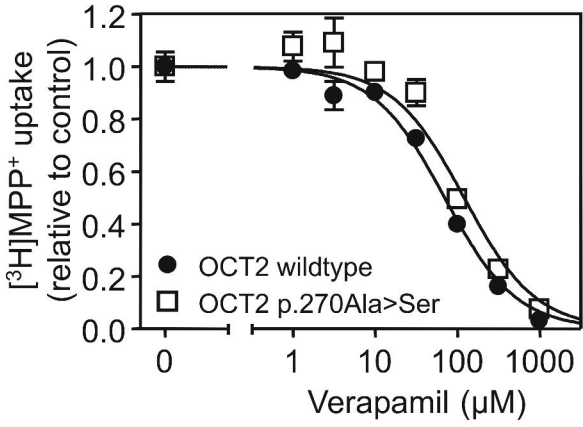
Fig. 4

a


[3H]MPP+ uptake (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OCT2 wildtype
OCT2 p.270Ala>Ser
0
0.1
1
10
100
1000
Cimetidine (µM)


[3H]MPP+ uptake (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OCT2 wildtype
OCT2 p.270Ala>Ser
0
0.01
0.1
1
10
Metformin (mM)


[3H]MPP+ uptake (relative to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OCT2 wildtype
OCT2 p.270Ala>Ser
0
10
100
1000
Propranolol (µM)


[3H]MPP+ uptake (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OCT2 wildtype
OCT2 p.270Ala>Ser
0
1
10
100
1000
Verapamil (µM)

b

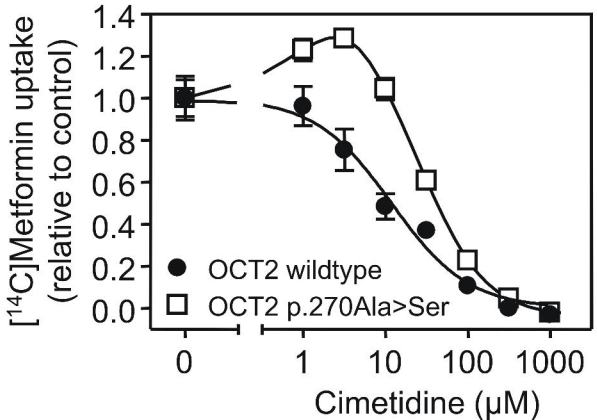
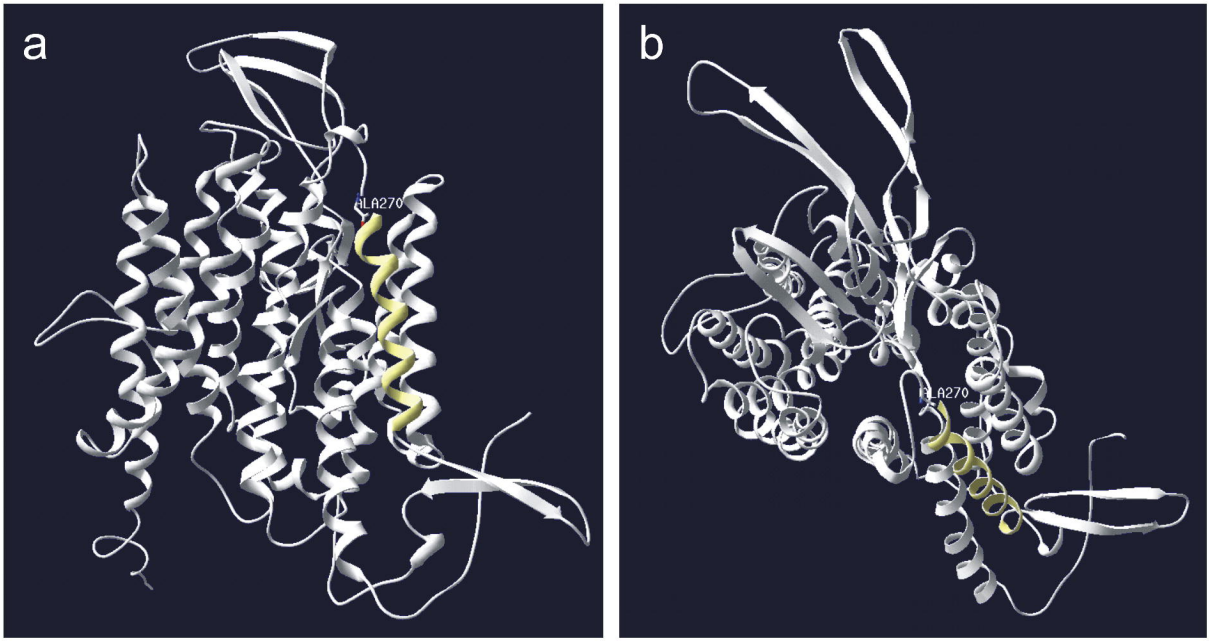

[14C]Metformin uptake (relative to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OCT2 wildtype
OCT2 p.270Ala>Ser
0
1
10
100
1000
Cimetidine (µM)

Fig. 5



c

```
NP_003049.1|  Homo sapiens                  LVLAGVAYALPHWRWLQFTVALPNFFFLLYYWCIPESPRWL 290
XP_518840.2|  Pan troglodytes               LVLAGVAYALPHWRWLQFTVALPNFFFLLYYWCIPESPRWL 290
Q5R5H7.1|     Pongo pygmaeus               LVLAGVAYALPHWRWLQFTVTLPNFFFLLYYWCIPESPRWL 290
NP_038695.1|  Mus musculus                 LILAGVAYALPNWRWLQFAVTLPNFCFLLYFWCIPESPRWL 290
NP_113772.1|  Rattus norvegicus             LILAGVAYVIPNWRWLQFAVTLPNFCFLLYFWCIPESPRWL 290
NP_999067.1|  Sus scrofa                    LVLAGVAYALPHWRWLQFTVTLPNFCFLFYYWCVPESPRWL 289
XP_862848.1|  Canis lupus familiaris        LLLAGVAYVLPHWRWLQVTVTLPNICFLLYYWCIPESPRWL 294
NP_001075584|Oryctolagus cuniculus          LVLAGVAYALPRWRWLQLTVTLPYFCFLLYYWCIPESPRWL 289
XP_599284.2|  Bos taurus                    LVLAGVAYVLPHWRWLQFTVTLPNFCFLLYYWCVPESPRWL 284
XP_419621.2|  Gallus gallus                 LILTAVAYVLPHWRWLQLAVTLPNFFFLLYHWCLPESPRWL 289
                                            *:*:.***.:*.*****.:*:** : **:*.**:*******
```