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Functional and structural relevance of conserved positively charged lysine residues in organic anion transporting polypeptide 1B3

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Running title:

Importance of Lys361 and Lys399 in OATP1B3

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OATP/Oatp, organic anion transporting polypeptide; BSP, sulfobromophthalein; PGE_2, prostaglandin E_2; E3S, estron-3-sulfate; E17βG, estradiol-17β-glucuronide; CCK-8, cholecystokinin 8; SEM, standard error mean; ANOVA, analysis of variance; HUSAR, Heidelberg UNIX Sequences Analysis Resource; TMH, transmembrane helix; VC, vector control; a.u., arbitrary units

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Abstract

The human organic anion transporting polypeptide 1B3 (OATP1B3), located in the basolateral membrane of hepatocytes, mediates the uptake of endogenous substrates such as taurocholate and drugs from blood into hepatocytes. The transport activity of OATP1B3 is influenced by positively charged amino acids, which are facing the central pore. Molecular modeling was performed to select conserved positively charged amino acids, which may influence transport activity and anchoring of OATP1B3 in the plasma membrane. The modeling revealed that Lys361 faces the pore and Lys399 is oriented to the plasma membrane. Therefore, the mutants Lys361>Ala, Lys361>Arg, Lys399>Ala and Lys399>Arg were generated using site-directed mutagenesis in order to investigate the impact of the positive charges on transport activity and anchoring in the membrane. Transport kinetic analyses for the substrates sulfobromophthalein and taurocholate showed a loss of function for the Lys361>Ala mutant, whereas the transport activity was maintained by the Lys361>Arg mutant, indicating that the positive charge at position 361 is important for transport activity of OATP1B3. Comparative modeling with OATP1A2 and OATP2B1 revealed that the pore size around this lysine residue is larger in OATP1A2 and smaller in OATP2B1 compared to OATP1B3, which could be related to the respective substrate spectra. Cell surface expression of Lys399>Ala and Lys399>Arg was decreased to 16% and 72% compared to wild type OATP1B3 ($p<0.001$), respectively indicating that the positive charge of lysine at position 399 is necessary for an unimpaired cell surface expression. Furthermore, we provide a summary of amino acids, which influence the transport activity of OATP1B3.

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Introduction

In the last decade, the relevance of transmembrane transporters for the disposition of endogenous compounds (e.g. bile acids and hormones) and drugs was increasingly recognized. The members of the organic anion transporting polypeptides (OATPs/Oatps) family are responsible for the uptake of anionic endogenous and xenobiotic substrates from the extracellular space into the cytosol (König, 2011). The human OATP family is composed of six subfamilies (OATP1-6) with 11 members. OATPs are expressed in different tissues such as liver, kidney, brain and intestine. Many in vitro and in vivo studies characterized in detail the function of individual OATPs. However, only a few studies investigated the relevance of certain molecular structures, such as amino acids or transmembrane domains, for the transport mechanism of OATPs/Oatps.

It was shown that four amino acid residues in the transmembrane helix X of rOatp2a1 are involved in the interaction with the substrate prostaglandin E_2 (PGE_2) (Chan et al., 1998). In a further study the role of cationic amino acids for the transport activity of rOatp2a1 and binding affinity of the substrate PGE_2 was investigated (Chan et al., 2002). The replacement of a positively charged amino acid such as arginine 560 and lysine 613 by lysine and arginine, respectively, revealed that the substrate affinity was maintained. With this approach, it was possible to show that the positive charges at these positions are involved in substrate binding.

Another approach for identifying molecular structures that are involved in the transport mode of OATPs was the construction of chimeras (Gui and Hagenbuch, 2008; Gui and Hagenbuch, 2010; Miyagawa et al., 2009). In one study regarding the liver specific

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OATP1B1 it was demonstrated that the transmembrane domain 8 is critical for the substrate recognition of estrone-3-sulfate (E3S) and estradiol-17β-glucuronide (E17βG) (Miyagawa et al., 2009). A later study with OATP1B1 identified transmembrane domain X as a critical structure for the transport of E3S. For OATP1B3, which is highly homologous to OATP1B1, similar studies were performed (Glaeser et al., 2010; Gui and Hagenbuch, 2008). Gui and Hagenbuch showed that the transmembrane domain X in OATP1B3 is critical for the transport of cholecystokinin 8 (CCK-8) (Gui and Hagenbuch, 2008). Using molecular modeling they proposed, that two amino acids might form hydrogen bonds with CCK-8. In a further study a molecular model of OATP1B3 was generated (Meier-Abt et al., 2005). Based on the model it was proposed, that OATP1B3 contains a positively charged central pore, which would be consistent with the transport of anionic substrates.

In our previous study, we demonstrated that the positive charge of the conserved Lys41 and Arg580 in OATP1B3 is important for the transport activity (Glaeser et al., 2010). Furthermore, our structural modeling indicated that in addition to the positive charge also the orientation and fixation of these amino acids in the pore is important for the transport activity. Using this model, we aimed to select further conserved positively charged amino acids, which may influence the transport activity of OATP1B3 or may have an influence on the structural anchoring in the plasma membrane. After the selection based on our model, different mutants of these amino acids were generated in order to test our hypothesis. In this study, we investigated the relevance of lysine 361, located in transmembrane helix VII, for the transport activity of sulfobromophthalein (BSP) and taurocholate. Furthermore, we also investigated the relevance of Lys399 for the anchoring of OATP1B3 in the plasma membrane. Based on a sequence alignment,

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we also performed modeling of other human OATPs such as OATP1A2 and OATP2B1 to investigate the structural differences in the pore region of these transporters.

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Material and Methods

Chemicals and antibodies- [^{3}H]sulfobromophthalein ([^{3}H]BSP; 14 Ci/mmol) was obtained from Hartmann Analytic GmbH (Braunschweig, Germany). [^{3}H]taurocholate, (10 Ci/mmol) was purchased from Biotrend GmbH (Cologne, Germany). Unlabeled sulfobromophthalein was obtained from Applichem GmbH (Darmstadt, Germany) and unlabeled taurocholate was obtained from Avocado Research Ltd. (Lancashire, England). Poly-D-lysine hydrobromide was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Sodium butyrate was purchased from Merck KGaA (Darmstadt, Germany). The polyclonal anti human OATP1B3 antiserum (SKT3fr2) was already characterized and purified as previously published (Glaeser et al., 2010). All other chemicals and reagents, unless stated otherwise, were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and were of the highest grade available.

Computer-based alignment of human OATP sequences- The identification of conserved lysine residues within the human OATP family was performed as previously described (Glaeser et al., 2010).

Structural modeling- Suitable templates for homology modeling were detected using the protein structure prediction server at the BioInfoBank Institute (Ginalski et al., 2003). The individual prediction methods combined by the server consistently predicted that the glycerol-3-phosphate transporter [PDB code 1pw4; (Huang et al., 2003) and the lactose permease structure [PDB code 1pv6; (Abramson et al., 2003) share a significant

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structural similarity to OATP1B3. OATP1B3 modeling was performed based on these templates using Modeller 6.2 (Sanchez and Sali, 2000). Due to the limited sequence homology of the long loop regions, modeling was mainly restricted to the transmembrane helices. Hydrogen atoms were added to the final model followed by 100 steps of conjugate gradient minimization using Sybyl7.3 (Tripos Inc.). The quality of the structure was assessed using Procheck (Laskowski et al., 1993) and Whatcheck (Hooft et al., 1996). Finally, mutations were inserted using Sybyl7.3 and Swiss-PdbViewer (Guex and Peitsch, 1997), selecting the lowest-energy side chain conformer for each mutated residue. The models of OATP1A2 and OATP2B1 were generated by the same strategy using the OATP1B3 structure as a template.

Mutagenesis of SLCO1B3- The mutagenesis of the investigated lysine residues 361 and 399 was performed using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene Europe, The Netherlands) according to manufacturer's instructions. The wild type (WT) expression plasmid pcDNA3.1/Hygro(-)-OATP1B3 containing the reference sequence NM_019844 (http://www.ncbi.nlm.nih.gov) (Cui et al., 2001) was kindly provided by Professor Dietrich Keppler (DKFZ, Heidelberg, Germany). The following primers were used for the mutagenesis reactions:

Lys361>Ala: 5'-tattggttcttttacttacgtctttgcatatatggagcaacagtacggtcag-3',

Lys361>Arg: 5'-ggttcttttacttacgtctttagatatatggagcaacagtacggt-3',

Lys399>Ala: 5'-actggaatgtttttaggaggatttatcattaaagcattcaaattgtctttagttggaattgc-3',

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Lys399>Arg: 5'-gcaactggaatgtttttaggaggatttatcattaaaagattcaaattgtctttagttg-3'. All mutagenesis products were verified by sequencing (Agowa, Berlin; Germany).

Cell culture- Human embryonic kidney (HEK) 293 cells were cultured in minimum essential medium, containing 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5 % CO_2. The cells were routinely subcultivated by trypsinization using trypsin (0.05%)-EDTA (0.02 %) solution. All cell culture media supplements were obtained from Invitrogen GmbH (Karlsruhe, Germany).

Transient transfection and uptake experiments- The transient transfection for the functional characterization of lysine mutants was performed as previously described (Glaeser et al., 2010). In brief, cells were incubated with different concentrations of a mix of radiolabeled and non-radiolabeled BSP (0.1 µM – 10.0 µM and taurocholate (0.25 µM – 125.0 µM) for 10 min at 37°C (König et al., 2000). The intracellular accumulation of radioactivity was measured using a liquid scintillation counter (Tricarb2800, PerkinElmer Life Sciences GmbH) and normalized to the protein concentration in each well. All experiments were performed at least in triplicates. Michaelis-Menten-type nonlinear curve fitting was performed to calculate maximal uptake rate (V_{max}) and the concentration at which half of maximal uptake occurs (K_m) using GraphPad Prism 5® software version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com). The obtained V_{max}-values were normalized

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to the cell surface expression of the respective mutants. Transport rates are given in pmol*milligrams of protein^{-1}*minute^{-1}.

Transient transfection and cell surface expression- The influence of the OATP1B3 WT and generated mutants on the cell surface expression was performed as previously described (Glaeser et al., 2010) using the cell surface biotinylation technique with subsequent immunoblot analysis. The biotinylation and isolation of cell surface proteins were performed with the cell surface labeling kit (Pierce) according to manufacturer's instructions. Briefly, the HEK293 cells were seeded at a cell density of 750,000 cells per well in a poly-D-lysine coated 6-well cell culture plate. After 24 h the cells were transfected with 4000 ng/well of the respective expression plasmids using Lipofectamine™ 2000 (Invitrogen) according the supplier's instructions. After further 24 h of incubation with the transfection reagent, the cells were treated with 10 mM sodium butyrate for additional 24 hours before the treatment with the biotinylation reagent EZ-Link® Sulfo-NHS-SS-Biotin (Pierce). The isolation of OATP1B3 WT and mutants was performed using NeutrAvidin agarose according to the manufacturer's instructions. The subsequent immunoblot and densitometric analyses were performed as previously described (Glaeser et al., 2007; Glaeser et al., 2002; Glaeser et al., 2010).

Statistical analysis- All data are presented as mean ± standard error mean (SEM). The impact of the investigated mutants on the protein expression was analyzed using one-way analysis of variance (ANOVA) with the Dunnett's multiple comparison test. The

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comparison of the K_m and V_{max}-values between wild type and Lys361>Arg was performed with an unpaired t-test. A p-value ≤ 0.05 was required for statistical significance. The calculations were performed using GraphPad Prism 5® software version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com).

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Results

Alignment of human OATP family members- The alignment (Fig. 1) revealed that the lysine residues 361 and 399 of OATP1B3 are highly conserved at the corresponding positions in the other human OATP family members. Furthermore, OATP2A1 and OATP2B1 have the positively charged amino acid arginine at position 399 indicating that the positive charge at this position may be important for the structure or function of human OATPs. Therefore, we selected these two amino acids for further studies in order to investigate their role for the function and structure of OATP1B3.

Structural modeling- The structural modeling (Fig. 2) shows that Lys361 is located at the end of transmembrane helix VII and its positive charge is located within the extracellular region forming the pore of OATP1B3. This suggests that the positive charge of Lys361 may contribute to the functional activity of OATP1B3 because it is already known that the positively charged amino acids Lys41 and Arg580 in OATP1B3 which are facing the pore contribute to the functional activity of OATP1B3 (Glaeser et al., 2010). Lysine 399 is located in the fourth intracellular loop and is not facing the pore. In addition, no intra-molecular interactions with other amino acids could be observed. Therefore, we concluded that Lys399 is most likely not involved in the transport mode of OATP1B3 and may contribute to the phospholipid-mediated anchoring in the plasma membrane.

Based on the structural modeling we investigated the role of Lys361 for the transport activity of OATP1B3 and the relevance of Lys399 for the anchoring of OATP1B3 in the plasma membrane. Lys361 was mutated to Lys361>Ala and Lys361>Arg in order to replace the positive charge at this position by either a neutral (Ala) or positive charge

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(Arg). In case that the positive charge at the position 361 is important for the activity of OATP1B3, the transport activity should be preserved by the replacement of lysine with arginine. For Lys399, the mutants Lys399>Ala and Lys399>Arg were generated to investigate the importance of the positive charge of Lys399 for the anchorage in the plasma membrane. We speculated that the replacement with alanine leads to a pronounced reduction in the cell surface expression of OATP1B3 whereas the replacement of Lys399 with arginine rescues the anchoring in the plasma membrane so that cell surface expression of this OATP1B3 mutant is only reduced to a minor extent compared to Lys399>Ala.

Additionally, we performed modeling of further human OATP-family members such as OATP1A2 and OATP2B1 to address the role of Lys341 and Lys396, respectively, which correspond to Lys361 in OATP1B3 (Fig. 3). These conserved lysine residues are located at the entry of the pore opposite to a loop which is variable between the investigated OATPs. The loop in OATP1B3 contains an arginine (Arg58) and a serine (Ser62) residue, which are oriented to the pore and therefore limit their size. In contrast, in OATP1A2 both residues are replaced by glutamine and proline, respectively, which leads to a reduced positive charge of the pore due to the lack of arginine. A kink, which is caused by proline results in a widened entry of the pore. In OATP2B1, the amino acid corresponding to serine 64 in OATP1B3, is replaced by glutamine resulting in a narrower pore due to the longer side chain of glutamine. Furthermore, the modeling revealed no difference in the pore region around Lys361 between OATP1B3 and OATP1B1 (data not shown).

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Cell surface expression and functional activity of Lys361>Ala and Lys361>Arg mutants- The cell surface expression of OATP1B3 WT and the Lys361>Ala and Lys361>Arg mutants are shown in Fig. 4. The Lys361>Ala mutant showed a modest increase of 15 ± 5 % in the cell surface expression compared to WT (100 %, $p<0.05$), whereas the Lys361>Arg led to a reduction by 33 ± 2 % in cell surface expression compared to WT (100 %, $p<0.001$).

The kinetic analysis of the Lys361>Arg mutant for the BSP and taurocholate uptake revealed no significant change in the V_{max} (normalized to cell surface expression)- and K_m-values compared to OATP1B3 WT (Fig. 5, table 1) indicating that function of the positively charged lysine can be maintained by the positively charged amino acid arginine. For the Lys361>Ala mutant the respective K_m- and V_{max}-values could not be reliably determined, which reflects the loss of activity when lysine at position 361 is replaced by alanine.

Cell surface expression of Lys399>Ala and Lys399>Arg mutants- The cell surface expression of OATP1B3 WT and the Lys399>Ala and Lys399>Arg mutants are shown in Fig. 6. The densitometric analyses revealed that the Lys399>Ala mutant was characterized by a cell surface expression of only 16 ± 1 % compared to OATP1B3 WT (100 %, $p<0.001$). The Lys399>Arg mutant showed a moderately reduced cell surface expression of 72 ± 4 % compared to OATP1B3 WT (100 %, $p<0.001$).

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Discussion

The present study investigated the importance of Lys361 and Lys399 in OATP1B3 for its function and anchoring in the plasma membrane, respectively. These two amino acids were selected because they are highly conserved within the human OATP family. Only in OATP6A1, the non-charged amino acids isoleucine and threonine, respectively, are present at the corresponding positions to 361 and 399 in OATP1B3. In all other OATPs lysine can be found at the corresponding positions to Lys361. This implies that the positive charge and the amino acid lysine at this position are important for the function and/or structure of OATP1B3. For the corresponding positions to Lys399 in OATP1B3, lysine is also present in most of the other OATP family members. In OATP2A1 and OATP2B1, the positively charged amino acid arginine is present at this position. This finding implies that the positive charge at this position may be important for the function and/or structure of OATP1B3.

Combining the results of the alignment and the molecular modeling (Fig. 2), we were able to specify our working hypothesis. Therefore, we proposed that Lys361 might be involved in the transport activity of OATP1B3 because our model shows that it is directed to the positively charged pore, which is consistent with the transport of anionic substrates. A positively charged pore was already proposed by the molecular modeling of Meier-Abt and colleagues (Meier-Abt et al., 2005). The results of the mutagenesis and molecular modeling of Lys361 show that the positive charge at this position is important for the transport activity. Surprisingly, the transport activities of OATP1B3 were maintained by the replacement with arginine at this position. The molecular modeling supports this because the properties of the pore region are only marginally

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affected by this amino acid exchange. The maintenance of the transport activity in our experiments was not expected due to the high conservation of lysine at this position. In contrary, Ala361 drastically affects the shape of the pore, which leads with the additional loss of the positive charge to a reduction of activity. It can be concluded, that rather the positive charge at position 361 is important for the transport activity of OATP1B3 than the amino acid lysine. Furthermore, Lys361 may be involved in substrate recognition and translocation because K_m and V_{max} did not change significantly following the replacement with arginine.

In OATP1B1, which shows an amino acid identity of 80 % to OATP1B3 (König et al., 2000), the influence of Lys361 on the function of OATP1B1 was investigated by replacing this amino acid with arginine and alanine, respectively (Weaver and Hagenbuch, 2010). For the substrates BSP, E17βG and E3S, no significant changes in the V_{max} were observed. These findings are in agreement with the results of our study. Interestingly, the Lys361>Arg mutant in OATP1B1 resulted in an increase of the K_m-values for all three substrates (Weaver and Hagenbuch, 2010). The Lys361>Ala mutant of OATP1B1 led to an elevated K_m and no change in V_{max} for E17βG and BSP as well as a decreased V_{max} and no change in K_m for E3S. Therefore, the authors concluded that the conserved lysine rather than simply the positive charge at this position seems to be important for the OATP1B1 function (Weaver and Hagenbuch, 2010). From both studies it could be summarized, that in OATP1B1 the positive charge from lysine at position 361 seems to be crucial for substrate recognition whereas in OATP1B3 mainly the positive charge at 361 is crucial for substrate recognition and transport rates. However, in OATP1B3 both processes are generally driven by the positive charge and not mainly by the presence of lysine.

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Due to our modeling, no difference between OATP1B1 and OATP1B3 is expected with regard to Lys361. Therefore, the slight difference between the study from Weaver et al. and our study may be related to differences in experimental design. However, further comparative studies are necessary in order to clarify the role of Lys361 in OATP1B1 and OATP1B3 for transport of substrates.

For Lys399 we hypothesized that this amino acid may be involved in the anchoring in the plasma membrane because it is directed to the edge of the transporter, which could be due to an interaction with negatively charged phospholipids. Our modeling supports this notion because Phe395 and Phe400 always stabilize the turn between the helices VIII and IX independently from the mutation at position 399. Thus, the amino acid at position 399 evidently plays no role for the stabilization of the protein fold itself, but rather for the interaction of OATP1B3 with the membrane. Even though it is not possible to show the interaction of lysine 399 with phospholipids by molecular modeling, the results support our hypothesis that Lys399 is involved in the anchoring to plasma membrane. The replacement of Lys399 with Ala lead to a drastic reduction in membrane localization clearly indicating that the positive charge in the fourth intracellular loop is crucial for the localization and fixation in the plasma membrane. Arginine at position 399 was able to maintain the plasma membrane localization with a moderate reduction compared to WT. This suggests that for an optimal membrane expression of OATP1B3 the positive charge has to be provided from lysine 399.

Since our studies above indicate that Lys361 is involved in substrate transport, we additionally investigated the structure and vicinity of this residue in related transporters. An identification of those residues that form the extracellular side of the pore is

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hampered by the fact that the template crystal structure of the glycerol-3-phosphate transporter used for modeling was solved in a conformation with the pore opening toward the intracellular side. Consequently, the OATP1B3 model is rather closed on the extracellular side. We have therefore focused our investigation on those residues in the vicinity of Lys361 that differ between various OATPs and might therefore play a role for substrate specificity.

Our comparison of the model of OATP1B3 with that of OATP1A2 and OATP2B1 concerning the vicinity of Lys361 and the corresponding lysine residues indicate that OATP1A2 exhibits a larger pore size compared to OATP1B3, whereas OATP2B1 is characterized by a smaller pore size compared to OATP1B3. These findings can be correlated to the fact that OATP1A2 exhibits the largest substrate spectrum and OATP2B1 the smallest substrate spectrum within the human OATP-family (Hagenbuch and Meier, 2004; König, 2011). Furthermore, our proposed less positively charged pore of OATP1A2 is in line with the fact that OATP1A2 transports the most amphipathic compounds such as fexofenadine and bile salts (Hagenbuch and Meier, 2004; König, 2011). Therefore, it can be concluded that the conserved residue at position 361 in OATP1B3 and the opposite loop to Lys361 influence the substrate spectrum of OATP1B3. Differences in the opposite loop to the conserved lysine residue at the end of transmembrane helix VII could be a reason for the differences in the substrate spectrum within the OATP-family.

The recent studies that also investigated the role of molecular structures of OATP1B1 and OATP1B3 indicate that amino acids from transmembrane helix VII to X have a significant influence on the transport mode of these transporters. However, the studies

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are different regarding the used substrates and experimental endpoints (K_m, V_{max}, etc.). Therefore, a direct comparison of these studies seems to be difficult. Nevertheless, all studies were able to show that some of the investigated amino acids are relevant for transport or expression. Previous studies showed, that for OATP1B3 the amino acids Lys41 (Glaeser et al., 2010), Tyr537, Ser545, Thr559 and Arg580 (Glaeser et al., 2010) are important for transport of different substrates such as BSP, CCK-8 and pravastatin. Therefore, we provide a summary of all these amino acids using our molecular model of OATP1B3 (Fig. 7) which shows that all these amino acids, which are influencing the transport activity, are directed towards the pore.

Taken together, our study shows that the positive charge of lysine 361 is important for the transport of the substrates BSP and taurocholate and that the positive charge of lysine 399 is important for the expression of OATP1B3 in the cell surface.

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Authorship Contributions

Participated in research design: Kathrin Mandery, Hartmut Glaeser

Conducted experiments: Kathrin Mandery, Heinrich Sticht, Krystyna Bujok, Ingrid Schmidt, Christina Fahrmayr, Bettina Balk, Hartmut Glaeser

Contributed new reagents or analytic tools: n.a.

Performed data analysis: Kathrin Mandery, Heinrich Sticht, Hartmut Glaeser

Wrote or contributed to the writing of the manuscript: Kathrin Mandery, Heinrich Sticht, Martin F. Fromm, Hartmut Glaeser

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Footnotes

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Legends for figures

Figure 1: Partial alignment of amino acid sequences of 11 human OATPs. The alignment was performed using the HUSAR (Heidelberg UNIX Sequences Analysis Resource) program. The highlighted lysine residues at positions 361 and 399 in OATP1B3 are highly conserved within the human OATP family and are located in the predicted pore-facing transmembrane helix (TMH) VII and in the fourth intracellular loop, respectively.

Figure 2: Structural modeling of OATP1B3. Side view (a) and top view (b) of the model showing the positions of the investigated residues. The positively charged amino acids are shown as ball presentation and are labeled (red balls and sticks: oxygen; blue balls and sticks: nitrogen)

Panels (c) to (e): Effect of the Lys361>Arg and Lys361>Ala mutations: (c) Lys361 is located in the extracellular side of the pore region. All amino acids, which are located in a distance smaller than 7 Angstrom of Lys361 are shown in space-filled presentation. (d) Arg361 has almost no influence on the local structure and the charge is conserved. (e) Ala361 influences the charge and the shape of the pore region (indicated by a green circle).

Panels (f) to (h): Effect of the Lys399>Arg and Lys399>Ala mutations: The orientation of Lys399, Arg399, and Ala399 is always directed to the solvent. The turn is always stabilized by two adjacent phenylalanine amino acids. The adjacent amino acids Lys398 and Lys401 are labeled.

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Figure 3: Structural comparison of different OATPs in vicinity of the pore region. The conserved lysine residues located at the entry of the pore are shown in space-filled presentation and the variable loop opposite to the lysine side chains (residues 58-64 in OATP1B3) are colored in green. Key residues of this loop that differ between the OATPs are marked. Panels (a) and (b) show the comparison of OATP1B3 (a) and OATP1A2 (b); panels (c) and (d) show the comparison of OATP1B3 (c) and OATP2B1 (d)

(a) Location of Arg58 and Ser62 in OATP1B3. The side chains of both residues are oriented towards the pore resulting in a narrow pore with a large positive charge. (b) In OATP1A2, Arg58 and Ser62 are replaced by glutamine and proline respectively. The lack of an arginine results in a reduced positive charge of the pore and Pro54 introduces a kink (red arrow) in the respective loop resulting in a widened entry of the pore.

(c) Location of Ser62 to Ser64 in OATP1B3. The Ser64 side chain is oriented towards the pore, but is not in direct proximity to Lys361. (d) In OATP2B1, Ser64 is replaced by glutamine. Due to the increased length of the glutamine side chain compared to serine, the pore gets narrowed (red arrow).

Figure 4: Representative immunoblot analysis of the cell surface protein expression of OATP1B3 mutants at position Lys361 compared to WT and (VC) vector control cells are shown. The results of densitometric analyses are given below. The data are expressed as mean ± SEM in arbitrary units (a.u.). A significant increase (+15 %, $p<0.05$) for

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Lys361>Ala and a significant decrease (-33 %, $p<0.001$) for Lys361>Arg in the cell surface expression compared to WT (100 %) was observed (one-way analysis of variance (ANOVA) with the Dunnett's multiple comparison test).

Figure 5: Michaelis-Menten kinetics of BSP (a) and taurocholate (b) net uptake mediated by OATP1B3 WT and the mutants Lys361>Ala and Lys361>Arg. The kinetic analysis of the Lys361>Arg mutant for the BSP and taurocholate uptake revealed no significant change in the V_{max} (normalized to cell surface expression) - and K_m-values compared to OATP1B3 WT. For the Lys361>Ala mutant the respective K_m- and V_{max}-values could not be reliably determined

Figure 6: Representative immunoblot analysis of the cell surface protein expression of OATP1B3 mutants at position Lys399 compared to WT and VC cells are shown. The results of densitometric analyses are given below. The data are expressed as mean ± SEM in arbitrary units (a.u.). Significant differences in the protein expression of the mutants compared to OATP1B3 WT were observed (one-way analysis of variance (ANOVA) with the Dunnett's multiple comparison test). Lys399>Ala and Lys399>Arg showed decreased protein expression of 16 % and 72 % compared to OATP1B3 WT (100 %, $p<0.001$), respectively.

Figure 7: Structural modeling of OATP1B3. Side view of the model showing the positions of all investigated residues [this study: Lys361; previous studies: Lys41 and

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Arg580 (Glaeser et al., 2010), Tyr537, Ser545, and Thr559 (Gui and Hagenbuch, 2008)], that show an influence on OATP1B3 transport activity. The amino acids are shown as ball presentation.

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Tables

Table 1: Kinetic parameters of OATP1B3 wild type (WT), Lys361>Ala mutant and Lys361>Arg mutant. (V_{max} normalized to expression in plasma membrane; n.d. not determinable)

	BSP		**taurocholate**	
	K_m (µM)	V_{max} (pmol*mg protein^{-1}*min^{-1})	K_m (µM)	V_{max} (pmol*mg protein^{-1}*min^{-1})
WT	2.3 ± 1.4	73.7 ± 15.2	22.5 ±19.3	29.1 ± 7.1
Lys361>Ala	n.d.	n.d.	n.d.	n.d.
Lys361>Arg	3.1 ± 1.3	106.5 ± 16.8	24.1 ± 15.1	39.2 ± 7.3

figure 1

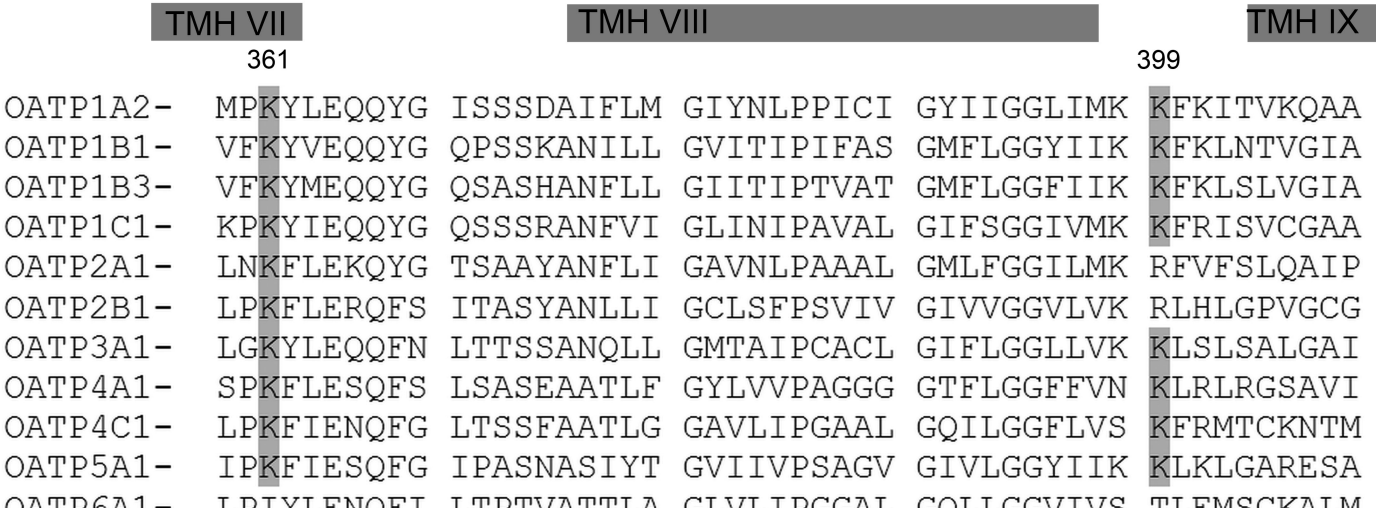
TMH VII
TMH VIII
TMH IX
361
399
OATP1A2- MPKYLEQQYG ISSSDAIFLM GIYNLPPICI GYIIGGLIMK KFKITVKQAA
OATP1B1- VFKYVEQQYG QPSSKANILL GVITIPIFAS GMFLGGYIIK KFKLNTVGIA
OATP1B3- VFKYMEQQYG QSASHANFLL GIITIPTVAT GMFLGGFIIK KFKLSLVGIA
OATP1C1- KPKYIEQQYG QSSSRANFVI GLINIPAVAL GIFSGGIVMK KFRISVCGAA
OATP2A1- LNKFLEKQYG TSAAYANFLI GAVNLPAAAL GMLFGGILMK RFVFSLQAIP
OATP2B1- LPKFLERQFS ITASYANLLI GCLSFPSVIV GIVVGGVLVK RLHLGPVGCG
OATP3A1- LGKYLEQQFN LTTSSANQLL GMTAIPCACL GIFLGGLLVK KLSLSALGAI
OATP4A1- SPKFLESQFS LSASEAATLF GYLVVPAGGG GTFLGGFFVN KLRLRGSAVI
OATP4C1- LPKFIENQFG LTSSFAATLG GAVLIPGAAL GQILGGFLVS KFRMTCKNTM
OATP5A1- IPKFIESQFG IPASNASIYT GVIIVPSAGV GIVLGGYIIK KLKLGARESA
OATP6A1- LPIYLENQFI LTPTVATTLA GLVLIPGGAL GQLLGGVIVS TLEMSCKALM

figure 2

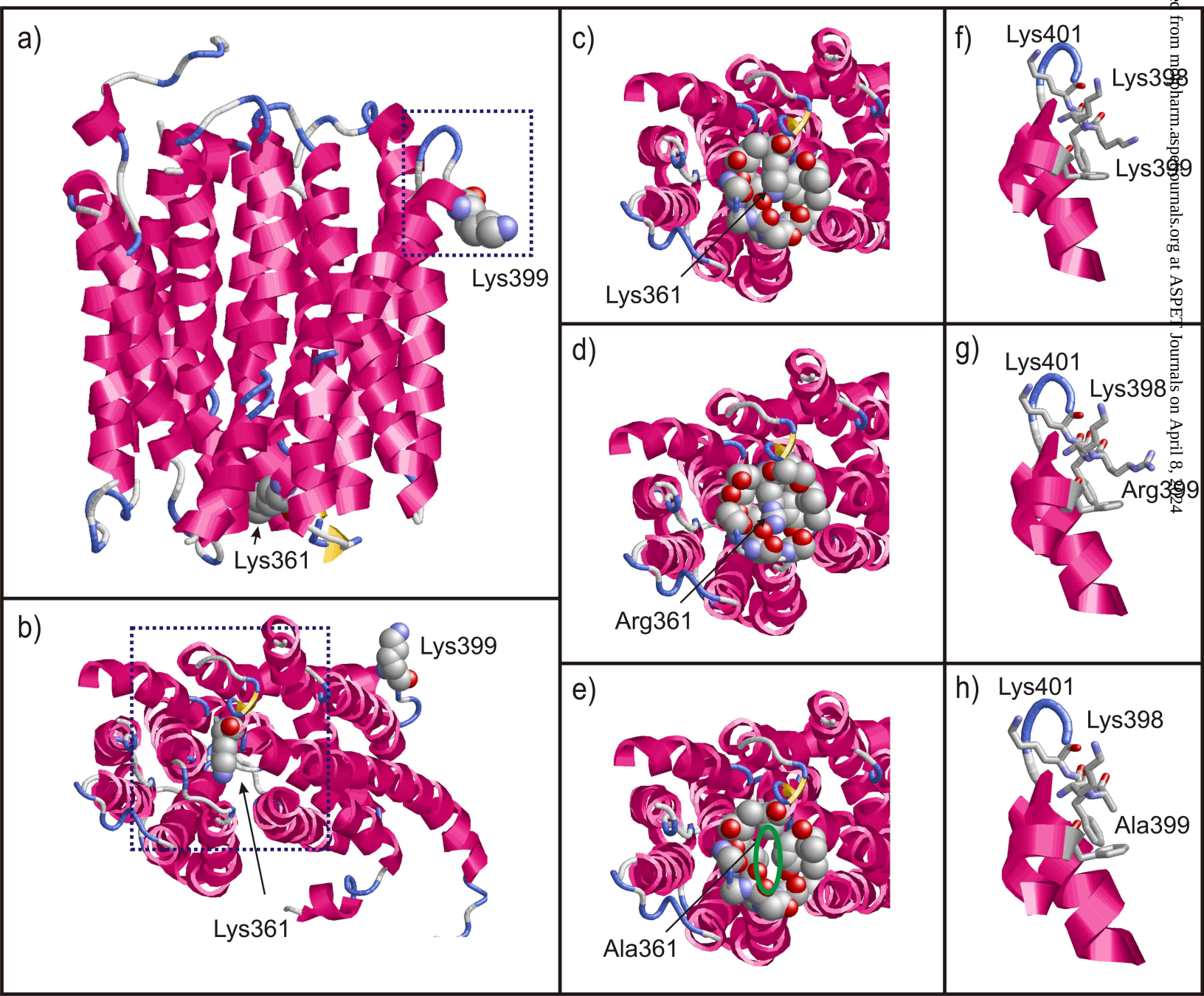

figure 3

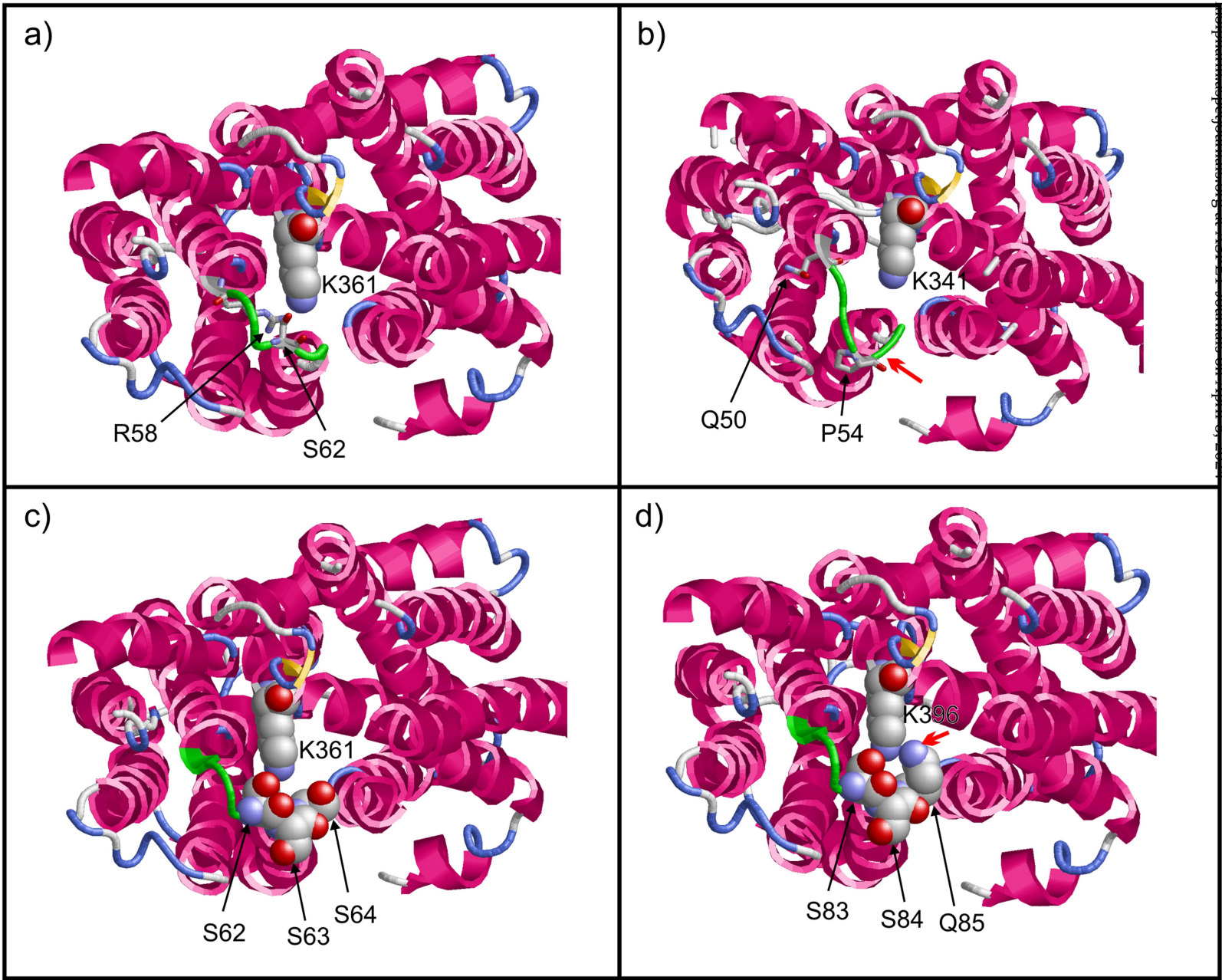

figure 4

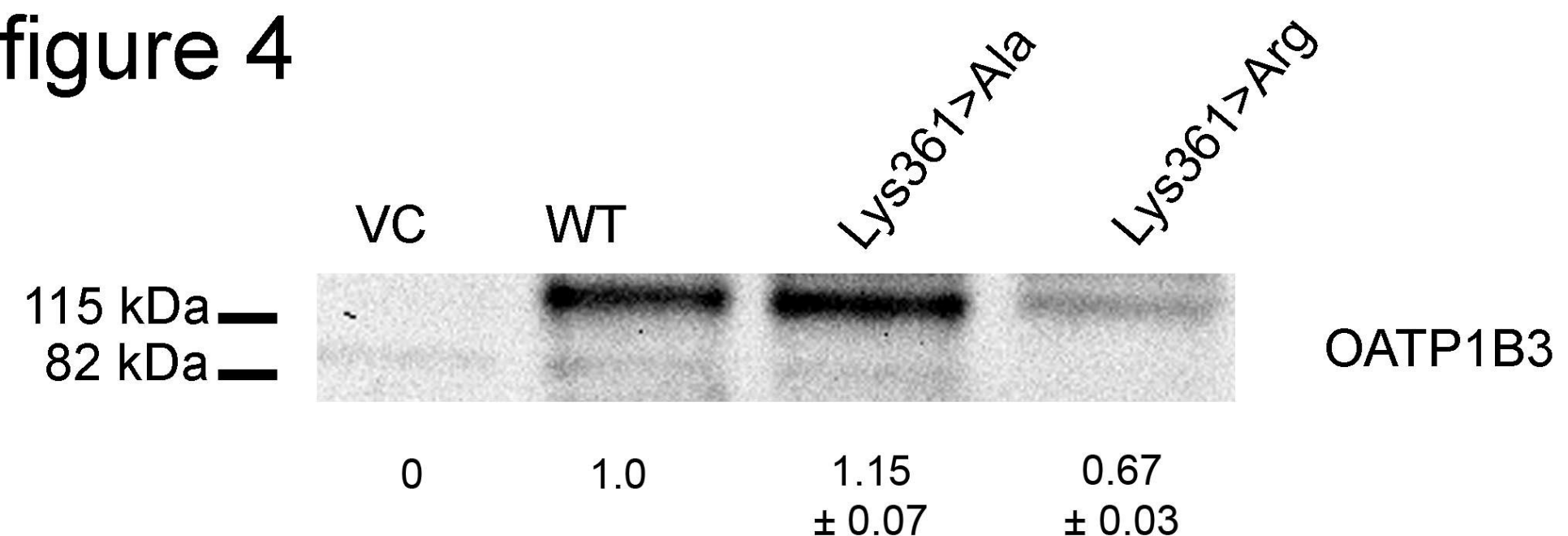
VC
WT
Lys361>Ala
Lys361>Arg
115 kDa
82 kDa
OATP1B3
0
1.0
1.15
± 0.07
0.67
± 0.03

figure 5

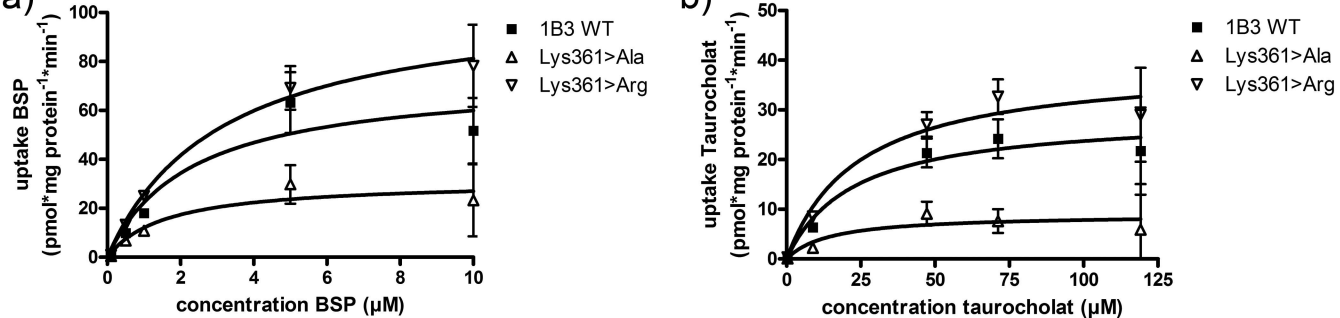
a)
uptake BSP
(pmol*mg protein-1*min-1)
100
80
60
40
20
0
0
2
4
6
8
10
concentration BSP (µM)
1B3 WT
Lys361>Ala
Lys361>Arg
b)
uptake Taurocholat
(pmol*mg protein-1*min-1)
50
40
30
20
10
0
0
25
50
75
100
125
concentration taurocholat (µM)
1B3 WT
Lys361>Ala
Lys361>Arg

figure 6

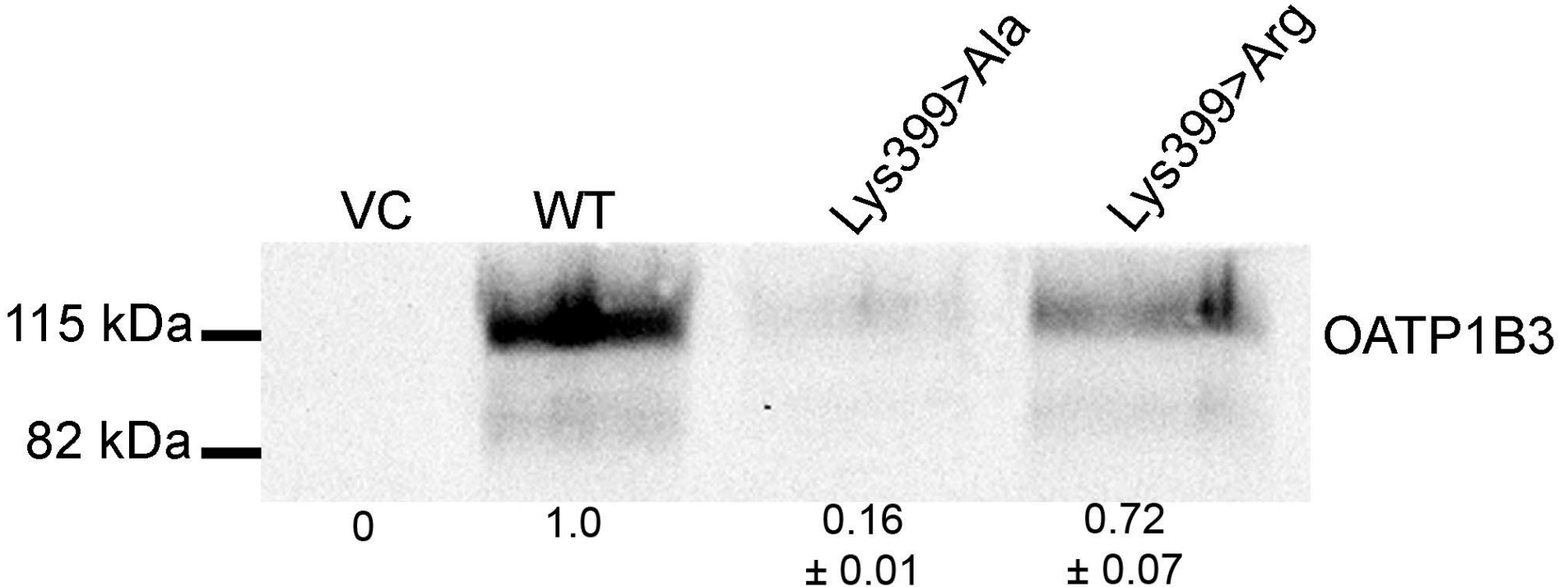
VC
WT
Lys399>Ala
Lys399>Arg
115 kDa
82 kDa
OATP1B3
0
1.0
0.16
± 0.01
0.72
± 0.07

figure 7