# ORGANIC CATION TRANSPORTER 1 (OCT1): NOT VITAL FOR LIFE, BUT OF SUBSTANTIAL BIOMEDICAL RELEVANCE

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# ORGANIC CATION TRANSPORTER 1 (OCT1): NOT VITAL FOR LIFE, BUT OF SUBSTANTIAL BIOMEDICAL RELEVANCE

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Around one third of all biologically relevant small molecules are organic cations. These include endogenous substances like catecholamines and other neurotransmitters, toxins and drugs designed to affect signaling processes. The organic cation transporter 1 (OCT1) is among the strongest expressed membrane transporters at the sinusoidal (blood-facing) side of liver cells and contributes substantially to the clearance of the blood from numerous organic cations. A most striking feature of OCT1 is its pronounced genetic diversity. Between 1 and 10% of all human populations have little to no OCT1 activity. With several of the OCT1 substrates up to 10% of Europeans are functionally OCT1 deficient. Apparently, the lack of OCT1 do not lead to apparent substantial pathological changes in these individuals. It thus appears that this transporter is not essential to human life, but does it means that OCT1 is irrelevant?

In the last 25 years since the first cloning of this transporter, data on its pharmacological and physiological relevance is steadily accumulating. Numerous clinically relevant drugs (e.g. metformin, morphine, fenoterol, sumatriptan, tramadol and tropisetron) have been shown to be substrates of OCT1, and OCT1 deficiency has been shown to affect the pharmacokinetics, efficacy, or toxicity of these drugs. Also vitamin B1 has been shown to be a substrate of OCT1, and in genetically modified mice OCT1 substantially modulated hepatic lipid metabolism, total body fat and systemic glucose and lipid concentrations.

Still, numerous important questions remain unsolved: For which drugs, toxins, or other endogenous or exogenous substances is OCT1 relevant? How can we predict the relevance of OCT1 from in vitro studies? What determines the substrate selectivity of OCT1 in comparison to other transporters or transport processes for organic cations? What regulates the expression of OCT1 in the liver and possibly in other tissues? What is the impact of OCT1 variation in different areas of medicine, including the therapies for cancer as well as for pulmonary, cardiovascular, or neurological diseases? How can evolutionary biology contribute to a better understanding of the roles of OCT1? And, importantly, what types of research are likely to significantly further the knowledge on OCT1 in the next decades?

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# Cellular Uptake of Psychostimulants – Are High- and Low-Affinity Organic Cation Transporters Drug Traffickers?

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Psychostimulants are used therapeutically and for illegal recreational purposes. Many of these are inhibitors of the presynaptic noradrenaline, dopamine, and serotonin transporters (NET, DAT, and SERT). According to their physicochemical properties, some might also be substrates of polyspecific organic cation transporters (OCTs) that mediate uptake in liver and kidneys for metabolism and excretion. OCT1 is genetically highly polymorphic, with strong effects on transporter activity and expression. To study potential interindividual differences in their pharmacokinetics, 18 psychostimulants and hallucinogens were assessed *in vitro* for transport by different OCTs as well as by the high-affinity monoamine transporters NET, DAT, and SERT. The hallucinogenic natural compound mescaline was found to be strongly transported by wild-type OCT1 with a $K_m$ of 24.3 µM and a $v_{max}$ of 642 pmol × mg protein$^{-1}$ × min$^{-1}$. Transport was modestly reduced in variants *2 and *7, more strongly reduced in *3 and *4, and lowest in *5 and *6, while *8 showed a moderately increased transport capacity. The other phenylethylamine derivatives methamphetamine, *para*-methoxymethamphetamine, (-)-ephedrine, and cathine ((+)-norpseudoephedrine), as well as dimethyltryptamine, were substrates of OCT2 with $K_m$ values in the range of 7.9–46.0 µM and $v_{max}$ values between 70.7 and 570 pmol × mg protein$^{-1}$ × min$^{-1}$. Affinities were similar or modestly reduced and the transport capacities were reduced down to half in the naturally occurring variant A270S. Cathine was found to be a substrate for NET and DAT, with the $K_m$ being 21-fold and the $v_{max}$ 10-fold higher for DAT but still significantly lower compared to OCT2. This study has shown that several psychostimulants and hallucinogens are substrates for OCTs. Given the extensive cellular uptake of mescaline by the genetically highly polymorphic OCT1, strong interindividual variation in the pharmacokinetics of mescaline might be possible, which could be a reason for highly variable adverse reactions. The involvement of the polymorphic OCT2 in the renal excretion of several psychostimulants could be one reason for individual differences in toxicity.

**Keywords: membrane transport, monoamine transporter, OCT1, organic cation transporter, psychostimulant, SLC22A1, solute carrier, hallucinogen**

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**Abbreviations:** CYP, cytochrome P450; DAT, dopamine transporter; DET, diethyltryptamine; DMT, dimethyltryptamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; MATE2-K, multidrug and toxin extrusion protein 2 kidney-specific; MBDB, *N*-methyl-1,3-benzodioxolylbutanamine; MDAI, 5,6-methylenedioxy-2-aminoindane; MDEA, 3,4-methylenedioxy-*N*-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; $MPP^+$, 1-methyl-4-phenylpyridinium; NET, noradrenaline (norepinephrine) transporter; OCT, organic cation transporter; PCR, polymerase chain reaction; PMA, *para*-methoxyamphetamine; PMMA, *para*-methoxymethamphetamine; SERT, serotonin transporter; SLC, solute carrier; WT, wild-type.

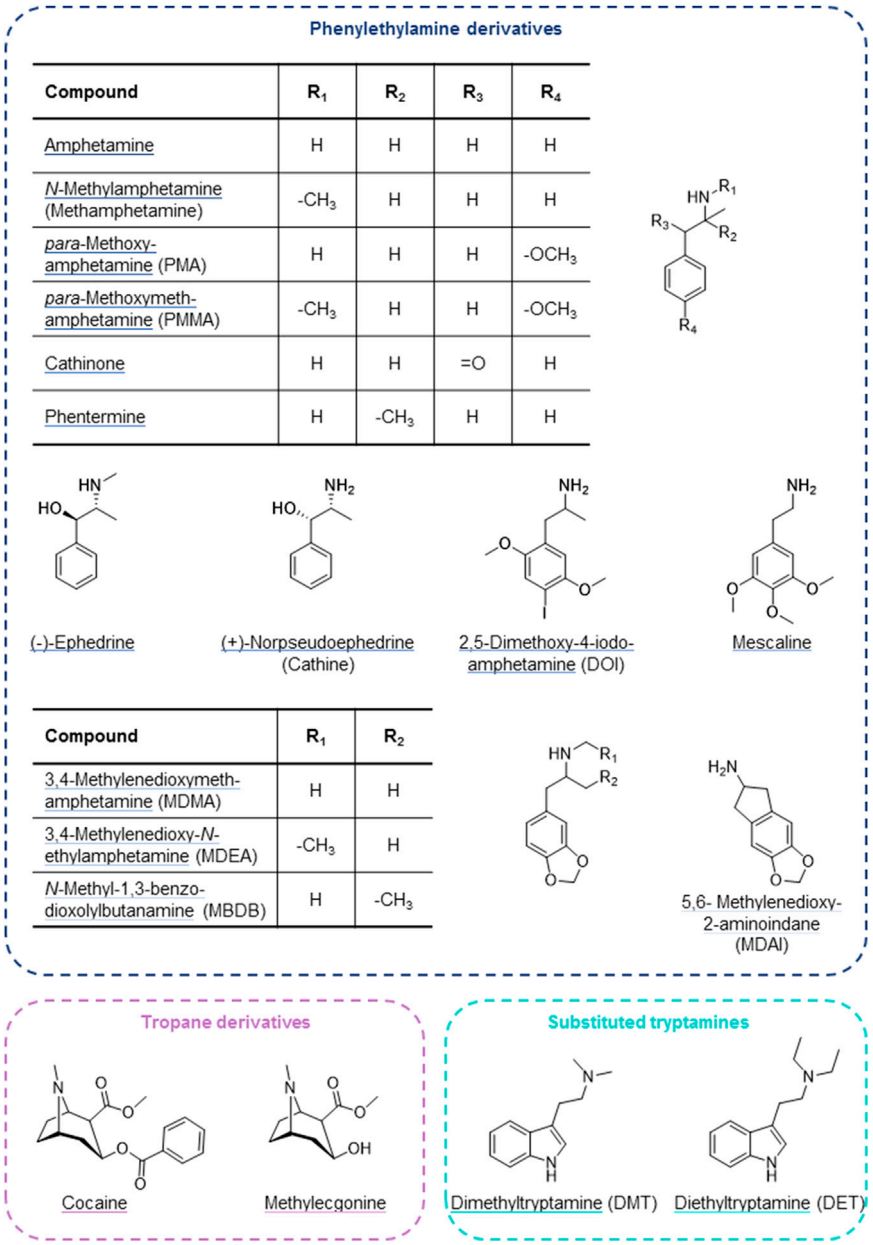



**FIGURE 1 |** Psychostimulant and hallucinogenic drugs assessed for cell uptake by OCTs and high-affinity monoamine transporters.

## INTRODUCTION

Psychostimulants modulate wakefulness and mental performance. They function as indirect sympathomimetics by raising synaptic concentrations of monoamine neurotransmitters through stimulating their release from presynaptic vesicles and/or inhibiting reuptake. Psychostimulants can also interfere with monoaminergic neurotransmitter metabolism and interact with monoaminergic receptors and other targets (Luethi and Liechti, 2020; Reith and Gnegy, 2020). Amphetamine and other phenylethylamine derivatives (**Figure 1** top) form a large group of such indirect sympathomimetics. They are used in the treatment of attention deficit hyperactivity disorder and narcolepsy but are also frequently found in illicit drugs (e.g., "speed", "ecstasy", "crystal meth") (Sharma and Couture, 2014; Luethi and Liechti, 2020). Another indirect sympathomimetic is cocaine (**Figure 1** bottom left), a tropa-alkaloid and, historically, the first local anesthetic. Its (widely illegal) use as a psychostimulant nowadays far exceeds its therapeutic application in local anesthesia. Psychostimulants are among the most popular drugs of abuse. A related and partially overlapping class of psychoactive substances are the hallucinogens (psychedelics), which alter perception, cognition, and mood. These include tryptamine derivatives, such as the alkaloid dimethyltryptamine (DMT). It

**TABLE 1 |** Physicochemical properties of investigated psychoactive compounds (predicted using MarvinSketch, version 19.8, ChemAxon, Budapest, Hungary).

| Test compound | $LogD_{pH\ 7.4}$ | $pK_a$ | % Positively charged at pH 7.4 |
|---|---|---|---|
| Amphetamine | −0.67 | 10.01 | 99.76 |
| Methylamphetamine | −0.44 | 10.21 | 99.85 |
| PMA | −0.85 | 10.04 | 99.77 |
| PMMA | −0.52 | 10.03 | 99.76 |
| Cathinone | 0.79 | 7.55 | 58.59 |
| Phentermine | −0.55 | 10.25 | 99.78 |
| (-)-Ephedrine | −0.78 | 9.53 | 99.26 |
| Cathine | −1.05 | 9.37 | 98.94 |
| DOI | 0.02 | 9.90 | 99.69 |
| Mescaline | −1.37 | 9.77 | 99.58 |
| MDMA | −0.76 | 10.14 | 99.82 |
| MDEA | −0.46 | 10.22 | 99.85 |
| MBDB | −0.34 | 10.28 | 99.87 |
| MDAI | −1.33 | 9.96 | 99.73 |
| Cocaine | 0.82 | 8.85 | 96.54 |
| Methylecgonine | −1.86 | 9.04 | 97.76 |
| DMT | 0.17 | 9.55 | 99.29 |
| DET | 0.39 | 10.08 | 99.79 |

is a main constituent of ayahuasca, the plant brew used traditionally by indigenous inhabitants of the Amazon region for spiritual and religious ceremonies. DMT and its diethyl analogue (**Figure 1** bottom right) show structural resemblance to the neurotransmitter serotonin and thereby function as agonists at 5-$HT_{2A}$ and related receptors (Nichols, 2016; Luethi and Liechti, 2020). Another traditional hallucinogen is mescaline, a phenethylamine alkaloid found in cacti (Ogunbodede et al., 2010; Nichols, 2016; Luethi and Liechti, 2020). It is a partial agonist at 5-$HT_{2A}$ and 5-$HT_{2B}$ receptors and a full agonist at the 5-$HT_{2C}$ receptor (Dinis-Oliveira et al., 2019).

Many psychoactive substances are substrates or inhibitors of the noradrenaline (norepinephrine) transporter (NET), the dopamine transporter (DAT), and/or the serotonin transporter (SERT) (Luethi and Liechti, 2020). These high-affinity transport proteins are expressed at presynaptic neurons, where they mediate the reuptake of monoamine neurotransmitters from the synaptic cleft to terminate synaptic signal transmission and for recycling (Torres et al., 2003). They are members of the large Solute Carrier (SLC) superfamily and coded for by the genes *SLC6A2* (NET), *SLC6A3* (DAT), and *SLC6A4* (SERT).

Organic cation transporters (OCTs) are also SLCs with a broad, partially overlapping substrate spectrum that is predominantly comprised of hydrophilic, organic cationic substances (including monoamine neurotransmitters as well as many drugs) (Busch et al., 1998; Gründemann et al., 1998; Wu et al., 1998; Koepsell et al., 2007). OCT1 (*SLC22A1*) and, to a lesser extent, OCT3 (*SLC22A3*) are expressed on the sinusoidal membrane of hepatocytes, where they mediate cellular uptake for hepatic metabolism (Nishimura and Naito, 2005; Nies et al., 2009). A high degree of genetic variation exists for *SLC22A1*, and several of these variants strongly impact transporter expression and function (Koepsell et al., 2007; Seitz et al., 2015). This may affect the pharmacokinetics of compounds that are substrates of OCT1, as has been shown, for example, for the opioid analgesics morphine and *O*-desmethyltramadol (Tzvetkov et al., 2011; Tzvetkov et al., 2013; Venkatasubramanian et al., 2014; Stamer et al., 2016), the antimalarial prodrug proguanil (Matthaei et al., 2019), the anti-asthma drug fenoterol (Tzvetkov et al., 2018), sumatriptan that is used for the treatment of migraine (Matthaei et al., 2016), and, to a minor extent, for the antidiabetic drug metformin (Tzvetkov et al., 2009; Yee et al., 2018). The psychoactive substances studied here (**Figure 1**) were selected based on physicochemical properties (organic cations with $pK_a$ > 8.4 and relatively hydrophilic substances with a $\log D_{pH\ 7.4}$ < 2; **Table 1**) that make them potential substrates for OCTs. Consequently, their pharmacokinetics could potentially be affected by OCT polymorphism as well. OCT2 (*SLC22A2*) is mainly found on the basolateral membrane of kidney epithelial cells (Motohashi et al., 2002; Motohashi et al., 2013). Together with multidrug and toxin extrusion protein 2 kidney-specific (MATE2-K, *SLC47A2*), an efflux transporter expressed on the brush-border membrane of the proximal tubule, it mediates transport across the epithelium for renal excretion (Motohashi et al., 2013). *SLC22A2* variants are less frequent compared to the gene coding for OCT1, and only a few affect OCT2 expression or function. The most frequent of these is Ala270Ser, which causes a moderate decrease in OCT2 activity (Zolk et al., 2009). As many psychoactive substances are structurally related to the neurotransmitters and OCT substrates noradrenaline, dopamine, and serotonin and have physicochemical properties in line with typical OCT substrates, their pharmacokinetics may be determined by OCTs and influenced by OCT1 (and possibly OCT2) polymorphism.

Although mainly expressed in peripheral tissues, OCT2 and OCT3 are also found on postsynaptic neurons (and OCT3 in astrocytes) predominantly in aminergic regions of the central nervous system. There, they may be involved in reuptake of monoamine neurotransmitters in brain areas lacking the high-affinity transporters, at distance from the aminergic nerve endings, or as an alternative when the high-affinity transporters are saturated or inhibited (Wu et al., 1998; Vialou et al., 2008; Bacq et al., 2012; Couroussé and Gautron, 2015). OCT2 appears to be involved in the uptake of noradrenaline and serotonin in particular, while OCT3 was found to be more strongly responsible for dopamine clearance (Vialou et al., 2008; Bacq et al., 2012). Interestingly, it has also been shown that amphetamines can induce neurotransmitter release through OCT3, which is capable of bi-directional transport (Mayer et al., 2018; Mayer et al., 2019). Thus, OCTs may not only determine the pharmacokinetics of psychoactive drugs but appear to be also involved in their actions.

Given the potential dual role of OCTs with respect to psychoactive drugs and the current lack of understanding of the pharmacokinetics and pharmacogenetics for these compounds, we characterised the transmembrane transport by polyspecific OCTs as well as high-affinity monoamine reuptake transporters. Of particular interest are those psychostimulants that are stereoisomers of one another (ephedrine, norephedrine, their enantiomers and diastereomers), because the impact of

stereospecificity on membrane transport is as yet not well understood but previous results suggest partially strong enantiopreferences (Jensen et al., 2020).

# MATERIALS AND METHODS

## Test Compounds

The psychoactive compounds studied here were selected based on their physicochemical properties that would make them likely substrates for OCTs. Selection criteria included hydrophilicity (logD at pH 7.4 of less than 2), at least 90% positively charged at physiological pH ($pK_a$ > 8.4), and molecular mass not higher than 500 Da. The reasons for these were that lipophilic compounds permeate membranes mostly by diffusion, while membrane transport is mostly relevant for more hydrophilic compounds, as well as the observation that typical OCT1 substrates are usually positively charged and of low to moderate size. Cathinone ($pK_a$ of 7.55) did not meet our selection criteria but was nonetheless included due to a low renal elimination (2–7% unchanged in urine) and, consequently, high rate of metabolism which, if taking place in the liver, might depend on hepatic uptake via OCT1 (Kalix and Braenden, 1985; Toennes and Kauert, 2002). Ranitidine-d6 was purchased from Toronto Research Chemicals (Toronto, Canada) and Tulobuterol from Santa Cruz Biotechnology (Darmstadt, Germany); all other test compounds and internal standards were bought from Sigma-Aldrich (Taufkirchen, Germany).

## Generation of Transporter-Overexpressing Cell Lines

Transport experiments were done using HEK293 cells stably transfected to overexpress OCT1*1 (wild-type), OCT1*2 (M420del), OCT1*3 (R61C), OCT1*4 (G401S), OCT1*5 (M420del, G465R), OCT1*6 (C88R, M420del), OCT1*7 (S14F), OCT1*8 (R488M), as well as wild-type OCT2, OCT3, NET, DAT, SERT, or MATE2-K. All cell lines were generated using the Flp-In system (Thermo Fisher Scientific, Darmstadt, Germany) as previously described (Saadatmand et al., 2012; Seitz et al., 2015; Chen et al., 2017), except for the OCT3-overexpressing HEK293 cells that were a kind gift from Drs. Koepsell and Gorbulev (University of Würzburg, Germany). The cells were kept in culture for no more than 30 passages.

The high-affinity monoamine transporters were also stably transfected into HEK293 cells by use of the Flp-In system (Thermo Fisher Scientific, Darmstadt, Germany). Coding sequences of *SLC6A2* (NET), *SLC6A3* (DAT), and *SLC6A4* (SERT) were obtained from Source BioScience (Nottingham, United Kingdom; pBluescriptR:SLC6A2) or Addgene (Watertown, MA, United States; pcDNA3.1-hDAT was a gift from Susan Amara, Addgene plasmid # 32810, http://n2t.net/addgene:32810, RRID:Addgene_32810 and hSERT pcDNA3 was a gift from Randy Blakely, Addgene plasmid # 15483, http://n2t.net/addgene:15483, RRID:Addgene_15483 (Ramamoorthy et al., 1993)). After sequence correction and cloning into the pcDNA5 vector, generation and characterization of the cell lines were carried out as described before for the above-mentioned cell lines (Saadatmand et al., 2012; Seitz et al., 2015). Genomic integration was validated for two independent cell clones by three polymerase chain reactions (PCR; **Figure 2**) to verify proper integration (integration PCR) and exclude multiple integration (multiple integration PCR). The presence of the gene of interest was verified by Sanger sequencing of the product of the third PCR (gene-of-interest PCR) after gel extraction (**Figure 2**). Overexpression of monoamine transporters was compared between cell clones by TaqMan® gene expression assays (Thermo Fisher Scientific, Darmstadt, Germany; **Figure 2**). Functional validation of newly generated cell clones was performed using noradrenaline and serotonin as probe drugs and one clone for each transporter was chosen for further transport studies.

## *In vitro* Cellular Uptake Experiments

The HEK293 cells were cultered in DMEM medium supplemented with 10% (v/v) fetal bovine serum as well as penicillin (100 U/ml) and streptomycin (100 µg/ml) obtained from Thermo Fisher Scientific (Darmstadt, Germany). Cells were seeded on 12-well plates coated with poly-D-lysine (Sigma-Aldrich, Taufkirchen, Germany) 48 h before the transport experiments and incubated at 37°C, 95% relative humidity, and 5% $CO_2$. Cell lines overexpressing MATE2-K were incubated with 30 mM $NH_4Cl$ in HBSS+ (10 mM HEPES in HBSS, pH 7.4; Thermo Fisher Scientific, Darmstadt, Germany) for 30 min prior to the assay to invert the direction of transport. All cell lines were washed with 37°C HBSS+ and subsequently incubated with the pre-warmed substrate in HBSS+ at 37°C. The time points for measuring substrate uptake were 1 min for MATE2-K and 2 min for the other SLCs. The uptake rate was experimentally determined to be linear for at least 10 min for OCT1*1. It was assumed to be linear for the other transporters as well, based on previous experience with these expression systems. The reaction was stopped by adding ice-cold HBSS+, and the cells were washed twice with ice-cold HBSS+ before lysis with 80% acetonitrile (LGC Standards, Wesel, Germany) including an internal standard. Subsequently, the intracellular substrate accumulation was determined using LC-MS/MS.

## Concentration Analyses

Intracellular accumulation was measured by HPLC-MS/MS using a Shimadzu Nexera HPLC system with a LC-30AD pump, a SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A controller (Shimadzu, Kyoto, Japan). Separation was done on a Brownlee SPP RP-Amide column (4.6 × 100 mm inner dimension with 2.7 µm particle size) with a C18 pre-column. The aqueous mobile phase contained 0.1% (v/v) formic acid and either 3% (v/v) organic additive (acetonitrile:methanol 6:1 (v/v)) for methylecgonine, 8% for amphetamine, methylamphetamine, cathinone, cathine, (-)-ephedrine, mescaline, MDAI, and DMT, or 20% for PMA, PMMA, DOI, phentermine, MDMA, MDEA, MBDB, cocaine, and DET. Chromatography was done at a flow rate of 0.3 ml/min. For detection, an API 4000 tandem mass spectrometer (AB SCIEX, Darmstadt, Germany) was used in MRM mode. The analytes, corresponding internal standards, and detection parameters are listed in the **Supplementary Table S1**. Peak integration and quantification of the analytes was done using the Analyst software

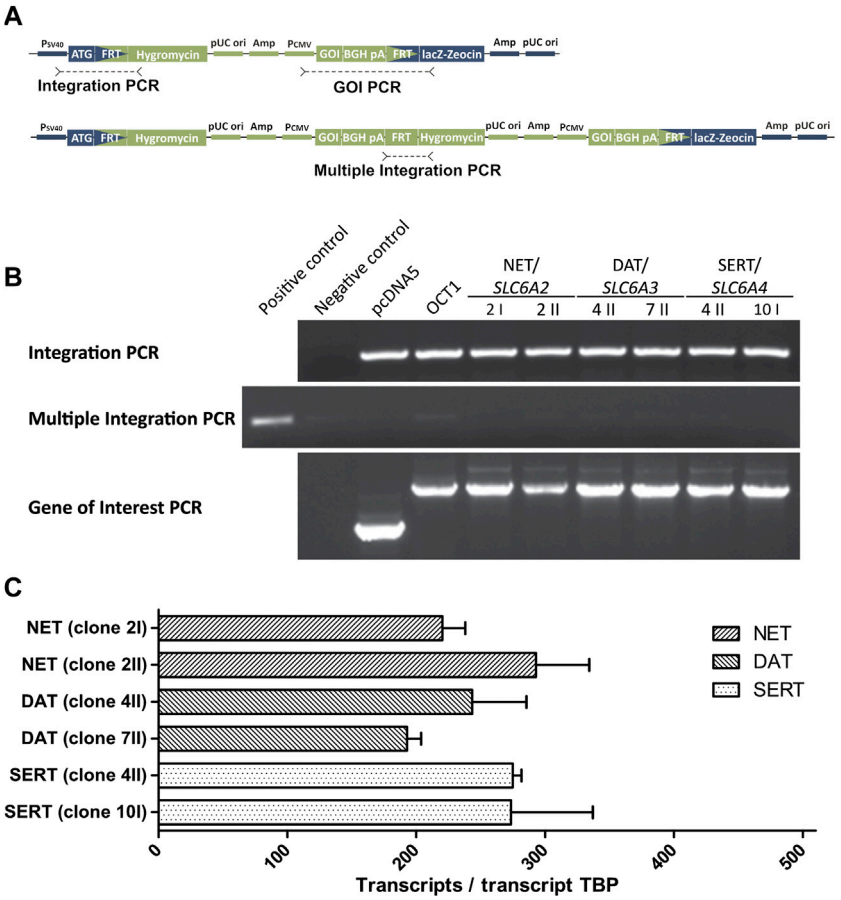



**FIGURE 2 |** Validation of HEK293 cell clones overexpressing monoamine neurotransmitter transporters **(A)** Schematic representation of the expression plasmid pcDNA5 (green) and the host cell line genome (blue) at the FRT site showing the target positions of the three conducted PCRs **(B)** Results of the three validation PCRs that show a successful integration (Integration PCR) for all newly created cell clones that overexpress the high-affinity monoamine transporters. The absence of amplicons in the Multiple Integration PCR indicate a single integration of the pcDNA5 plasmid. The correctness of amplified genes in the Gene of Interest (GOI) PCR was validated by Sanger sequencing **(C)** Quantitative real-time PCR results to confirm comparable overexpression of monoamine transporters, shown as transcripts per transcript of the TATA-binding protein. Only one cell clone was selected per transporter for experiments.

(Version 1.6.2, AB SCIEX, Darmstadt, Germany) and determined by simultaneous measurement of standard curves with known concentrations.

## Calculations

For the screenings, cellular uptake measured in cell lines overexpressing the respective transporter was divided by the uptake measured in an empty vector control cell line to calculate normalised ratios to enable comparisons between test compounds. For studying transport kinetics, the net transport mediated by the overexpressed transporters was calculated by subtracting the cellular uptake measured in an empty vector control cell line from the uptake in cell lines overexpressing the respective transporter. The parameters $K_m$ and $v_{max}$ were estimated by regression analysis using the Michaelis-Menten equation (GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla, CA, United States). Means and standard errors were calculated from individual $K_m$ and $v_{max}$ values of at least three independent experiments. The kinetic parameters $v_{max}$ and $K_m$ were tested for statistical significance over empty vector control cells using Student's t-test with an alpha value of 0.05.

# RESULTS

## Screening of Transport Activity at OCTs, Monoamine Transporters, and MATE2-K

Eighteen psychostimulants and hallucinogens were initially screened for their potential to be substrates for different polyspecific OCTs and high-affinity monoamine neurotransmitter transporters (**Figure 3**), as well as for the efflux transporter MATE2-K (**Supplementary Figure S1**). The compounds were assessed at a concentration of 1 μM, because it is unlikely that low-affinity transport at higher concentrations may have any medical relevance and the relative contribution of

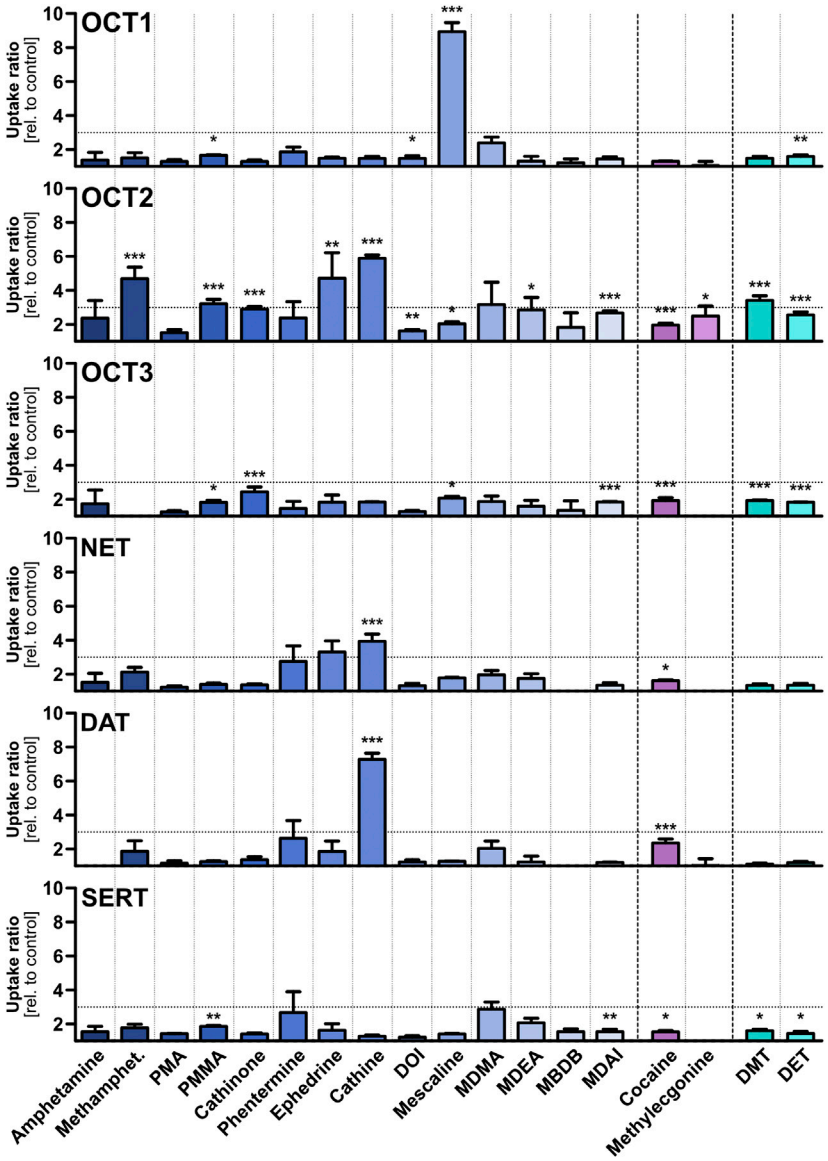



**FIGURE 3 |** Transport of different psychostimulant and hallucinogenic substances at a concentration of 1 μM by OCTs and high-affinity monoamine transporters, shown as the ratios of uptake after 2 min in transporter-transfected cells over empty vector control cells. Shown are the mean values of ≥3 independent experiments +SEM. The horizontal dotted line indicates an uptake ratio of 3, which was set as the minimum threshold for more detailed characterisation. Statistical significance over empty vector control cells was determined using Student's t-test with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

carrier-mediated transport over passive diffusion is significantly greater at lower compared to higher substrate concentrations, as was previously shown for morphine (Tzvetkov et al., 2013). Although the test compounds were selected based on physicochemical properties that are in accordance with those of typical OCT substrates, OCT1 showed high transport activity at this concentration only for mescaline. A cellular uptake in transporter-transfected cells of at least 3-fold higher than in non-overexpressing control cells was selected as the threshold for further studies, as this ratio is suitable to distinguish substrates from non-substrates. Cellular uptake of mescaline was more than 8-fold higher in OCT1-overexpressing cells, which was the highest transport activity that was observed altogether in this study. Interestingly, mescaline was not transported much at 1 μM by any of the other transporters. In contrast to the substrate-specific but very strong transport activity exhibited by OCT1, moderate (4- to 6-fold) cellular uptake by OCT2 was seen for methamphetamine, (-)-ephedrine, and cathine ((+)-norpseudoephedrine) and approximately 3-fold for *para*-methoxymethamphetamine (PMMA) and DMT. OCT3 and MATE2-K (**Supplementary Figure S1**) showed little or no transport activity with any of the 18 psychoactive compounds studied here at 1 μM. Our observation, that amphetamine does not appear to be a substrate of OCT3, is in accordance with previous reports (Zhu et al., 2010).

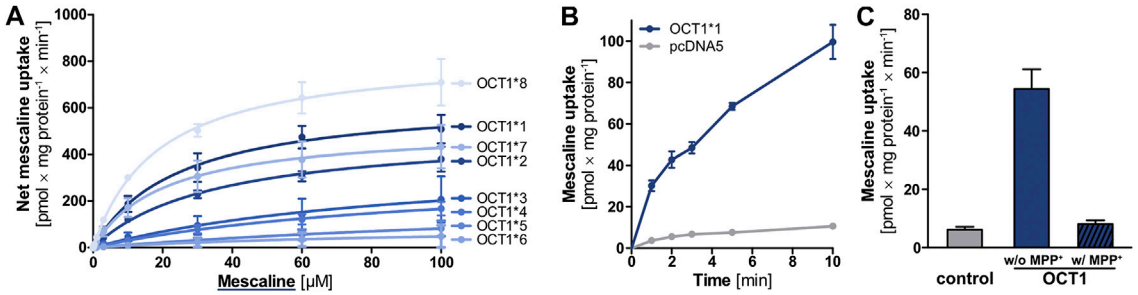



**FIGURE 4 | (A)** Transport of mescaline at different concentrations by OCT1*1 (wild-type) and genetic variants, shown as the cellular uptake in transporter-overexpressing cells with substracted uptake in non-overexpressing control cells. Shown are the mean values of ≥3 independent experiments ±SEM. $K_m$ and $v_{max}$ values are given in **Table 2**. **(B)** Time-dependent uptake of 1 µM mescaline in OCT1*1-overexpressing (blue) and non-overexpressing control (pcDNA5, gray) cells, shown as the mean values of 3 independent experiments ±SEM **(C)** Mescaline transport by OCT1 could be completely inhibited by 1 mM $MPP^+$ to values not significantly different from the unspecific cellular uptake observed in empty vector-transfected cells (control).

**TABLE 2 |** Kinetic parameters for the transport of mescaline by different OCT1 genetic variants.

| Variant | $K_m$ [µM] | $v_{max}$ [pmol × mg protein$^{-1}$ × min$^{-1}$] |
|---|---|---|
| OCT1*1 (WT) | 24.3 (±6.3) | 641.7 (±57.1) |
| OCT1*2 (M420del) | 34.7 (±7.4) | 500.7 (±42.1) |
| OCT1*3 (R61C) | 93.6 (±110.8) | 390.7 (±265.8) |
| OCT1*4 (G401S) | 98.2 (±46.7) | 329.4 (±91.6) |
| OCT1*5 (M420del, G465R) | Not determinable | Not determinable |
| OCT1*6 (M420del, C88R) | Not determinable | Not determinable |
| OCT1*7 (S14F) | 20.2 (±7.9) | 514.6 (±63.8) |
| OCT1*8 (R488M) | 18.6 (±3.7) | 837.2 (±51.5) |

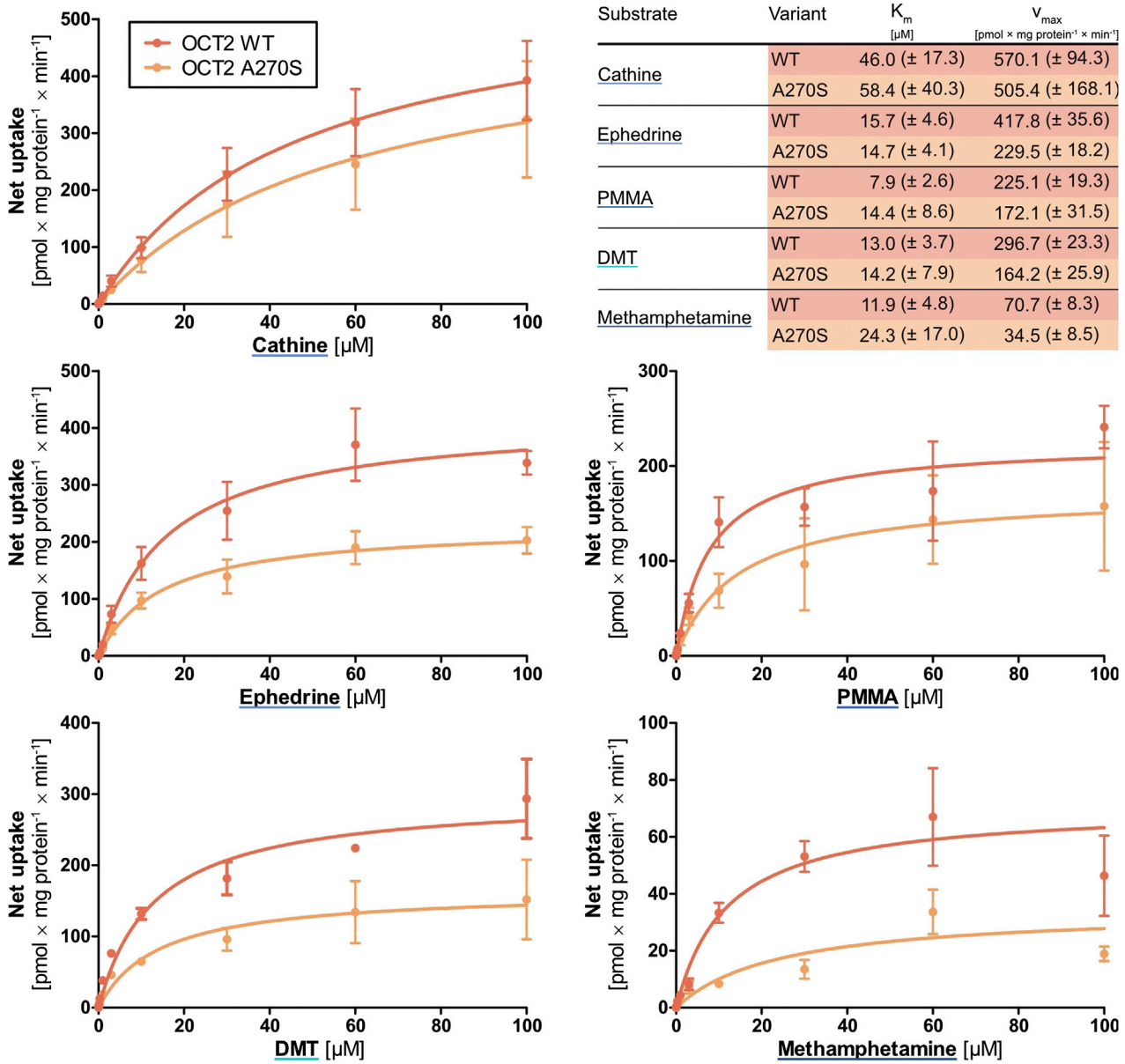
The OCTs are known as low-affinity, high-capacity solute carriers with a very broad substrate spectrum that comprises structurally diverse compounds. In contrast, the monoamine neurotransmitter reuptake transporters NET, DAT, and SERT show high affinities to their respective endogenous substrates and a more narrow substrate profile than the OCTs. Cathine was transported modestly (4-fold) by NET and higher (7-fold) by DAT. No notable transport activity was observed for the other compounds, and none by SERT altogether. Cathine and (-)-ephedrine (as well as their stereoisomers) have been described previously as substrates for NET and DAT in *vitro* experiments with very different setup (Rothman et al., 2003). The slightly higher (albeit still low) uptake of PMA and PMMA by SERT compared to DAT is in line with literature reports that substitution in *para*-position of the phenyl ring of amphetamine derivatives shifts substrate preference toward SERT (Simmler et al., 2014).

## Concentration-dependent Transport of Mescaline by OCT1 Genetic Variants

Mescaline was found in our substrate screenings to be strongly transported by OCT1 and, therefore, it was studied in more detail. Given the high degree of genetic polymorphism and the large differences in transporter activity and expression for some variants, cellular uptake of mescaline was not only characterised for wild-type (OCT1*1) but for OCT1 variants *2 to *8 as well. OCT1*1 transported mescaline with a $K_m$ of 24.3 ± 6.3 µM and a $v_{max}$ of 642 ± 57 pmol × mg protein$^{-1}$ × min$^{-1}$ (**Figure 4A**, **Table 2**). Time-dependent uptake of 1 µM mescaline showed a faster uptake rate within the first minute of incubation and a constant, linear uptake rate for 2 to at least 10 min (**Figure 4B**). The apparently more rapid initial uptake rate is likely a result of high-affinity binding to OCT1, but a short-lived more rapid transport might also be possible. The constant transport rate after 2 min of incubation might be the more relevant transport rate for pharmacokinetics because the exposure of the liver and other organs to drugs and other substances usually occurs for several hours. Mescaline uptake could be completely inhibited by the competitive OCT1 inhibitor 1-methyl-4-phenylpyridinium ($MPP^+$; **Figure 4C**). The $K_m$ was slightly higher and the $v_{max}$ slightly lower for *2, which is analogous to literature data on reduced transport activity for *2 (Seitz et al., 2015; Koepsell, 2020). This was even more pronounced ($K_m$ of 93.6 ± 110.8 and 98.2 ± 46.7 µM; $v_{max}$ of 391 ± 266 and 329 ± 92 pmol × mg protein$^{-1}$ × min$^{-1}$) for *3 and *4, which are known to have strongly reduced transport activity (Seitz et al., 2015; Koepsell, 2020). For the variants *5 and *6 that result in impaired translocation to the plasma membrane (Seitz et al., 2015), very low transport activity was observed. Consequently, $K_m$ and $v_{max}$ values could not be reliably calculated. OCT1*7 exhibited a similar $K_m$ and a modestly reduced $v_{max}$ than OCT1*1. OCT1*8, on the other hand, showed a higher $v_{max}$ than the wild-type, which has been reported previously for a number of substrates as well (Seitz et al., 2015; Koepsell, 2020). To summarise, transport activity of mescaline was slightly lower than wild-type OCT1 in variants *2 and *7, more drastically reduced in *3 and *4, and lowest in *5 and *6, while *8 showed a moderately higher $v_{max}$ than wild-type OCT1.

## Concentration-dependent Transport of Methamphetamine, PMMA, (-)-Ephedrine, Cathine, and DMT by OCT2 Wild-type and A270S Variant

Whereas only mescaline appeared to be a substrate for OCT1, transport *via* OCT2 was seen for methamphetamine, PMMA,




| Substrate | Variant | $K_m$ [μM] | $v_{max}$ [pmol × mg protein$^{-1}$ × min$^{-1}$] |
|---|---|---|---|
| Cathine | WT | 46.0 (± 17.3) | 570.1 (± 94.3) |
| | A270S | 58.4 (± 40.3) | 505.4 (± 168.1) |
| Ephedrine | WT | 15.7 (± 4.6) | 417.8 (± 35.6) |
| | A270S | 14.7 (± 4.1) | 229.5 (± 18.2) |
| PMMA | WT | 7.9 (± 2.6) | 225.1 (± 19.3) |
| | A270S | 14.4 (± 8.6) | 172.1 (± 31.5) |
| DMT | WT | 13.0 (± 3.7) | 296.7 (± 23.3) |
| | A270S | 14.2 (± 7.9) | 164.2 (± 25.9) |
| Methamphetamine | WT | 11.9 (± 4.8) | 70.7 (± 8.3) |
| | A270S | 24.3 (± 17.0) | 34.5 (± 8.5) |

**FIGURE 5 |** Transport of cathine, (-)-ephedrine, PMMA, DMT, and methamphetamine at different concentrations by wild-type OCT2 (red) and the variant A270S (orange), shown as the cellular uptake in transporter-overexpressing cells with substracted uptake in non-overexpressing control cells. Shown are the mean values of ≥3 independent experiments ±SEM.

(-)-ephedrine, cathine, and DMT. These compounds were subsequently assessed in greater detail (**Figure 5**). For methamphetamine, the $v_{max}$ for wild-type OCT2 was only 70.7 ± 8.3 pmol × mg protein$^{-1}$ × min$^{-1}$, whereas it was between 225 and 570 pmol × mg protein$^{-1}$ × min$^{-1}$ for the other four compounds. The $K_m$ values were around 10 μM except for cathine (46.0 ± 17.3 μM). For the A270S variant, the $v_{max}$ values were slightly to moderately lower (except for PMMA) and the $K_m$ values either similar ((-)-ephedrine and DMT) or up to 4-fold higher (methamphetamine, PMMA, cathine) compared to wild-type OCT2, in agreement with literature reports that the A270S exchange can lead to a moderate decrease in OCT2 activity (Zolk et al., 2009).

## Concentration-dependent Transport of Cathine by NET and DAT

Cathine was the only compound for which notable cellular uptake was observed by the high-affinity monoamine transporters NET and DAT. Further characterisation and a comparison between NET and DAT revealed that the $K_m$ was 21-fold and the $v_{max}$ 10-fold higher for DAT (**Figure 6**). Yet, both $K_m$ and $v_{max}$ were still

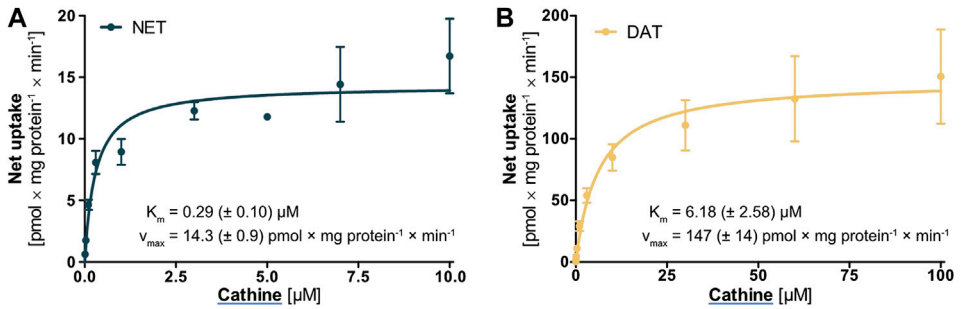



**FIGURE 6 |** Transport of cathine at different concentrations by **(A)** NET and **(B)** DAT, shown as the cellular uptake in transporter-overexpressing cells with substracted uptake in non-overexpressing control cells. Shown are the mean values of ≥3 independent experiments ±SEM.

significantly lower compared to OCT2, in line with the general description of NET and DAT as high-affinity and low-capacity transporters.

## DISCUSSION

In this study, three groups of psychostimulants and hallucinogens (14 phenylethylamine derivatives, the tropanes cocaine and methylecgonine, and the substituted tryptamines dimethyl- and diethyltryptamine) were assessed for their substrate properties for OCTs as well as for high-affinity monoamine transporters. OCTs are known to have a very broad substrate profile that comprises many different structural classes. It is therefore surprising that only relatively few of the 18 psychoactive compounds studied here were moderate or good OCT substrates, especially because these were selected based on physicochemical properties that were in accordance with those of typical OCT substrates. Other transporters, such as OCTN1 and OCTN2, the proposed $H^+$-organic cation antiporter, or ATP-binding cassette efflux transporters might potentially be more relevant for some of the tested psychoactive compounds.

Only mescaline was transported significantly at 1 µM by OCT1, and that this was the highest transport activity observed here altogether. With a $pK_a$ of 9.77, a $logD_{pH\ 7.4}$ of -1.37, and a molecular mass of 211.3 g/mol, its physicochemical properties are not significantly different from those of the other compounds (**Table 1**). It is thus reasonable to wonder what properties make mescaline the only substrate at this concentration compared to the 17 other compounds studied here. Possible explanations are not evident from its chemical structure, as it is an amphetamine derivative structurally relatively similar to many of the other phenylethylamines.

Mescaline is an alkaloid biosynthesised from tyrosine in different cacti, where it is found at concentrations of 0.05–4.7% by dry weight (Ogunbodede et al., 2010). *Lophophora williamsii* (peyote cactus) and several *Echinopsis* species (e.g., *Echinopsis pachanoi* and *Echinopsis peruvianus*, also known as the San Pedro and the Peruvian torch cacti) have a long-standing use in religious ceremonies and traditional medicine of South American indigenous populations. The hallucinogenic effects of these cacti were attributed to their relatively high mescaline contents (Ogunbodede et al., 2010; Dinis-Oliveira et al., 2019; da Silveira Agostini-Costa, 2020). Interestingly, OCT1 deficiency or reduced activity is more frequently found in Central and South American populations than in most other parts of the

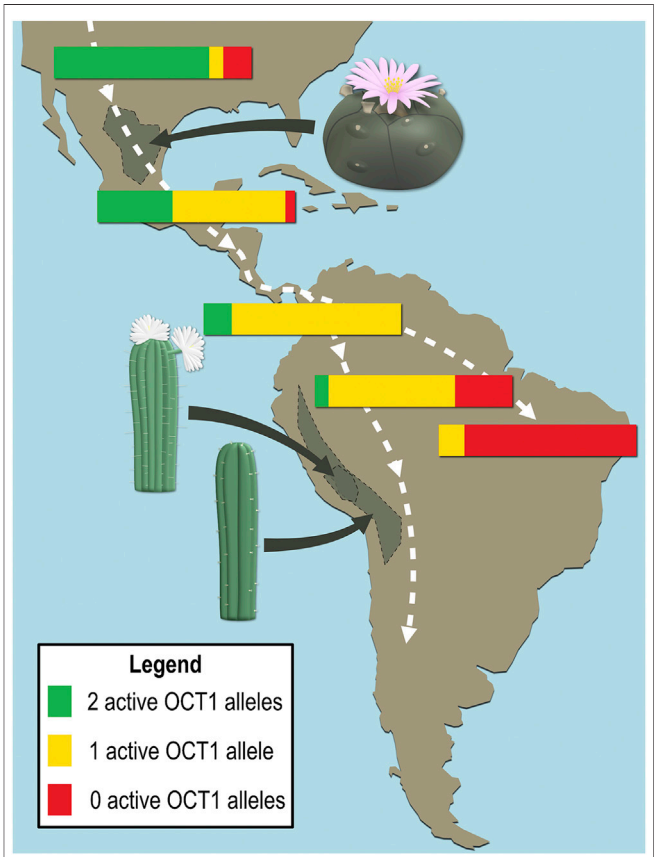



**FIGURE 7 |** Schematic representation of the frequency distributions of active and inactive OCT1 alleles in local populations and natural habitats of the high mescaline-containing cacti *Lophophora williamsii* (peyote), *Echinopsis pachanoi* (San Pedro), and *Echinopsis peruvianus* (Peruvian torch). The white dashed lines broadly illustrate the migration pattern during the first population of the continent by humans.

world and the prevalence of inactive alleles generally increases further south on the American continent (**Figure 7**) (Seitz et al., 2015). It is likely that OCT1 deficiency was somehow advantageous, e.g., in connection with dietary ingredients that are OCT1 substrates (or perhaps mescaline?), and inactive alleles thus dominated as the first human inhabitants of the continent migrated south.

Typical mescaline dosages are in the range of 170–400 mg, which induce a psychedelic state that may involve visual hallucinations, altered perception, synesthaesia, and euphoria. The lifetime prevalence of mescaline use over the past 3 decades was estimated to be between 3–4% in the United States (Dinis-Oliveira et al., 2019; Johnson et al., 2019). Being a high-affinity partial agonist for the 5-$HT_{2A}$ receptor, potential therapeutic uses for mescaline were proposed for disorders associated with serotonin deficiency, such as addiction, anxiety, and depression (Kyzar et al., 2017; Johnson et al., 2019). Based on the key finding of this study, that mescaline is a strong substrate of the genetically highly polymorphic OCT1, large interindividual variations in mescaline pharmacokinetics might be possible. This could lead to intoxication and other adverse effects due to decreased elimination in carriers of alleles with reduced or absent OCT1 activity (e.g., OCT1 variants *2 to *6, which are particularly common in European populations, or OCT1*7 that is frequently found in Africans and Afro-Americans (Seitz et al., 2015)). However, a substance being identified as OCT1 substrate in vitro may not necessarily be affected by OCT1 genetic polymorphism in vivo, as illustrated by the example of the indirect sympathomimetic compound tyramine (Rafehi et al., 2019). Thus, the effects of OCT1 genotype on mescaline should be studied in vivo and its clinical implications taken into consideration when developing therapeutic interventions involving mescaline.

Another key result of this study was that methamphetamine, PMMA, (-)-ephedrine, cathine, and DMT were substrates of OCT2 and that their transport was moderately reduced in the A270S variant. OCT2 is strongly expressed in the kidneys, where it contributes to transepithelial transport of usually hydrophilic substances and thereby renal elimination. Cathine was excreted unchanged in urine to 46–65% in four healthy volunteers and the renal elimination was reported to be 70% for (-)-ephedrine and 30–54% for methamphetamine (Toennes and Kauert, 2002; www.dosing.de and www.drugbank.ca, both accessed on September 16, 2020). The reduced transport by the A270S variant of OCT2 might thus possibly result in a decreased elimination of these compounds. Besides variation due to inherited polymorphisms, variation in renal elimination of these psychostimulants may additionally arise from drug-drug interactions or conditions associated with increased blood concentrations of endogenous organic cations. DMT, on the other hand, is extensively metabolised and excreted unchanged in urine only to a very low extent (e.g., 0.16% following intramuscular administration) (Sitaram et al., 1987; Barker, 2018). OCT2 polymorphism is thus unlikely to have any significant effects on DMT pharmacokinetics but might still influence local concentrations of DMT as well as of methamphetamine, PMMA, (-)-ephedrine, and cathine in the central nervous system due to OCT2 expression in postsynaptic neurons.

OCT1 and OCT2 polymorphism is not the only form of genetic variation that may affect the above-mentioned compounds. Metabolising enzymes and target receptors may also be polymorphic. A few examples regarding the pharmacogenetics of these compounds are given in **Table 3**. A good example for discussing the general importance of genetic polymorphism is MDMA, as this psychostimulant has been studied in greater detail. MDMA is widely used as the recreational drug "ecstasy" but therapeutic use for the treatment of posttraumatic stress disorder has also been proposed (Mithoefer et al., 2011; Mithoefer et al., 2013; Amoroso and Workman, 2016; Mithoefer et al., 2016). It is a substrate of the polymorphic enzymes cytochrome P450 (CYP) 2C19, 2B6, and 1A2, which catalyze the conversion to 3,4-methylenedioxyamphetamine. Carriers of genetic variants that result in increased activity of these enzymes showed higher metabolism and CYP2C19 poor metabolisers had greater cardiovascular effects in response to MDMA consumption (Schindler et al., 2014; Vizeli et al., 2017). Poor metabolisers for the highly polymorphic CYP2D6 also showed higher cardiovascular responses, but only to a minor extent due to the inhibition of CYP2D6 (Schmid et al., 2016). Based on in vitro data, the effect of CYP2D6 polymorphism was previously predicted to be higher (La Torre et al., 2012). MDMA has a basic secondary amine group that is protonated to 99.8% at physiological pH (**Table 1**). It would thus require a transport mechanism for efficient passage across cell membranes and into hepatocytes for metabolism. Our results suggest that OCTs only contribute to a minor extent. Although MDMA is not a good OCT substrate, its metabolites might possibly be (as we had previously shown analogously for different opioids, where their more hydrophilic metabolites were better OCT substrates (Meyer et al., 2019)). For example, the main metabolites 3,4-dihydroxyamphetamine and 3,4-dihydroxymethamphetamine are more hydrophilic than MDMA and might thus potentially be better OCT substrates, as they would likely rely more strongly on transport mechanisms to traverse cell membranes. However, the present study has shown that substrate specificity cannot always be predicted based on physicochemical properties alone. Although a number of contributors to the serotonergic system are polymorphic, significant variation in MDMA effects were not seen in healthy humans (Vizeli et al., 2019). NET polymorphism also showed only minor effects on the cardiovascular response to MDMA in clinical studies (Vizeli et al., 2018). To summarise this, genetic polymorphism significantly determines the pharmacokinetics but not so much the pharmacodynamics of MDMA (and possibly of other psychostimulants as well).

A concept that has so far not received much attention is stereoselectivity in membrane transport. Recent results from our laboratory have shown that transmembrane transport of adrenergic drugs by OCTs can show strong enantiospecificity (Jensen et al., 2020). The phenylethylamine derivatives cathine (also referred to as (+)-norpseudoephedrine) and (-)-ephedrine that were assessed in this study are chiral compounds and structurally very closely related. If it were not for the methyl substitution at the amino group (**Figure 1**), both compounds would be stereoisomers of one another. With this in mind, it

**TABLE 3 |** Pharmacogenetics of methamphetamine, PMMA, (-)-ephedrine, cathine, mescaline, and DMT (this list is not exhaustive).

| Test compound | Substrate of | | | Polymorphic targets[a] | References |
|---|---|---|---|---|---|
| | OCT1 | OCT2 | Polymorphic enzymes[a] | | |
| Methamphetamine | – | ++ | CYP2D6, FMO3 | TAAR1, VMAT2, MAO | Cashman et al. (1999), Eiden and Weihe (2011), Miller 2011, Smith et al. (2012), and Matsusue et al. (2018) |
| PMMA | – | + | CYP2D6 | TAAR1, 5-$HT_{2A}$ | Simmler et al. (2014), and Vevelstad et al. (2017) |
| (-)-Ephedrine | – | ++ | | $\beta_2$-adrenoceptor | Rao et al. (2019) |
| Cathine | – | +++ | | $\beta_1$- and $a_{2A}$-adrenoceptors | Adeoya-Osiguwa and Fraser (2007) |
| Mescaline | +++ | – | Possibly MAO | 5-$HT_{2A}$,5-$HT_{2C}$,TAAR1 | Spector (1961), Lerer et al. (2001), Mulder et al. (2007), Kling et al. (2008), Hoekstra et al. (2010), Rickli et al. (2015), and Dinis-Oliveira et al. (2019) |
| DMT | – | + | MAO-A | 5-$HT_{2A}$,5-$HT_{2C}$,TAAR1 | Keiser et al. (2009), Rickli et al. (2016), and Barker (2018) |

[a]*Abbreviations: 5-HT, 5-hydroxytryptamine; CYP2D6, cytochrome P450 subtype 2D6; FMO3, Flavin-containing monooxygenase 3; MAO, monoamine oxidase; TAAR1, trace amine-associated receptor 1, VMAT2, vesicular monoamine transporter 2*

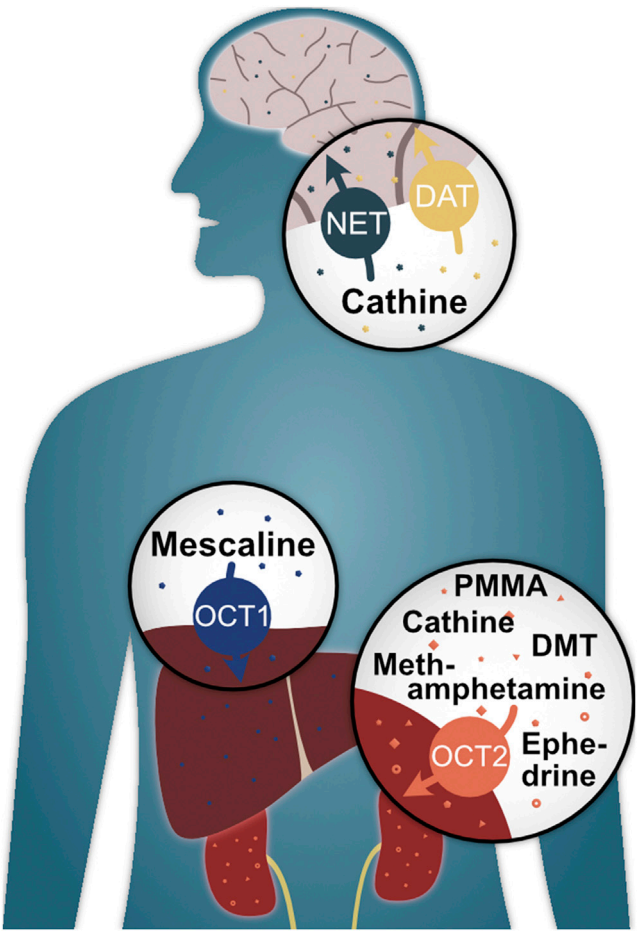



**FIGURE 8 |** Illustrated summary of key findings of this study and their potential biological relevance.

appears astonishing that cathine was found to be a good substrate of DAT whereas (-)-ephedrine was not, despite their close structural resemblance. Whether this difference in transport was due to the opposite steric orientation of the hydroxyl group or due to the methyl substitution at the amino group cannot be deduced from this study.

To summarise, this study has shown that the classic hallucinogen mescaline is a strong substrate of the genetically highly polymorphic OCT1 (**Figure 8**) and that genetic variants show altered cell uptake, which may have clinical implications. It was also found that the psychoactive compounds methamphetamine, PMMA, (-)-ephedrine, cathine, and DMT are substrates of OCT2 with partially moderate reductions in cell uptake in the A270S variant. Cathine was also discovered to be a substrate of NET and DAT. As to the question of whether OCT1 is a drug trafficker or not, we would argue that it is one indeed. However, it is a very selective one with a clear preference for the hallucinogenic compound mescaline, which is rather unusual for OCT1 given its generally broad substrate profile.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

Conceptualisation: OJ, MR, and JB; Funding acquisition: MR and JB; Investigation: OJ and LG; Methodology: OJ and JB; Project administration: OJ, MR, and JB; Supervision: JB; Visualisation: OJ and MR; Writing – original draft: OJ and MR; Writing – review and editing: MR and JB.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.609811/full#supplementary-material.

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MINI REVIEW
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# Regulation Mechanisms of Expression and Function of Organic Cation Transporter 1

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The organic cation transporter 1 (OCT1) belongs together with OCT2 and OCT3 to the solute carrier family 22 (SLC22). OCTs are involved in the movement of organic cations through the plasma membrane. In humans, OCT1 is mainly expressed in the sinusoidal membrane of hepatocytes, while in rodents, OCT1 is strongly represented also in the basolateral membrane of renal proximal tubule cells. Considering that organic cations of endogenous origin are important neurotransmitters and that those of exogenous origin are important drugs, these transporters have significant physiological and pharmacological implications. Because of the high expression of OCTs in excretory organs, their activity has the potential to significantly impact not only local but also systemic concentration of their substrates. Even though many aspects governing OCT function, interaction with substrates, and pharmacological role have been extensively investigated, less is known about regulation of OCTs. Possible mechanisms of regulation include genetic and epigenetic modifications, rapid regulation processes induced by kinases, regulation caused by protein–protein interaction, and long-term regulation induced by specific metabolic and pathological situations. In this mini-review, the known regulatory processes of OCT1 expression and function obtained from *in vitro* and *in vivo* studies are summarized. Further research should be addressed to integrate this knowledge to known aspects of OCT1 physiology and pharmacology.

**Keywords: transporter, regulation, excretion, liver, kidneys**

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## INTRODUCTION

Organic cations (OCs) are positively charged substances with multiple biological significances as neurotransmitters, metabolic waste products, xenobiotics, and drugs. Their movement through the plasma membrane is mediated by transporters, such as the organic cation transporters (OCTs), which for this reason have important physiological, toxicological, and pharmacological implications. Three OCT paralogs are known: OCT1–3, which work as polyspecific, pH- and $Na^+$-independent, bidirectional transporters (Ciarimboli, 2008). OCTs have a species-specific tissue distribution. For example, OCT1 in humans is mainly expressed in the liver (Gorboulev et al., 1997; Nies et al., 2009), while in rodents, it is also present in the kidneys (Jonker and Schinkel, 2004; Holle et al., 2011). Important substances of endogenous and exogenous origin are transported by OCT1. Acetylcholine and monoamine neurotransmitters, and the antidiabetic metformin and the anticholinergic trospium are examples for substrates of endogenous and exogenous origin, respectively (Busch et al., 1996; Wang et al., 2002; Lips et al., 2005; Wenge et al., 2011). Since OCT1 has a high level of expression in excretory organs, its activity has the potential to significantly impact not only local but

also systemic concentrations of its substrates. Many aspects of physiology and pharmacology of OCT1 are well known; however, less attention has been paid to regulation processes of this transporter. Therefore, this mini-review is aimed at collecting the information available in the literature about regulation of OCT1 and to underline the possible physiological, pharmacological, and pathological implications of such a regulation.

## CELLULAR PROCESSING OF OCTS

Generally, OCTs localize to the basolateral membrane domain of polarized cells, and, specifically, OCT1 is highly expressed in the sinusoidal membrane of the hepatocytes (Wright and Dantzler, 2004). However, immunohistochemical staining suggested that OCT1 may be also present in the apical domain of the plasma membrane of human renal tubules (Tzvetkov et al., 2009) and enterocytes (Han et al., 2013), suggesting that the localization signals determining polarized expression of OCTs are not inherent to the OCT structure, but probably depend on specific processing mechanisms of the cells, where the transporters are expressed. Human OCT1 (hOCT1), like all the other OCTs, contains potential *N*-linked glycosylation sites in the big extracellular loop (Zhang et al., 1997). This glycosylation may be important for the trafficking of OCTs to the plasma membrane, as demonstrated for rabbit OCT2 (Pelis et al., 2006). Cysteine residues in the big extracellular loop of rat OCT1 (rOCT1) are important for transporter homo-oligomerization, which influences its plasma membrane insertion, without changing the transport characteristics (Keller et al., 2011). In other OCT paralogs, cysteine residues in the extracellular loop seem to have a similar meaning for oligomerization processes and transporter cellular processing (Brast et al., 2012), suggesting that this is a common property of OCTs. Therefore, modifications in this part of OCTs can change their cellular expression pattern and activity.

Another possible mechanism to regulate protein activity derives from a direct interaction with other proteins. Such an interaction may be important for regulation of cellular processing of the transporter, like its trafficking to/from the plasma membrane, and for stabilization of its expression in the plasma membrane. For example, the importance of interaction partners for transporter regulation has been already shown for the $Na^+$-glucose cotransporter 2 (SGLT2) and for the $Na^+$-dependent neutral amino acid transporter B (0)AT1 in the kidneys. Here, a direct interaction with PDZK1-interacting protein 1 (PDZK1P1, also known as MAP17, a protein mainly expressed in the apical brush border membranes from renal proximal tubules) stimulates SGLT2 activity (Calado et al., 2018), and the interaction of B(0) AT1 with collectrin stabilizes the transporter in the apical plasma membrane of renal proximal tubules (Camargo et al., 2009). Focusing on OCT1, a specific interaction of OCT1 with another protein, may explain why the transporter has a clear basolateral cellular localization in some tissues, while in others, it appears to be expressed on the apical membrane domain. However, there are only few studies aimed at identifying OCT1 interaction partners. A screening performed using the mating-based split-ubiquitin system, a special yeast-two-hybrid technique, able to detect protein–protein interactions taking place in the plasma membrane, identified 24 potential interaction partners for hOCT1 (Snieder et al., 2019). According to gene ontology annotations, the interacting proteins are associated mainly with transport processes, vesicle-mediated transport, signaling pathways, protein modification, homeostatic processes, and cell adhesion (Snieder et al., 2019). The cellular distribution of the identified interaction partners may reflect hOCT1 cellular processing: they are localized in the plasma membrane (CD9 (tetraspanin-29), CYSTM1 (cysteine-rich and transmembrane domain containing protein 1), and PDZKP1), in the endoplasmic reticulum (KRTCAP2 (keratinocyte-associated protein 2), SERP1 (stress-associated endoplasmic reticulum protein 1), VAMPB (vesicle-associated membrane protein-associated protein B isoform 1), and TMEM147 (transmembrane protein 147), in the Golgi system (CHST12 (carbohydrate (chondroitin 4) sulfotransferase 12) and TMBIM4 (transmembrane protein 41B), in endosomes and lysosomes (CD63 (tetraspanin-30) and LAPTM4A (lysosomal associated protein transmembrane 4 α), and in mitochondria (FIS1 (fission, mitochondrial 1 protein), GHITM (growth hormone–inducible transmembrane protein), and SLC25A11 (solute carrier family 25, member 11)). Of special interest may be the hOCT1/PDZK1P1 interaction, which may explain why hOCT1 in the kidneys appears to be expressed in the apical plasma membrane domain (Tzvetkov et al., 2009). Of course, these interactions with hOCT1 should be confirmed using an independent system.

## SHORT-TERM REGULATION OF ORGANIC CATION TRANSPORTER 1 ACTIVITY

Substrate transport is one of the main functional performances of the liver and of the kidneys. These organs use transporters to secrete variable quantity of substances, depending on rapidly changing fluid and meal intake and metabolic activities. For this reason, a rapid regulation of hepatic and renal transport functions, adapting their activity to variable situations, is possible. Short-term transporter regulation can be achieved by posttranslational modifications like phosphorylation/dephosphorylation, which can alter transport kinetics. Indeed, both the liver and the kidneys are the target of several hormones, which regulate multiple signaling pathways. For example, insulin regulates in the liver glucose, lipid, and energy metabolism *via* binding to tyrosine kinase receptors, starting a cascade of phosphorylation reactions (Boucher et al., 2014). Conversely, glucagon, by binding to its hepatic receptors, activates adenylate cyclase, which stimulates protein kinase A (PKA) and cyclic AMP (cAMP) response element-binding (CREB) protein. The activation of this pathway leads to increased gluconeogenesis (Janah et al., 2019). In the kidneys, the peptide hormone angiotensin II (Ang II) regulates the most important $Na^+$ transporters of the proximal tubules (the apical $Na^+/H^+$ exchanger isoform 3, the basolateral $Na^+$-$HCO_3^-$

**TABLE 1 |** Summary of the short-term effects of selected regulation pathways on the organic cation transport mediated by specific organic cation transporter 1 (OCT1) orthologs (mouse (m), rat (r), and human (h) OCT1) in expression systems and in isolated murine proximal tubules (PT) freshly isolated from the kidneys of wild-type (WT) mice and of mice with genetic deletion of OCT2 ($OCT2^{-/-}$) (↑ indicates a stimulation of the transport activity; ↓ indicates an inhibition of the transport activity; 0 indicates no effect on the transport activity). Where measured, the regulation effect on transporter kinetic parameters (affinity, Km; or maximum velocity, Vmax) is also reported.

| Activated pathway | Transporter/freshly isolated proximal tubules (PT) | | | | |
|---|---|---|---|---|---|
| | mOCT1 | rOCT1 | hOCT1 | $OCT2^{-/-}$ mouse PT | WT mouse PT |
| PKA | ↑ (Schlatter et al., 2014) | ↑ (Mehrens et al., 2000), 0 and ↓* (Gerlyand and Sitar, 2009) | ↓ (Ciarimboli et al., 2004); $K_m$-effect | ↑ (Schlatter et al., 2014) | ↑ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $V_{max}$-effect (Guckel et al., 2012) |
| PKC | ↓ (Schlatter et al., 2014) | ↑ (Gerlyand & Sitar, 2009), (Mehrens et al., 2000); $K_m$-effect (Mehrens et al., 2000) | 0 (Ciarimboli et al., 2004) | ↓ (Schlatter et al., 2014) | ↓ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $V_{max}$-effect (Guckel et al., 2012) |
| $p56^{lck}$ | ↑ (Schlatter et al., 2014) | ↑ (Mehrens et al., 2000) | ↑ (Ciarimboli et al., 2004) | ↑ (Schlatter et al., 2014) | ↑ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $V_{max}$-effect (Guckel et al., 2012) |
| Tyrosine kinase | | 0 (Mehrens et al., 2000) | | | |
| cGMP | | ↓ (Schlatter et al., 2002) | 0 (Ciarimboli et al., 2004) | | |
| CaM | ↑ (Schlatter et al., 2014) | | ↑ (Ciarimboli et al., 2004); $K_m$-effect | ↑ (Schlatter et al., 2014) | ↑ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $K_m$-effect (Guckel et al., 2012) |
| CamKII | | | ↑ (Ciarimboli et al., 2004) | | |
| MLCK | | | 0 (Ciarimboli et al., 2004) | | |
| PI3K | ↑ (Schlatter et al., 2014) | | 0 (Ciarimboli et al., 2004) | ↑ (Schlatter et al., 2014) | ↑ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $V_{max}$-effect (Guckel et al., 2012) |
| Ang II | | | | ↑ (Schlatter et al., 2014) | ↑ (Guckel et al., 2012; Holle et al., 2011; Schlatter et al., 2014); $K_m$-effect (Guckel et al., 2012) |

**Effect magnitudev depends on forskolin concentration used in (Gerlyand and Sitar, 2009): with 1 μM forskolin, no significant regulation of rOCT1 activity was observed, and with 10 μM forskolin, a significant inhibition of rOCT1 was measured.*

*PKA, protein kinase A; PKC, protein kinase C; $p56^{lck}$, $p56^{lck}$ tyrosine kinase; cGMP, cyclic GMP; CaM, $Ca^{2+}$–calmodulin complex; CaMKII, multifunctional $Ca^{2+}$/CaM-dependent protein kinase II; MLCK, myosin light chain kinase; PI3K, phosphoinositide 3-kinase; Ang II, angiotensin II.*

cotransporter, and the basolateral $Na^+/K^+$-ATPase) in a concentration-dependent manner. For example, at low picomolar to nanomolar concentrations, Ang II stimulates these transporters by binding to the Ang II receptor type 1 (AT1R), activating the protein kinase C (PKC), and/or lowering intracellular cAMP concentration (Shirai et al., 2014).

Rapid regulation has been investigated in cells overexpressing mouse, rat, or human OCT1 (mOCT1 (Schlatter et al., 2014), rOCT1 (Mehrens et al., 2000; Ciarimboli et al., 2005), hOCT1 (Ciarimboli et al., 2004)), and in isolated proximal tubules (PT) from mouse (Holle et al., 2011; Guckel et al., 2012; Schlatter et al., 2014) and rabbit (Hohage et al., 1994) kidneys. In mouse and rabbit PT, both OCT1 and OCT2 are expressed (Kaewmokul et al., 2003; Schlatter et al., 2014); however, OCT1 seems to be the functionally predominant form in mouse PT (Schlatter et al., 2014).

Most studies on acute regulation of OCT1 have been performed measuring the effects of short-time (10 min) activation or inhibition of various kinase pathways on OCT1 orthologs (m, r, or hOCT1) overexpressed in human embryonic kidney (HEK293) cells using the fluorescent organic cation 4-(4-(dimethylamino) styryl-N-methyl-pyridinium ($ASP^+$) as a substrate. This technique allows a dynamic measurement of transporter function with high time resolution, as explained in detail elsewhere (Ciarimboli and Schlatter, 2016). In the following, the main results of these studies are summarized.

## Rapid Regulation of mOCT1

Transport mediated by mOCT1 was stimulated by the activity of $Ca^{2+}$–calmodulin complex (CaM), $p56^{lck}$ tyrosine kinase, PKA, and phosphoinositide 3-kinase (PI3K) (Schlatter et al., 2014) (**Table 1**). Only PKC activation inhibited mOCT1 transport (Schlatter et al., 2014) (**Table 1**).

## Rapid Regulation of rOCT1

Using the same experimental approach, transport of $ASP^+$ mediated by rOCT1 was demonstrated to be stimulated by PKA, PKC, CaM, and $p56^{lck}$ tyrosine kinase. Other tyrosine kinases did not influence rOCT1-mediated transport, while cGMP inhibited it (**Table 1**) (Mehrens et al., 2000; Schlatter et al., 2002; Ciarimboli et al., 2005). Importantly, it was demonstrated that PKC activation directly phosphorylates rOCT1 and changes transporter affinities (Ciarimboli et al., 2005). The potential PKC-phosphorylation sites at positions S286, S292, T296, S328, and T550 seem to be important for

this effect. Other studies showed a strong downregulation of rOCT1-mediated transport under PKC inhibition with staurosporine (Gerlyand and Sitar, 2009), confirming a possible PKC stimulation of rOCT1-mediated transport.

Interestingly, using tetraethylammonium ($TEA^+$) as a transport tracer, PKA stimulation did not change rOCT1 activity (Gerlyand and Sitar, 2009). These results may be explained admitting that OCTs have a large binding pocket, with partially overlapping interaction domains for different substrates (Ciarimboli et al., 2005; Popp et al., 2005). Therefore, PKA may induce conformational changes in the binding domain of $ASP^+$ and not in that of $TEA^+$, resulting in stimulation of $ASP^+$ uptake but not of $TEA^+$ transport.

## Rapid Regulation of hOCT1

$ASP^+$ microfluorimetry has been used also to characterize the rapid regulation of hOCT1 overexpressed in HEK293 cells or Chinese hamster ovary (CHO) cells (Ciarimboli et al., 2004). Interestingly, regulation patterns observed in the two cell systems were not different. The hOCT1 activity was downregulated by PKA stimulation. This regulation was different from what was observed for mOCT1 and rOCT1, where PKA activation stimulated the transporters (s above). Activity of PKC, myosin light chain kinase (MLCK), or PI3K did not regulate hOCT1. The $p56^{lck}$ tyrosine kinase, CaM, and the multifunctional $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII) stimulated hOCT1 activity, similarly to what was observed for m and rOCT1. Regulation of hOCT1 by PKA and CaM was associated with changes of the transporter apparent affinity for its substrates (Ciarimboli et al., 2004).

The presence of specific phosphorylation sites in the OCT1 orthologs can probably contribute to explain the specific regulation observed in the works cited above. For example, focusing on PKA effect on mOCT1, rOCT1, and hOCT1, using the group-based prediction system GPS 5.0 (Xue et al., 2008), the same 3 potential phosphorylation sites (S334, T348, and S537) are found in the primary structure of mOCT1 (NCBI sequence NP_033228.2) and rOCT1 (UniProtKB: Q63089.1). In hOCT1 primary structure (UniProtKB: O15245.2), only one potential PKA phosphorylation site (T347) is detected. Therefore, phosphorylation of S334 and/or S537 may be responsible for PKA upregulation of mOCT1 and rOCT1 activities measured by $ASP^+$ microfluorimetry.

## Rapid Regulation of $ASP^+$ Transport in Freshly Isolated Mouse Proximal Tubules

Comparing acute regulation of $ASP^+$ uptake in freshly isolated proximal tubules (PT) from wild-type (WT) mice and mice with genetic deletion of OCT2 ($OCT2^{-/-}$, in these mice, the predominant OCT in proximal tubules is OCT1 (Holle et al., 2011)), an identical regulation pattern was observed. Moreover, the regulation of $ASP^+$ uptake in PT isolated from WT- and $OCT2^{-/-}$- mice was the same as that observed in HEK cells overexpressing mOCT1. These results suggest that OCT1 is the main functional OCT paralog in this part of the mouse nephron (Schlatter et al., 2014). OCT regulation by $p56^{lck}$, PI3K, PKA, and PKC in mouse kidneys is linked to $V_{max}$ changes of $ASP^+$ transport (Guckel et al., 2012). Interestingly, the same qualitative regulation pattern of $ASP^+$ transport was observed in PT from male and female $OCT2^{-/-}$ mice. However, $p56^{lck}$ and PKC had an approximately 20 % stronger effects in female than in male animals (Schlatter et al., 2014). A dependence of OCT-mediated transport regulation on sex has been observed also in rats (Wilde et al., 2009), probably due to a stronger endogenous expression of regulatory enzymes, such as CaM (Wilde et al., 2009).

Activation *via* $p56^{lck}$ seems to be a rapid regulation pathway conserved along all OCT1 orthologs (s above) and the other OCT paralogs (Wilde et al., 2009; Massmann et al., 2014; Schlatter et al., 2014; Frenzel et al., 2019). Interestingly, according to the group-based prediction system GPS 5.0 (Xue et al., 2008), all OCT1 and OCT2 orthologs have a conserved potential lck phosphorylation site in the intracellular domain on tyrosine 543 (hOCT1), 545 (mOCT1 and rOCT1), or 544 (mOCT2, rOCT2, and hOCT2), close to the carboxy-terminus. The OCT3 orthologs have such a conserved potential lck phosphorylation site on tyrosine 456 (mOCT3 and rOCT3) or 461 (hOCT3) in the small intracellular loop between the transmembrane domains 10 and 11. Therefore, it can be supposed that a direct phosphorylation by $p56^{lck}$ regulates OCT activity. Mutagenesis of these tyrosines to, for example, alanine may help to reveal whether OCT regulation by $p56^{lck}$ is associated with phosphorylation of these sites. Such an experimental approach has been used to show that point mutations in the OCTs can have a specific influence on the binding characteristics of different substrates (Ciarimboli et al., 2005). These findings support the concept that OCTs have a large binding site with different interaction regions for diverse substrates (Koepsell, 2019; Sandoval et al., 2019). Therefore, transporter regulation studied with different OCT substrates may show different results, as outlined above for PKA regulation of rOCT1 activity.

In conclusion, the cellular processing of OCT1 may be regulated by a direct interaction with other proteins. Moreover, rapid regulation of OCT1 activity is probably necessary to adjust transporter activity to physiological requirements. This regulation is ortholog and paralog specific, and changes transport characteristics such as the affinity to known substrates and the maximum reaction velocity. Both in the liver and at least in the rodent kidneys, OCT1 mediates the first step of organic cation secretion, that is, the uptake of substrates into hepatocytes and into cells of the renal proximal tubules, respectively. For this reason, it should be investigated whether a regulation pathway able to stimulate the second step of secretion process, that is, the excretion of organic cations into the bile and urine, by, for example, the multidrug and toxin extrusion proteins (MATEs) (Kantauskaite et al., 2020), can work together with OCT regulation to globally modulated hepatic and renal substrate secretion.

# LONG-TERM REGULATION OF OCT1

## Factors Determining OCT1 Expression and Function in Humans and Mice

Tissue-specific processes activated by different pathophysiological conditions influence OCT1 expression and activity. As outlined above, OCT1 is mainly expressed in hepatocytes. However, there are few studies, which try to explain why OCT1 is highly expressed in the liver. The hepatic expression of many proteins is under the control of two transcription factors: the hepatocyte nuclear factor 4α (HNF4α) and the CCAAT/enhancer-binding protein (C/EBP) (Nishiyori et al., 1994). For this reason, the expression of mRNA coding for HNF4α and for C/EBP has been correlated with that of hOCT1 in the human liver. A significant correlation between hepatic mRNA expression of hOCT1 and that of HNF4α and C/EBP was found (Rulcova et al., 2013). Moreover, stimulation of HNF4α by dexamethasone in human primary hepatocytes increased hOCT1 expression. Two functionally important response elements for HNF4α have been found in the 5'-flanking region of the solute carrier 22A1 (*SLC22A1*) gene, the gene coding for hOCT1 (Saborowski et al., 2006; Hyrsova et al., 2016). These elements seem not to be conserved in rodents (Saborowski et al., 2006).

Another important regulator of drug transport and metabolism in humans is the hepatocyte nuclear factor 1 (HNF1), a transcription factor, which is highly expressed in the liver (Courtois et al., 1988). HNF1 has been identified as a potent regulator of hOCT1 expression. HNF1 increases *SLC22A1* promoter activity by binding to an evolutionary conserved region in intron 1 of the *SLC22A1* gene (O'Brien et al., 2013).

The presence of single nucleotide polymorphisms (SNPs) in *SLC22A1* is well known to modulate its function (Kerb et al., 2002; Shu et al., 2003; Shu et al., 2007; Tzvetkov et al., 2009; Tzvetkov et al., 2011; Tzvetkov et al., 2012; Tzvetkov et al., 2013). The SNPs of *SLC22A1* have been shown to influence hOCT1 transport characteristics (affinity $K_m$ and maximal velocity $V_{max}$) and the pharmacokinetics of drugs, which are substrates of hOCT1. This aspect of hOCT1 regulation has been already summarized in other excellent reviews (Yee et al., 2018; Zazuli et al., 2020) and will be further discussed in detail in other contributions to this special issue.

In mice, a transcriptional regulation of *Slc22a1* (murine genes are conventionally written in lowercase) by peroxisome proliferators activated receptors (PPAR) has been proposed. PPAR are transcription factors which play an important role in metabolic regulation and in determining liver function (Kersten, 2014). For example, in the liver, the nuclear receptor PPARα is activated in the fasted state, and its activation induces fatty acid oxidation and gluconeogenesis (Preidis et al., 2017). PPARγ stimulates several proteins associated with lipid uptake, triacylglycerol storage, and formation of lipid droplets (Wang et al., 2020). The physiological ligands of PPAR are fatty acids, which are mobilized during fasting or food restriction. Therefore, PPAR-α and PPAR-γ agonists are agents, which can modulate many hepatic metabolic and transport processes. In mice, feeding PPAR-α and -γ agonists increased transcriptional *Slc22a1* gene expression. In an *in vitro* model, the increased *Slc22a1* expression induced by PPAR-α and PPAR-γ agonists resulted in a stimulation of cellular organic cation uptake (Nie et al., 2005). Since OCT1 is a high-affinity choline transporter (Sinclair et al., 2000), and choline is essential for phosphatidylcholine synthesis, the stimulation of OCT1 by fatty acids may be useful to increase choline uptake when its portal blood concentrations are low (Nie et al., 2005).

There are several works demonstrating that sex can influence OCT1 expression at mRNA and protein levels. In mice and rats, renal OCT1 protein expression was higher in male than in female animals (Sabolic et al., 2011). However, renal OCT1 mRNA expression was higher in female than in male rats (Sabolic et al., 2011). Therefore, regulation of OCT1 mRNA and protein expression can be divergent, at least in rodents. It is not known whether OCT1 expression in humans is dependent on sex.

## Long-Term Effect of Kinase Activation on OCT1 Expression and Function

Regarding long-term effects of kinase activity on transporter function, 24 h incubation with 10 μM epinephrine has been demonstrated to down-regulate hOCT1 mRNA-expression *via* cAMP formation in primary human hepatocytes (Mayati et al., 2017a). These results confirm what was observed for rapid regulation of hOCT1 under stimulation of PKA activity (Ciarimboli et al., 2004), showing that this regulation axis has similar short- and long-term effects on hOCT1 function.

Long-time (24–48 h) exposure to PKC activators such as phorbol ester 12-myristate 13-acetate (PMA, 100 nM) reduced hOCT1 mRNA-expression and activity in human hepatoma HepaRG cells and primary human hepatocytes (Mayati et al., 2015). However, shorter incubation times with PMA did not change hOCT1 transport activity in HepaRG cells (Mayati et al., 2017b), confirming what was found for rapid hOCT1 regulation by PKC (s above). Therefore, it can be concluded that acute and chronic kinase activation may have also a different impact on hOCT1 activity.

How can the data be interpreted on OCT1 regulation in a physiological context? Focusing on hOCT1 and the liver, one can try to build a model integrating hOCT1 short- and long-term regulation and activation of a specific signaling pathway. For example, it is well known that activation of the cAMP/PKA pathway is important for regulation of hepatic energy metabolism. Glucagon and catecholamines stimulate in the liver the formation of cAMP and PKA, which leads to an increased glucose production, increased gluconeogenesis, and a decreased glycolysis. Activation of this pathway also influences lipid metabolism by decreasing biosynthesis of fatty acids and lipogenesis and increasing fatty acid oxidation. Moreover, activation of this pathway represses the expression of the PPAR-γ gene, which is a key regulator of lipogenic genes. Therefore, activation of the cAMP/PKA pathway leads to increased hepatic glucose production and decreased lipid accumulation (Wahlang et al., 2018). Therefore, since hOCT1 activity is rapidly inhibited by PKA activity and PPAR-γ agonists increased transcriptional *Slc22a1* gene expression, increased hepatic glucose production may be associated with immediate reduction of hOCT1 activity and decrease of hOCT1 gene expression.

## LONG-TERM REGULATION UNDER PATHOLOGICAL CONDITIONS AND BY ENVIRONMENTAL TOXINS

### Studies in Animal Models

Decreased protein expression of OCT1 in kidneys from diabetic rats was detected (Thomas et al., 2003; Grover et al., 2004), which was restored by inhibition of angiotensin-converting enzyme (Thomas et al., 2003) or insulin treatment (Grover et al., 2004). Since these changes are evident at the protein but not at the mRNA level (Grover et al., 2004), it was speculated that they are due to posttranscriptional alterations (Grover et al., 2004).

Ischemia–reperfusion injury (IRI) down-regulated mRNA and protein expression of OCT1 in rat kidneys. In this model, IRI increased NO generation by stimulation of inducible nitric oxide synthases (iNOS). Inhibition of iNOS at the end of ischemia restored OCT1 expression at normal levels, suggesting that NO is a negative regulator of OCT1 (Schneider et al., 2011).

Syngeneic and allogeneic rat kidney transplantation downregulated the mRNA and protein expression of OCT1 in the transplanted kidneys. Immunosuppression with cyclosporine A partially restored OCT1 mRNA expression in the allogeneic model (Ciarimboli et al., 2013).

Hepatic cholestasis, studied in a bile duct ligation (BDL) model in the rat, down-regulates the expression and function of rOCT1 in the liver, probably as a protection mechanism, to decrease hepatic accumulation of potentially toxic substances (Denk et al., 2004).

### Studies in Human Tissues and Human Cells in Culture

Epigenetic modifications (e.g., DNA methylation and histone modification) are heritable variations that regulate chromatin structure and DNA accessibility and can change gene expression without changing its DNA sequence. In human hepatocellular carcinoma (HCC), the mRNA and protein expression of hOCT1 were found to be significantly reduced compared with normal adjacent liver tissue (Schaeffeler et al., 2011). Methylation of *SLC22A1* seems to be associated with a lower expression of hOCT1 in HCC (Schaeffeler et al., 2011). Interestingly, the downregulation of hOCT1 in HCC has been confirmed in an independent study and was found to be associated with tumor progression and a worse patient survival (Heise et al., 2012). The same relationship between hOCT1 expression and consequences for tumor progression and patient survival has been observed in cholangiocellular carcinoma (CCA), a hepatic malignancy derived from cholangiocytes (Lautem et al., 2013). It has been observed that in diabetic patients treated only with metformin, methylation of liver *SLC22A1* decreases (Garcia-Calzon et al., 2017), suggesting that diabetes may decrease OCT1 expression by increasing *SLC22A1* methylation. However, to my knowledge, there is still no quantitative measurement of OCT1 expression in diabetic patients.

Liver function deterioration (e.g., induced by hepatitis C, primary biliary cholangitis, primary sclerosing cholangitis, alcoholic liver disease, and autoimmune hepatitis) decreased the amount of hOCT1 mRNA and protein in the liver (Drozdzik et al., 2020).

Cigarette smoking is well known to have an important pharmacological impact because it can change drug pharmacokinetics and drug–drug interactions. Cigarette smoke condensate decreases mRNA expression and activity of hOCT1 in human hepatoma HepaRG cells, probably *via* activation of the aryl hydrocarbon receptor (AhR) signaling pathway (Sayyed et al., 2016). Activation of the AhR signaling pathway may be also the mechanism by which exposure to the diesel exhaust particles (25 μg/ml, 48 h) decreases hOCT1 mRNA expression in HepaRG cells (Le et al., 2015).

Taken together, these results suggest that pathological insults and environmental toxins down-regulate OCT1 expression.

## CONCLUSION

In conclusion, OCT1 is subjected to a multifaceted regulation, which can change its function. Therefore, modulation of its expression and activity may have important physiological and pharmacological consequences due to the role of OCT1 for handling of endogenous and exogenous substrates such as neurotransmitters and drugs. It would be important to investigate whether and at which position OCT1 is phosphorylated by rapid regulation processes and to define the exact role of interacting proteins for transporter cellular processing. In this way, new functional meaning of SNPs in hOCT1 and/or interacting proteins can be detected. Regulation of OCT1 may change excretion of its substrates and modify toxicity of drugs and environmental toxins. Further research is necessary to clarify these important issues of OCT-mediated transport.

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The author confirms being the sole contributor of this work and has approved it for publication.

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# Expansion of Knowledge on OCT1 Variant Activity *In Vitro* and *In Vivo* Using Oct1/2$^{-/-}$ Mice

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The role of organic cation transporter 1 (OCT1) in humans is gaining attention as data emerges regarding its role in physiology, drug exposure, and drug response. OCT1 variants with decreased *in vitro* function correlate well with altered exposure of multiple OCT1 substrates in variant carriers. In the current research, we investigate mechanisms behind activity of OCT1 variants *in vitro* by generating cell lines expressing known OCT1 variants and quantifying membrane OCT1 protein expression with corresponding OCT1 activity and kinetics. Oct knockout mice have provided additional insight into the role of Oct1 in the liver and have reproduced effects of altered OCT1 activity observed in the clinic. To assess the complex effect of Oct1 depletion on pharmacokinetics of prodrug proguanil and its active moiety cycloguanil, both of which are OCT1 substrates, Oct1/2$^{-/-}$ mice were used. Decreased membrane expression of OCT1 was demonstrated for all variant cell lines, although activity was substrate-dependent, as reported previously. Lack of change in activity for OCT1*2 resulted in increased intrinsic activity per pmol of OCT1 protein, particularly for sumatriptan but also for proguanil and cycloguanil. Similar to that reported in humans with decreased OCT1 function, systemic exposure of proguanil was minimally affected in Oct1/2$^{-/-}$ mice. However, proguanil liver partitioning and exposure decreased. Cycloguanil exposure decreased following proguanil administration in Oct1/2$^{-/-}$ mice, as did the systemic metabolite:parent ratio. When administered directly, systemic exposure of cycloguanil decreased slightly; however liver partitioning and exposure were decreased in Oct1/2$^{-/-}$ mice. Unexpectedly, following proguanil administration, the metabolite ratio in the liver changed only minimally, and liver partitioning of cycloguanil was affected in Oct1/2$^{-/-}$ mice to a lesser extent following proguanil administration than direct administration of cycloguanil. In conclusion, these *in vitro* and *in vivo* data offer additional complexity in understanding mechanisms of OCT1 variant activity as well as the effects of these variants *in vivo*. From cell lines, it is apparent that intrinsic activity is not directly related to OCT1 membrane expression. Additionally, in situations with a more complicated role of OCT1 in drug pharmacokinetics there is difficulty translating *in vivo* impact simply from intrinsic activity from cellular data.

**Keywords: drug transport, organic cation transporter 1 (OCT1), pharmacokinetics, knockout mice, metabolite kinetics, targeted proteomics, pharmacogenetics**

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## INTRODUCTION

In humans, organic cation transporter 1 (OCT1) is localized in the liver and intestine, organs pertaining to drug absorption, distribution, metabolism and excretion (Drozdzik et al., 2019). As many therapeutic agents are demonstrated OCT1 substrates (Koepsell, 2020), the potential exists for a role of OCT1-mediated transport on the disposition of therapeutic drugs. This prospect was propagated by the identification of OCT1 variants with varying degrees of transport activity impacting cellular exposure, initially on metformin uptake but followed by sumatriptan, fenoterol, proguanil, ranitidine and others (Shu et al., 2007; Matthaei et al., 2015; Meyer et al., 2017; Tzvetkov et al., 2018; Matthaei et al., 2019; Jensen et al., 2020). Subsequent clinical evaluation in subjects carrying these variant alleles has demonstrated clinically relevant effects on the exposure of these therapeutic agents (Matthaei et al., 2015; Tzvetkov et al., 2018; Matthaei et al., 2019). Interestingly, OCT1 pharmacogenetics demonstrate substrate-specificity, most notably OCT1*2, for which uptake of some substrates, such as fenoterol and metformin, is impaired compared to wildtype, while for sumatriptan, proguanil and cycloguanil activity was relatively maintained (Shu et al., 2007; Matthaei et al., 2015; Matthaei et al., 2019).

Due to the observed clinical relevance of OCT1 variants on the pharmacokinetics of OCT1 substrates, tools for identifying the role of OCT1 in the pharmacokinetics of an investigational drug have become important. Notably, *in vitro* data on the uptake activity of various OCT1 variants has correlated quite well with *in vivo* observations. A prominent example is the effect of OCT1 activity on the metabolite ratio for cycloguanil:proguanil, in which the authors were able to demonstrate a continuous correlation of *in vitro* activity to the relationship observed *in vivo* (Matthaei et al., 2019). Oct knockout mice have also provided insight into the role of Oct1 in hepatic clearance and partitioning, as well as its physiologic role in lipid metabolism (Higgins et al., 2012; Liang et al., 2018; Morse et al., 2020). We previously assessed Oct1/2$^{-/-}$ mice as a model for reproducing or predicting the effect of OCT1 variants in the clinic. In these studies, hepatic clearance of sumatriptan and fenoterol was significantly decreased, and the change in oral and IV clearance was similar to that reported in human carriers of OCT1 null variants (Morse et al., 2020). In these mice, a corresponding decrease in liver partitioning was determined for sumatriptan and fenoterol and was also previously demonstrated for metformin (Higgins et al., 2012). We did not find this Oct1/2$^{-/-}$ mice model to be as robust for the effects of Oct1 deficiency on ondansetron or tropisetron pharmacokinetics, although the clinical data for comparison in humans is also not as robust as that for other OCT1 substrates mentioned above (Tzvetkov et al., 2012).

In the current research, we generated cell lines expressing 8 OCT1 variants proteins using a novel stable lentiviral transfection method and confirm previous results for activity toward OCT1 substrates sumatriptan, fenoterol, metformin, proguanil and cycloguanil. We additionally quantitated the OCT1 membrane protein expression level of each one of these variants, which has not previously been reported, to understand changes in substrate kinetics relative to protein OCT1 expression. To follow-up on previous application of the Oct1/2$^{-/-}$ mouse model for sumatriptan, fenoterol and metformin, we assess the pharmacokinetics of proguanil and cycloguanil. A considerable advantage to the use of rodent models is the ability to assess tissue concentrations and to assess the effect of altered transport activity of pharmacokinetics of a metabolite administered directly. Therefore, we utilize the Oct1/2$^{-/-}$ mouse model to assess the plasma pharmacokinetics of proguanil for comparison to that in humans, as well as cycloguanil following proguanil administration and administered directly. Additionally, we use the model to assess the liver exposure changes in these agents, as this is a site of action and may add to hypotheses on liver exposure of these therapeutic agents in patients with decreased OCT1 activity.

## MATERIALS AND METHODS

### Generation of OCT1 Variant Cell Lines and Uptake of OCT1 Substrates

Generation of OCT1 variant cell lines was performed as reported previously for wildtype OCT1*1 (Morse et al., 2020). OCT1*2 (Met420del), OCT1*3 (Arg61Cys), OCT1*4 (Gly401Ser), OCT1*5 (Met420del and Gly465Arg), OCT1*6 (Cys88Arg and Met420del), OCT1*8 (Arg488Met) and OCT1*10 (Ser189Leu) were synthesized and cloned into the pLenti6.3 vector. pLenti6.3 empty vector and pLenti6.3- OCT1 variants were transfected into a lentiviral package cell line Lenti-X-293T to produce lentivirus supernatants. HEK293 cells were then transfected with these nine lentivirus supernatants respectively and OCT1 variants was selected by blasticidin (5 μg/ml) to generate stable cell lines. OCT1 expression in HEK293-OCT1*1 was confirmed by flow cytometry using antibody staining (Novusbio Cat#NBP1-51684). HEK-293 stably transfected cells with empty vector, OCT1 variants were grown in 5% CO2 at 37 °C in DMEM supplemented with 10% FBS, 50 μg/ml gentamicin, and 5 μg/ml blasticidin. Cell lines were maintained in T-75 flasks, reaching approximately 80% confluence before being passaged twice weekly at 1:10 ratio (volume: volume).

HEK293-VC (vector control) and -OCT1 expressing cells were seeded onto 12-well poly-D lysine plates at concentrations ranging from $1.7 \times 10^5$ to $2.7 \times 10^5$ cells/mL. Three days post-seeding, the cells were washed twice with prewarmed pH 7.4 HBSS buffer and preincubated with assay buffer for 10 min at 37 °C. Following the preincubation, cells were treated with the desired substrate for one or 2 min at 37 °C. After one or 2 min at 37 °C, the cells were washed three times with ice-cold HBSS and extracted with 80% MeOH containing an internal standard mix for sample analysis via LC-MS/MS. A separate set of cells were used to determine protein concentration by bicinchoninic acid method. Uptake was assessed in triplicate in two separate experiments. Using the same experimental method, a range of substrate concentrations was used to assess the kinetics of sumatriptan and fenoterol in OCT1*1 and OCT1*2 expressing cells, using a time point of 1 min at each concentration.

For OCT1 protein quantitation, the membrane fraction from each sample was extracted using an adapted differential surfactant extraction method (Qasem et al., 2020). For each variant, 2 separate samples were analyzed in duplicate, from cell passages one week apart. Quantitation of transporter expression was performed by nanoLC-MS/MS using SIL (stable isotope labeled) peptide standards as previously described (Khatri et al., 2019; Morse et al., 2020). The reporting peptide for the (human) OCT1 concentrations is LSPSFADLFR (the UniProt accession # is O15245). The peptide ENTIYLK was used as confirmatory. Concentrations of $Na^+/K^+$-ATPase were also measured by nanoLC-MS/MS, for use as a membrane marker control.

## Pharmacokinetics in Oct1/2$^{-/-}$ Mice

The pharmacokinetics of proguanil and cycloguanil were assessed in Oct1/2$^{-/-}$ mice as described previously for other OCT1 substrates (Morse et al., 2020). Studies were carried out at Covance (Greenfield, IN) and were approved by the Institutional Animal Care and Use Committee. Male Oct1/2$^{-/-}$ mice were taken from a breeding colony, maintained by Taconic. Age-matched FVB male mice were also purchased from Taconic. To evaluate the blood pharmacokinetics, groups of mice (n = 5, ages 8–14 weeks) were administered proguanil (2, 10, and 30 mg/kg) or cycloguanil (2 mg/kg) intravenously via the tail vein and serial blood samples collected as dried blood spots. To evaluate tissue partitioning, groups of mice ($n$ = four to five, ages 8–14 weeks) were administered proguanil (2, 10 and 30 mg/kg) or cycloguanil (2 mg/kg) and blood, plasma, and 4 tissues (liver, kidney, spleen, duodenum) collected at 0.75, 1.5, 2, 4 or 8 h post-dose. Tissues and plasma were kept at <60 °C until analysis for concentrations of proguanil and cycloguanil by LC/MS-MS (details in Supplemental Material). Both cycloguanil and proguanil were quantified in animals administered proguanil.

## Data Analysis

Uptake of OCT1 substrates in cells expressing OCT1 variants were normalized first by total protein, then represented as fold uptake of OCT1*1. The data were further analyzed by normalizing uptake by total amount of membrane OCT1 protein in each variant, then again represented as fold uptake of OCT1*1. $K_m$ and $V_{max}$ values for sumatriptan and fenoterol were determined using the equation below:

$$\text{Uptake} = \frac{V_{max} \cdot [S]}{K_m + [S]} + P_d \cdot [S],$$

where $V_{max}$ and $K_m$ represent the maximal rate of uptake and the concentration and half maximal rate of uptake, $P_d$ represents passive diffusion and [S] represents substrate concentration.

*In vivo* blood parameters in mice were determined by noncompartmental analysis using Watson 7.2. Clearance and liver partitioning of proguanil was dose-proportional from 2 to 30 mg/kg, therefore these groups were combined and data dose-normalized to 10 mg/kg. Metabolite ratio (M:P) in the plasma was calculated as cycloguanil AUC/proguanil AUC. Renal clearance (CLR) was determined as Ae/AUC, where Ae represents the amount recovered in urine, and AUC represents the area under the blood concentration–time curve (the mean AUC of animals administered the same dose of compound IV). The CLR was then corrected for creatinine recovery as described previously (Morse et al., 2020). Mean hepatic clearance was calculated as total clearance-CLR (determined as one value for each compound, due to pooled nature of urine samples). Tissue partitioning coefficients (Kp) at single timepoints were calculated as tissue/plasma concentrations. AUC in the liver was determined by noncompartmental analysis using the sparse sampling function in Phoenix 64. Liver metabolite ratio (M:P) was calculated as cycloguanil AUC/proguanil AUC. Student's t-tests were used to determine significant differences in pharmacokinetic parameters or tissue Kp values between wildtype and knockout mice using GraphPad 9.3.

# RESULTS

The uptake of known clinical OCT1 substrates in cell lines expressing wildtype and variant OCT1 protein are shown in **Figure 1**; uptake values are shown both before (A) and after (B) normalizing for measured membrane expressed OCT1 protein. In general, uptake for the substrates in the respective variants reproduce well those reported previously (Shu et al., 2007; Matthaei et al., 2015; Tzvetkov et al., 2018; Matthaei et al., 2019). The substrate-dependence of OCT1*2 previously demonstrated is clearly observed. Membrane OCT1 protein expression was lower in all variants tested compared to OCT1*1; absolute OCT1 concentrations from membranes of each variant cell line are shown in **Supplementary Table S1**, as well as the concentrations of membrane marker $Na^+/K^+$-ATPase in the cell samples, which were very similar for samples from each variant. Interestingly, after normalizing for measured OCT1 protein, increased intrinsic activity per pmol of OCT1 for certain substrates was evident, most notably sumatriptan for OCT1*2, whereas intrinsic activity for fenoterol and metformin in OCT1 variants appeared to be reconciled by normalization of membrane OCT1 expression. As two substrates with differing activity for OCT1*2, kinetics of sumatriptan and fenoterol in OCT1*1 and *2 were assessed. Prior to normalizing for membrane expressed OCT1 protein, for sumatriptan neither $K_m$ nor $V_{max}$ were dramatically different in OCT1*2 compared to OCT1*1 (mean $K_m$ of 68.3 vs 98.9 μM and $V_{max}$ of 5,360 vs. 4,300 pmol/min/mg, data not shown). However, as shown in **Figure 2**, after normalizing for OCT1 protein, the $V_{max}$ of sumatriptan in OCT1*2 was 2.6-fold higher than that for OCT1*1 (43.6 vs. 17.0 pmol/min/mg,OCT1) while $K_m$ values remained similar (93.4 vs 71.0 μM), consistent with higher intrinsic activity of OCT1*2 when normalized for OCT1 protein (**Figure 1**). For fenoterol, the $V_{max}$ for OCT1*2 was decreased substantially compared to OCT1*1 prior to normalizing for membrane expressed OCT1 protein (351 vs 32.0 pmol/min/mg), while the $K_m$ was affected to a lesser extent (3.24 vs. 0.85 μM). After normalizing for OCT1 protein, the $K_m$ and $V_{max}$ were similarly ~3-fold lower in OCT1*2 compared to OCT1*1 (mean $K_m$ of 3.55 vs 0.98 μM and $V_{max}$

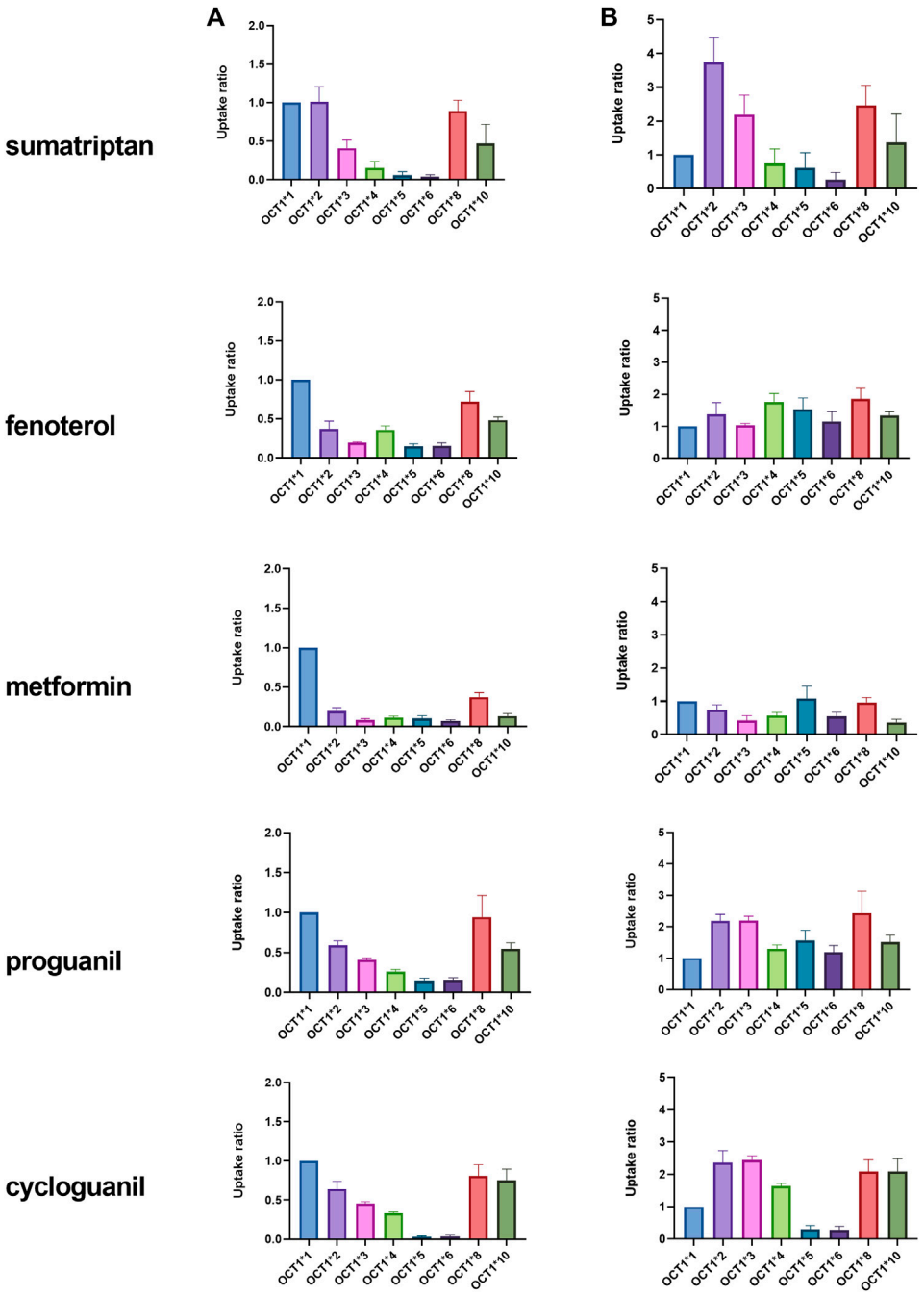


**FIGURE 1 | Uptake of OCT1 substrates in HEK cells expressing wildtype (*1) and variant OCT1 protein.** Uptake was assessed over 1 or 2 min, depending on substrate, in triplicate, at substrate concentration of 3 μM, with the exception of metformin, which was assessed at 22 μM. Data are presented as fold uptake compared to OCT1*1 (mean ± SD). Data in column **(A)** represent uptake prior to normalization for absolute OCT1 membrane protein expression. Data in column **(B)** represent uptake after normalization for absolute OCT1 membrane protein expression measured in cells expressing each variant.

of 1.15 vs. 0.39 pmol/min/mg,OCT1), consistent with maintained intrinsic activity of OCT1*2 when normalized for OCT1 protein (**Figure 1**).

The pharmacokinetic parameters of OCT1 substrates proguanil and cycloguanil in WT and Oct1/2$^{-/-}$ mice are given in **Table 1**. The blood concentration-time profiles are shown in **Figure 3A**. The clearance of proguanil was minimally, but significantly decreased in wildtype mice compared to Oct1/2$^{-/-}$ mice. Accordingly, proguanil was found to be excreted primarily in the urine; renal clearance was similar between wildtype and knockout mice. Liver partitioning and exposure of proguanil was,

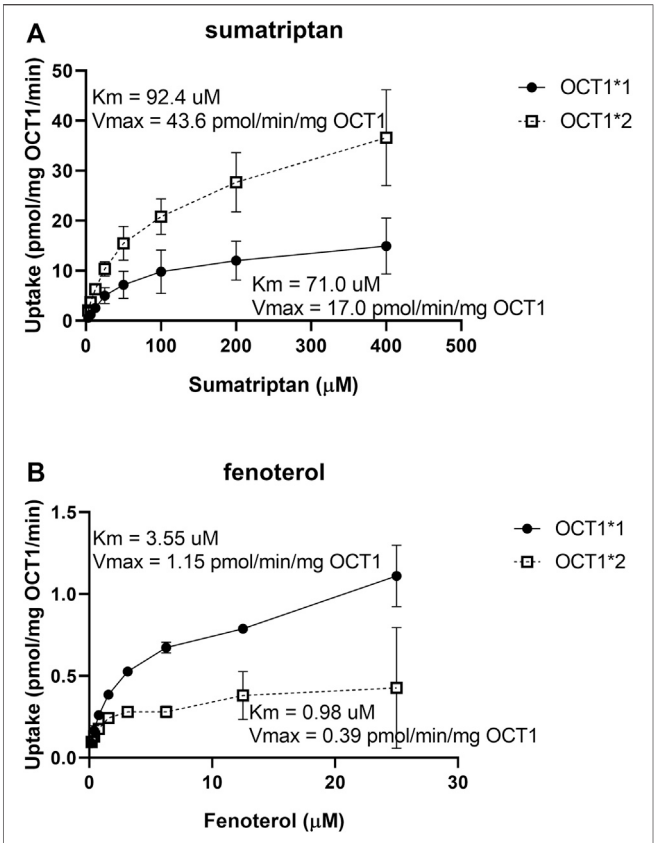


**FIGURE 2 | Kinetics of sumatriptan (A) and fenoterol (B) in HEK cells expressing OCT1*1 or OCT1*2.** Uptake was assessed over 1 min, in triplicate, at substrate concentration of 3 μM. Data are presented as mean ± SD.

however, substantially affected by Oct knockout, as shown in **Figures 3B,C**, and liver Kp and AUC decreased by ~3-fold (**Table 1**).

Following administration of proguanil, the exposure of cycloguanil in the blood was lower in knockout mice compared to wildtype, as shown in **Figure 3A**. As shown in **Table 1**, the metabolite:parent ratio (M:P) in the blood also decreased in knockout mice. Liver AUC of cycloguanil decreased, however the liver partitioning of cycloguanil between wildtype and knockout mice was only minorly affected following administration of proguanil (**Figure 3D**). The liver metabolite ratio of cycloguanil:proguanil did not change in knockout compared to wildtype mice, as shown in **Figure 3E**.

Following the administration of cycloguanil, the clearance of cycloguanil changed minimally, though significantly in Oct1/2$^{-/-}$ mice compared to wildtype (**Table 1**). The blood concentration-time profiles are shown in **Figure 4A**. Similarly to the parent proguanil, cycloguanil was found to be excreted primarily in the urine in wildtype and knockout mice and renal clearance was unchanged between the strains. Liver partitioning and exposure of cycloguanil decreased substantially, ~3-fold in knockout compared to wildtype mice (**Figures 4B,C**).

Partitioning in organs other than the liver are shown in **Figures 5**,**6**, following proguanil and cycloguanil administration, respectively. Proguanil partitioning was decreased in the duodenum but not spleen or kidney. The cycloguanil:proguanil ratio was lower in knockout mice, at the timepoints in which cycloguanil concentrations could be detected in these tissues (**Figure 5**), which was consistent with the change in the systemic cycloguanil:proguanil ratio. Following cycloguanil administration, the partitioning of cycloguanil was decreased in the duodenum but not spleen or kidney, similar to proguanil.

## DISCUSSION

There is now compelling evidence for the clinical relevance of OCT1-mediated transport in the liver, predominantly due to the

**TABLE 1 | Effect of Oct knockout on the pharmacokinetics of proguanil and cycloguanil following IV administration of both proguanil and cycloguanil.** Shown are dose-normalized blood and liver pharmacokinetic parameters for mice ($n$ = 5 or 5/timepoint) administered proguanil 2, 10 or 30 mg/kg or cycloguanil 2 mg/kg. Data presented as mean ± SD. Ratio of KO/WT are reported below mean values with significant changes.

| | Proguanil | | Cycloguanil | | Cycloguanil | |
|---|---|---|---|---|---|---|
| **Compound dosed** | | **Proguanil** | | | **Cycloguanil** | |
| | WT | KO | WT | KO | WT | KO |
| $AUC_{blood}$ (nM*Hr) | 46,300 (6,420) | 61600$^{ccc}$ (678) | 274 (33.5) | 92.8$^{bb}$ (12.4) | 3,315 (715) | 4,815 (1,570) |
| | 1.33 | | 0.34 | | 1.43 | |
| CL (ml/kg/min) | 13.8 (2.24) | 10.1$^{ccc}$ (1.15) | — | — | 41.2 (9.20) | 30.1$^{a}$ (8.96) |
| | 0.73 | | | | 0.73 | |
| M:P | — | — | 0.00504 (0.00041) | 0.00147$^{ccc}$ (0.000064) | — | — |
| | | | 0.29 | | | |
| CLrenal (ml/kg/min) | 10.7 | 12.6 | — | — | 40.4 | 33.1 |
| CLhepatic (ml/kg/min) | 3.1 | NC | — | — | 0.8 | NC |
| B:P | 2.61 (0.48) | 2.50 (0.42) | — | — | 1.15 (0.23) | 1.12 (0.13) |
| $AUC_{liver}$ (nM*Hr) | 368,000 | 137,000 | 8,380 | 2,680 | 29,400 | 14,300 |
| | 0.37 | | 0.32 | | 0.49 | |
| liver M:P | — | — | 0.0228 | 0.0200 | — | — |
| | | | 0.88 | | | |

*WT, wildtype; KO, knockout; AUC, area under the concentration-time curve, from time 0 extrapolated to infinity (blood) or until the last detectable concentration (liver); CL, clearance; M:P, metabolite:parent ratio; B:P, blood:plasma ratio; NC, not calculated. $^{a}p<0.05$ using student's t-test, compared to WT. $^{bb}p<0.01$ using student's t-test, compared to WT. $^{ccc}p<0.001$ using student's t-test, compared to WT.*

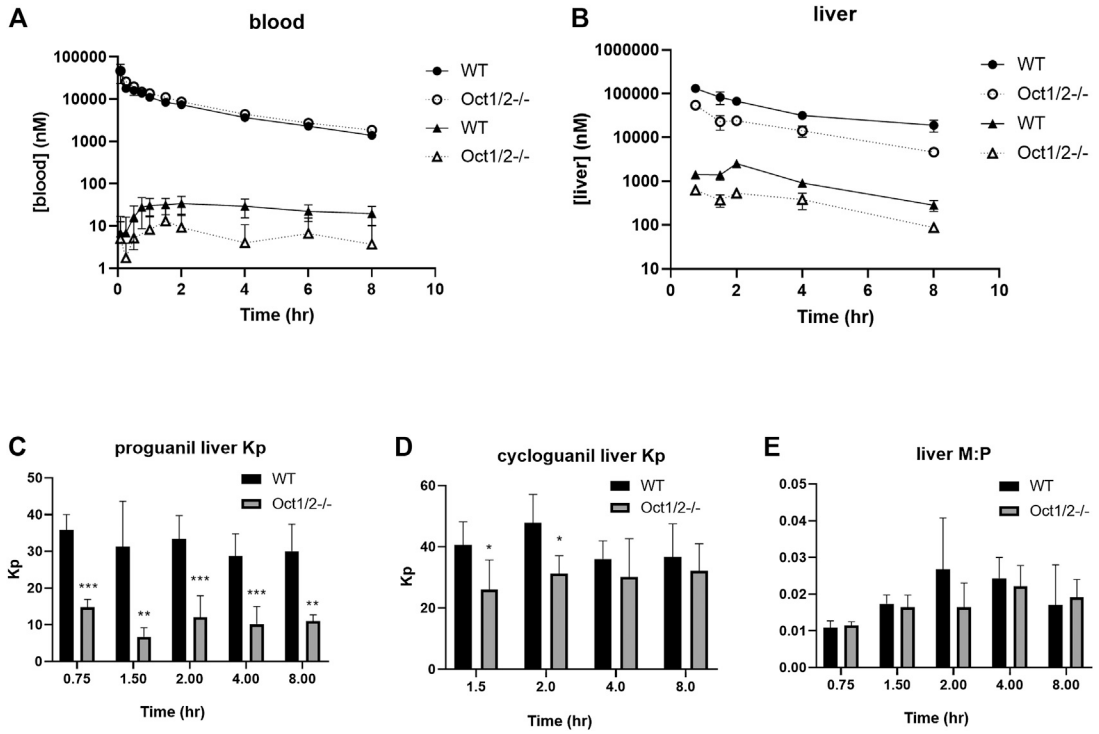


**FIGURE 3 | Pharmacokinetics of proguanil/cycloguanil in wildtype (WT) and Oct1/2$^{-/-}$ mice following IV administration of proguanil.** Shown are dose-normalized blood **(A)** and liver **(B)** concentrations for mice ($n$ = 5 or 5/timepoint) administered proguanil 2, 10 or 30 mg/kg. Filled symbols represent wildtype mice and open symbols represent knockout mice. Circles represent concentrations of proguanil and triangles represent concentrations of cycloguanil **(C** and **D, E)** Liver Kp and M:P values determined from tissues collected at 0.75 and 2 h (2 mg/kg), 1.5 and 4 h (10 mg/kg) or 8 h (30 mg/kg) post-dose. Data presented as mean ± SD. *$p$ < 0.05 using student's t-test, compared to WT. **$p$ < 0.01 using student's t-test, compared to WT. ***$p$ < 0.001 using student's t-test, compared to WT. Kp = tissue:plasma partition coefficient. M:P = metabolite:parent ratio.

extensive *in vitro* characterization of OCT1 variant activity and *in vivo* correlation with altered exposure of OCT1 substrates. In agreement with *in vitro* data generated in other laboratories, the current data with the OCT1 variants confirm the effect of the variant alleles on multiple OCT1 substrates. The substrate-dependence of OCT1*2 is particularly interesting and has been explored in detail (Seitz, 2016). Notably, the lack of effect of OCT1*2 on sumatriptan uptake has been demonstrated in the clinic, in agreement with maintained *in vitro* activity (Matthaei et al., 2015). Interestingly, however, the membrane OCT1 expression of all variants, including OCT1*2 was currently determined lower than for OCT1*1. In previous work, membrane localization of many OCT1 variants was explored qualitatively using confocal microscopy and results are again in general agreement with what we have measured using nanoLC-MS/MS (Seitz et al., 2015). Specifically, the variants which demonstrate loss-of-function across substrates, (e.g. OCT1*5 and *6) were lacking from the plasma membrane, and almost entirely localized in the endoplasmic reticulum using microscopy. We also determined that membrane expression in these variants is ~10% of that measured for OCT1*1. Conversely, maintenance of some plasma membrane localization for variants with substrate-dependent activity, (e.g. OCT1*2 and *10) was previously reported and we determined these variants to have 25–30% of the membrane expression compare to OCT1*1 (**Supplementary Table S1**).

When OCT1 membrane protein was not considered, no significant difference in the sumatriptan kinetics was previously observed (Seitz, 2016), which we similarly determined. However, we observe an apparent increase in sumatriptan $V_{max}$ when OCT1 membrane expression is considered, indicating increased intrinsic activity per mg of OCT1 protein. For fenoterol, a decrease in $V_{max}$ without considering OCT1 protein expression was previously reported (Seitz, 2016; Tzvetkov et al., 2018), as we also determined currently. The current data indicating an apparent maintenance of activity after normalization suggests that the decrease in fenoterol activity for OCT1*2 can be explained almost entirely by membrane expression level. However, after evaluating the kinetics of fenoterol and normalizing for membrane OCT1 protein expression, it appears that the maintenance of activity is due to increased affinity, which is offset by decreased $V_{max}$, in contrast to that of sumatriptan. Due to similarities in the kinetics of sumatriptan and fenoterol reported here and previously, prior to membrane protein normalization (Seitz, 2016), we

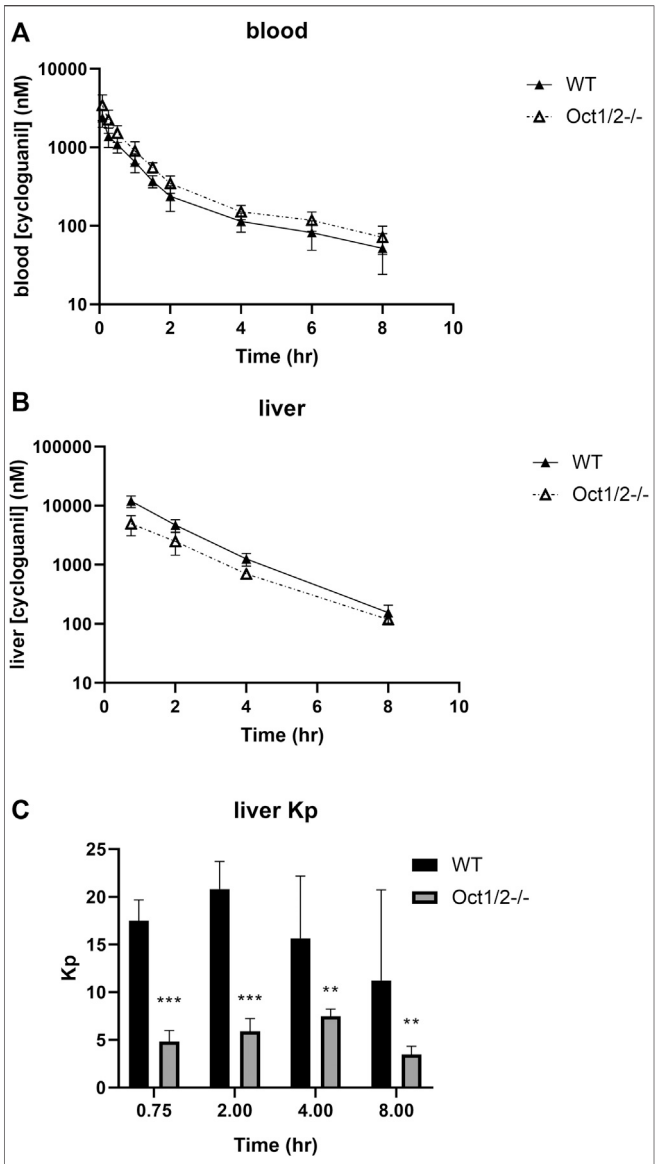


**FIGURE 4 | Pharmacokinetics of cycloguanil in wildtype (WT) and Oct1/2$^{-/-}$ mice following IV administration of cycloguanil**. Shown are blood **(A)** and liver **(B)** concentrations for mice ($n$ = 5 or 4–5/timepoint) administered cycloguanil 2 mg/kg. Filled symbols represent wildtype mice and open symbols represent knockout mice **(C)** Tissues were collected at 0.75, 2-, 4- and 8 h post-dose. Data presented as mean ± SD. $^{**}p < 0.01$ using student's t-test, compared to WT. $^{***}p < 0.001$ using student's t-test, compared to WT. Kp = tissue:plasma partition coefficient.

may use previous kinetic data to speculate on the protein-normalized kinetics of other OCT1 substrates. Interestingly, while protein normalization also appeared to explain the effects of different variants on metformin uptake in the current evaluation, a substantial increase in the $K_m$ and lack of change in the $V_{max}$ for metformin in OCT1*2 has been previously reported, when membrane OCT1 protein expression is not considered (Seitz, 2016). One would assume then that the $V_{max}$ for metformin per membrane expressed OCT1 protein must increase substantially for this variant. With regard to the effect of the variants on proguanil and cycloguanil, the current data are in agreement with the effects of OCT1*5 and *6, in that both variants have decreased uptake of both proguanil and cycloguanil. Conversely, while *2, *3 and *4 were previously reported to effect primarily uptake of proguanil, we found these variants to affect proguanil and cycloguanil similarly. For OCT1*2, we determined an apparent increase in the intrinsic activity for both proguanil and cycloguanil of ~2-fold when considering membrane OCT1 expression. From experiments not normalized for protein expression the $K_m$ and $V_{max}$ of proguanil decreased to a similar extent (Seitz, 2016). Therefore, similar to sumatriptan, for the apparent intrinsic activity to increase when normalized for protein expression, the $V_{max}$ for proguanil normalized to OCT1 protein would be expected to increase.

Previous data for OCT1 substrates sumatriptan and fenoterol in Oct1/2$^{-/-}$ mice indicate changes in hepatic clearance and overall exposure consistent with that reported in humans (Morse et al., 2020). Protein expression data indicate OCT1 to be the primary OCT expressed in mice and human liver (Drozdzik et al., 2019; Morse et al., 2020). In mice, the decrease in sumatriptan and fenoterol hepatic clearance was consistently associated with a decrease in liver partitioning, which would be expected to occur in humans lacking OCT1 function as well. In humans, while proguanil was clearly demonstrated an OCT1 substrate *in vitro*, the exposure of proguanil was not altered in subjects with null OCT1 activity (Matthaei et al., 2019), which can be explained by hepatic clearance not being the major clearance pathway for proguanil. While hepatic metabolism of proguanil may be the primary route of formation of its active metabolite, it is not necessarily the primary route of elimination of the parent. Indeed, following an oral dose of proguanil, 30–69% was found in urine (Somogyi et al., 1996), meaning that urinary excretion represents at least 30–69% of proguanil elimination, depending on the bioavailability of proguanil. This appears consistent between humans and mice from the current dataset. In humans, while proguanil plasma exposure was not significantly affected, the exposure of cycloguanil was decreased in subjects with decreased OCT1 activity, with a corresponding decrease in the metabolite ratio (Matthaei et al., 2019). We observe similar effects on systemic exposure in Oct1/2$^{-/-}$ mice.

Given that hepatocytes are a site of action/replication for malaria, an understanding of the potential liver exposures of proguanil and cycloguanil in subjects lacking OCT1 function is relevant as these may play a role in the pharmacodynamics, as discussed previously (Matthaei et al., 2019). The decrease in cycloguanil exposure indirectly supports a decrease in proguanil liver partitioning in subjects carrying OCT1 variants. The current data in mice directly indicate that proguanil liver exposure is decreased with depletion of Oct1 in mice. Given that the primary route of elimination for proguanil for humans and mice is renal clearance, it is

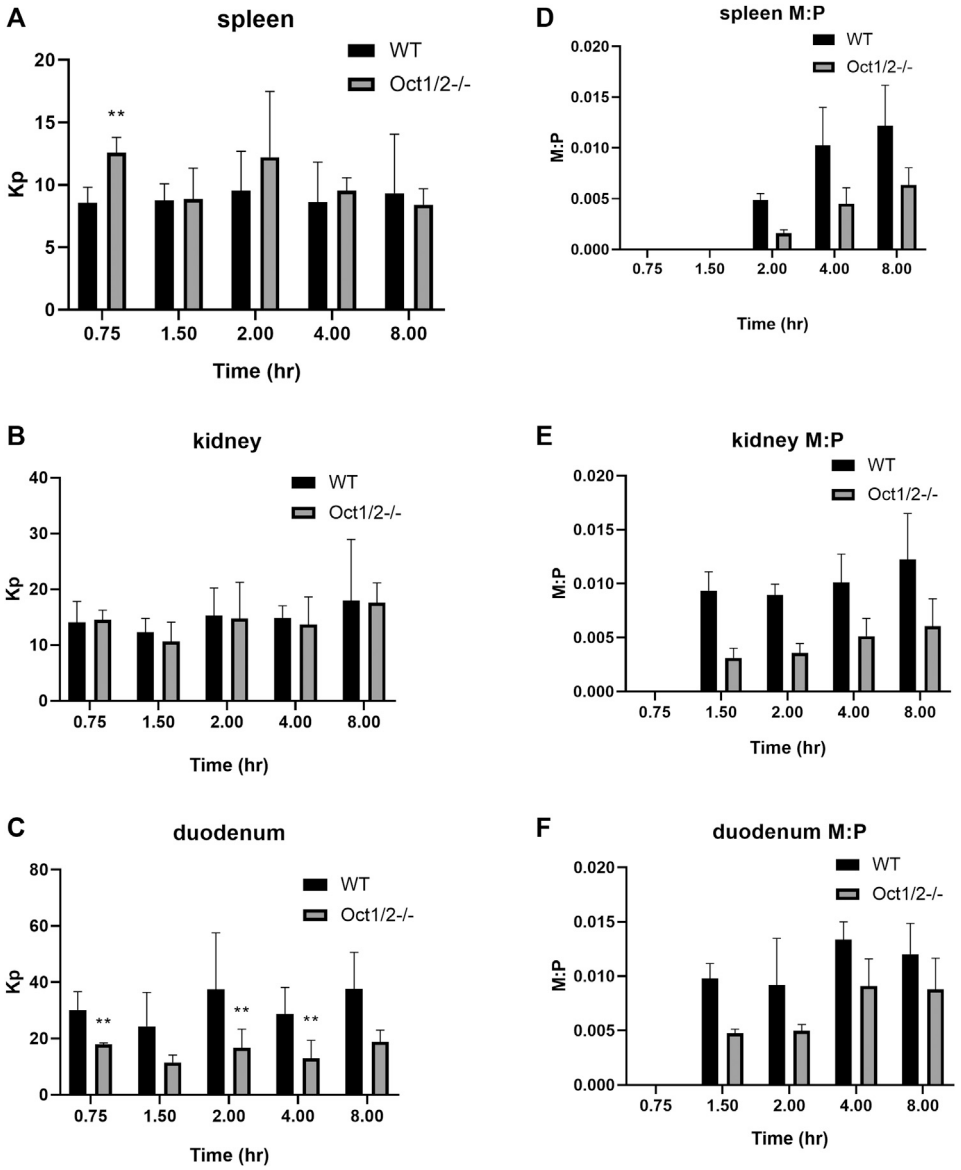


**FIGURE 5 | Partitioning of proguanil (A and B, C) and cycloguanil metabolite:parent ratio (D and E, F) in tissues other than liver in wildtype (WT) and Oct1/2$^{-/-}$ mice following IV administration of proguanil.** Mice ($n$ = 5/timepoint) were administered proguanil 2, 10 or 30 mg/kg and tissues collected at 0.75 and 2 h (2 mg/kg), 1.5 and 4 h (10 mg/kg) or 8 h (30 mg/kg) post-dose. Data presented as mean ± SD. **$p$ < 0.01 using student's t-test, compared to WT. Kp = tissue:plasma partition coefficient. M:P = metabolite:parent ratio.

likely that in humans the liver exposure is also decreased, as the authors of the clinical study hypothesized. The authors also determined cycloguanil to be a substrate of OCT1, therefore predicting the effect of decreased OCT1 activity on the liver exposure of cycloguanil is somewhat less straightforward. Indeed, this could mean than in subjects with null OCT1 activity, the exposure of cycloguanil may be decreased by two mechanisms, that being decreased formation due to decreased liver partitioning of proguanil, and by decreased uptake back into hepatocytes once effluxed. In the current mouse experiments, we did in fact determine lower exposure of cycloguanil in the liver of knockout mice compared to wildtype, following administration of proguanil or cycloguanil. We also confirmed that knockout of Oct1 led to decreased liver partitioning of cycloguanil, following dosing of cycloguanil. Therefore, what is unexpected in the current dataset is that when proguanil is directly administered compared to cycloguanil administration the lack of change in the metabolite:parent ratio in the liver, along with the small change in cycloguanil liver Kp in knockout compared to wildtype. This suggests that the effect of decreased OCT1 activity on liver partitioning of

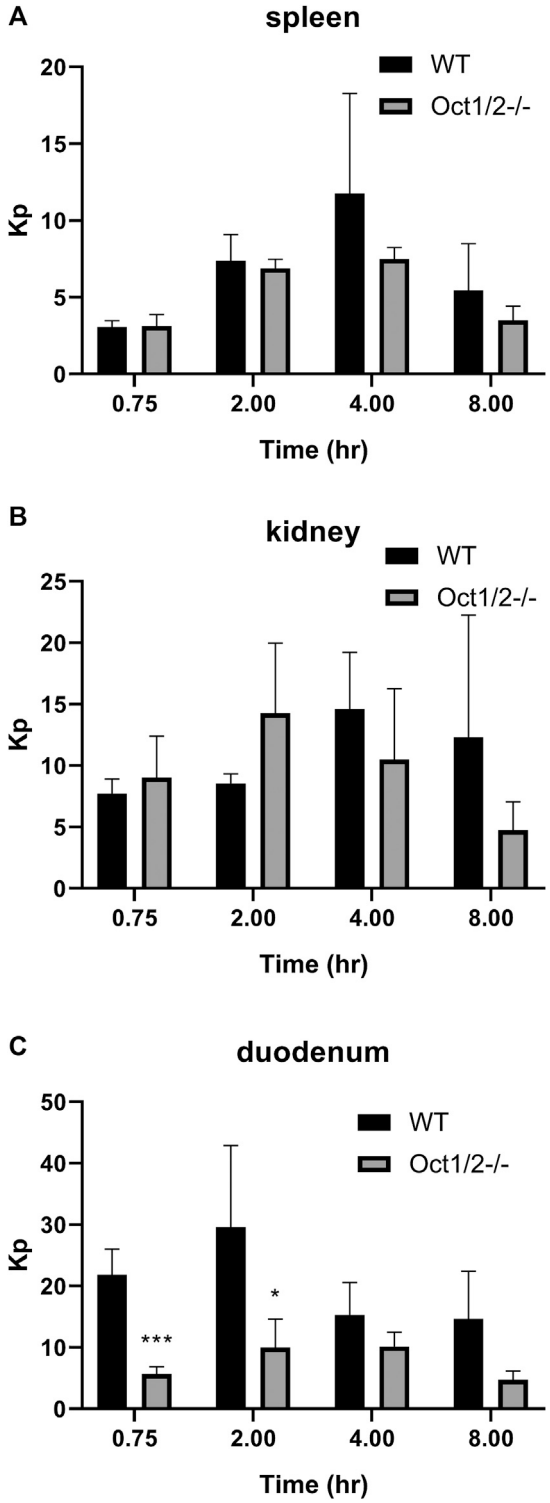


**FIGURE 6 | Partitioning of cycloguanil in tissues other than liver (A and B, C) in wildtype (WT) and Oct1/2$^{-/-}$ mice following IV administration of cycloguanil.** Mice ($n$ = 4–5/timepoint) were administered 2 mg/kg cycloguanil and sacrificed 0.75, 2, 4, or 8 h post-dose. Data presented as mean ± SD. *$p$ < 0.05 using student's t-test, compared to WT. ***$p$ < 0.001 using student's t-test, compared to WT. Kp = tissue:plasma partition coefficient.

an OCT1 substrate that is *formed* in the liver may differ from that ascertained by assessment of that taken up into the liver. This may occur if OCT1 is responsible for both the uptake and efflux into hepatocytes, and therefore depletion of OCT1 activity may affect both to a different degree due to differences in the electrochemical gradient and concentration gradient of the substrate when a metabolite is formed vs. administered.

One potential limitation to the current dataset is the use of commercially available Oct1/2 double knockout mice, and not a model specific for Oct1. However, in our previous work, we detected only mouse Oct1 in the liver (Morse et al., 2020), suggesting that any changes in the knockout model in the liver can be attributed to changes in Oct1, not Oct2. Oct2 is highly and primarily expressed the kidney in mice, therefore given the lack of change in renal clearance, kidney partitioning and only minimal change in total clearance of either proguanil or cycloguanil, it does not appear that Oct2 knockout significantly affected the pharmacokinetics in either compound in this study. Another limitation may be measurement of membrane-associated OCT1 protein, without consideration for expression on the membrane surface. Additional techniques, such as biotinylation, may refine measurements specifically at the membrane surface to determine if membrane localization vs. surface expression may differ. Furthermore, it is difficult to confirm the results of membrane OCT1 protein in cell lines to that in hepatocytes expressing the variants, given the difficulty in identifying hepatocyte samples homozygous for all of the variants, some of which exist at very low frequency in any population (Seitz et al., 2015).

In conclusion, it is clear that there is a reproducible effect of changes in OCT1 activity *in vitro* that can be observed on plasma pharmacokinetics *in vivo*, from both human and animal studies. The mechanisms behind the altered activity and substrate-dependence have been investigated here and elsewhere. In general, from the current and previous results, even when considering differences in protein expression and affinity, the $V_{max}$ for OCT1*2 changes in a substrate dependent manner, suggesting complex mechanisms behind activity by OCT1*2, possibilities of which have been discussed in detail (Seitz, 2016). Furthermore, the current dataset indicate that even though *in vitro* activity may reproduce changes in plasma pharmacokinetics, we are lacking in an understanding of what may be happening in sites other than plasma, particularly for metabolites that are transported by OCT1. Further work *in vitro* and *in vivo* are needed to understand these mechanisms and therefore the effects in subjects with decreased function of OCT1, especially when drug concentration at the site of action may be influenced by OCT1.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Covance IACUC.

## AUTHOR CONTRIBUTIONS

BLM, LHC, JKF, PCS, and KMH contributed to writing of the manuscript. BLM, LHC, JKF, PCS, KMH designed the research. BLM, LHC, JTC, and JKF performed the research. BLM, LHC, JTC, and JKF analyzed the data.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.631793/full#supplementary-material.

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REVIEW
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# Drug-Drug Interactions at Organic Cation Transporter 1

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The interaction between drugs and various transporters is one of the decisive factors that affect the pharmacokinetics and pharmacodynamics of drugs. The organic cation transporter 1 (OCT1) is a member of the Solute Carrier 22A (SLC22A) family that plays a vital role in the membrane transport of organic cations including endogenous substances and xenobiotics. This article mainly discusses the drug-drug interactions (DDIs) mediated by OCT1 and their clinical significance.

**Keywords: organic cation transporters, OCT1, substrate, inhibitor, drug-drug interaction**

## 1 INTRODUCTION

Drug-drug interactions (DDIs) are among the critical factors in determining clinical drug disposition and response. DDIs refer to the changes in toxicity, pharmacokinetics or pharmacodynamics of a drug when two or more drugs are applied simultaneously or sequentially (Palleria et al., 2013; Prueksaritanont et al., 2013; Koepsell, 2015). DDIs on one hand can enhance the efficacy of a drug and on the other hand may reduce the efficacy or even lead to toxic reactions to a drug (Palleria et al., 2013; Sun et al., 2016; Niu et al., 2019). Movement of endogenous and exogenous chemicals across the biological membrane is usually mediated by transporter proteins that play a central role in the physiological function, pharmacological action, and elimination fate of these compounds. Drug transporters usually have extensive binding affinity toward a broad spectrum of small molecule substrates and inhibitors, suggesting their important role in DDIs (Girardin, 2006; Koepsell, 2015; Liang et al., 2015). Nowadays, more and more attention has been paid to the DDIs mediated by drug transporters. Transporter-mediated DDIs affect pharmacokinetics and pharmacodynamics, especially drug absorption and elimination (Giacomini et al., 2010; Liu et al., 2015). Drug transporters exist in almost all organs of human body, mainly in the brain, intestinal tract, kidney, liver, and lung (Liu and Pan, 2019).

The human organic cation transporter 1 (hOCT1), encoded by the *SLC22A1* gene, is highly expressed in the liver and possesses a broad substrate specificity (Koepsell et al., 2003). Approximately 40% of prescription medicines are organic cations (Neuhoff et al., 2003; Koepsell, 2020). The disposition of more than 120 drugs has been related to the activity of OCTs including OCT1-3 (Nies and Schwab, 2010). OCT1 function is thus closely related to pharmacotherapy of various diseases including cancer, cardiovascular and cerebrovascular diseases, digestive system diseases, substance addiction and CNS diseases. Because OCT1 can also transport certain endogenous metabolites, its activity may also be of great significance to the maintenance of homeostasis in the body (Nies et al., 2011b; Lozano et al., 2013; Brosseau and Ramotar, 2019; Li et al., 2019). Herein the physiological and pharmacological effects of OCT1 are briefly introduced, followed by a focused review on DDIs mediated by OCT1.

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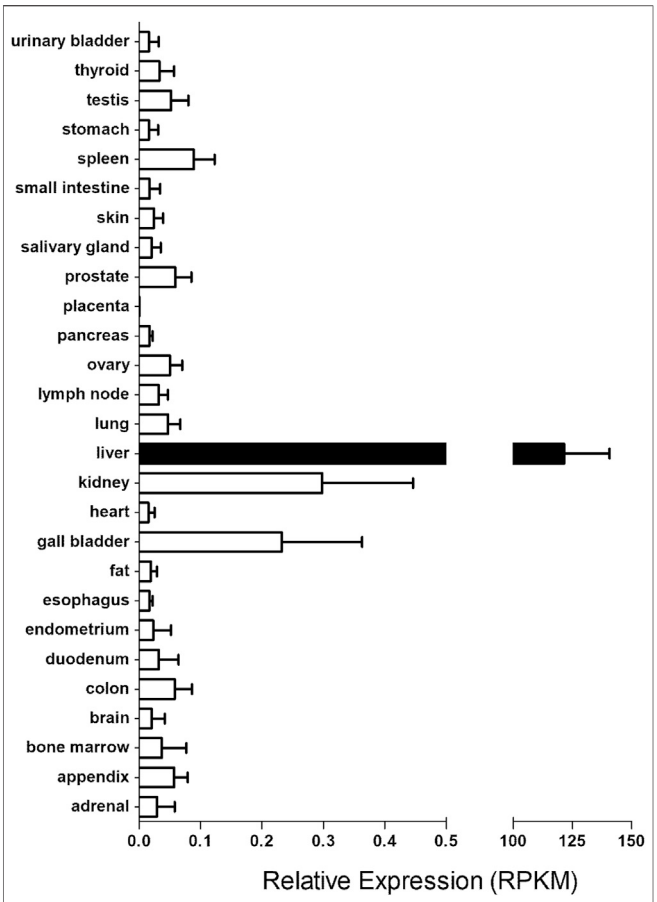


**FIGURE 1 |** The transcript levels of *SLC22A1* gene in major human tissues. The RNA sequencing data for human tissues were retrieved from https://www.ncbi.nlm.nih.gov/gene/6580. RPKM stands for the Reads Per kilobase of transcript, per Million mapped reads in RNA sequencing, which is a normalized unit of transcript expression.

## 2 MOLECULAR CLONING AND CHARACTERIZATION OF ORGANIC CATION TRANSPORTER 1

OCT1 is a member of the Solute Carrier (SLC) Family 22 responsible for the uptake of numerous organic cations, anions and zwitterions, across the plasma membrane (Koepsell, 2013). Rat OCT1 (rOCT1) was the first cloned member of the SLC22A family. rOCT1 was cloned in 1994, and hOCT1 in 1997 by Koepsell group (Grundemann et al., 1994; Gorboulev et al., 1997). The human *SLC22A1* gene encoding hOCT1 is located on chromosome 6q26 and consists of 11 exons and 10 introns (Koehler et al., 1997). The human OCT1 protein has 554 amino acids. Like most transporters in the SLC22A family, it is composed of 12 α-helical transmembrane domains (TMDs) with intracellular N- and C-termini (Shu et al., 2003; Koepsell, 2013; Lozano et al., 2013). There is a large glycosylated extracellular loop between the TMD 1 and TMD 2, which can form disulfide bonds for protein oligomerization. In addition, between the TMD 6 and TMD 7, there is an intracellular loop with consensus sites that can be phosphorylated by several protein kinases. These glycosylation and phosphorylation sites are associated with the regulation of transport functions by regulatory proteins such as protein kinases A&C (Ciarimboli and Schlatter, 2005; Shu, 2011; Brosseau and Ramotar, 2019).

## 3 DISTRIBUTION AND FUNCTION OF ORGANIC CATION TRANSPORTER 1 IN HUMAN TISSUES

The importance of hOCT1 in drug disposition and response is implicated by its tissue expression pattern and cellular location. Although hOCT1 is widely distributed in human tissues, it is primarily expressed in the liver (Koepsell et al., 2003) (**Figure 1**). In hepatocytes, it has been located at the sinusoidal (basolateral) membrane. Of note, it is less expressed in cholangiocytes as compared to hepatocytes in the liver (Nies et al., 2009). In the intestine, there is evidence from immunolocalization and pharmacokinetics (PK) studies in support of hOCT1 expression in the basolateral membrane (Muller et al., 2005). However, this has been challenged by other results which supported that hOCT1 and mouse OCT1 (mOCT1) were actually expressed in the apical membrane of intestinal epithelia cells (Han et al., 2013). Further investigation is needed to settle this dispute. In the kidney, while rOCT1 has been reported to be located to the basolateral membrane of epithelial cells in the proximal tubules (Karbach et al., 2000; Sugawara-Yokoo et al., 2000), there is immunohistochemistry evidence supporting the location of hOCT1 in the apical membranes of proximal and distal tubules (Tzvetkov et al., 2009). In the lung, OCT1 is located in the lumen (apical) membrane of ciliated cells (Lips et al., 2005) and bronchial epithelial cells (Mukherjee et al., 2012). In addition, OCT1 has been reported to be expressed on the luminal side of brain microvessel endothelial cells (BMECs) (Lin et al., 2010), olfactory and nasal respiratory tissues (Chemuturi and Donovan, 2007), ovary, prostate, testis (Jung et al., 2008), cardiomyocytes (Rossato et al., 2011) and $CD4^+$ cells of HIV-infected patients (Minuesa et al., 2008; Jung et al., 2013).

OCT1 is a poly-specific amphiphilic solute facilitator of transmembrane protein which bidirectionally mediates the transport of electrogenic organic cations across the plasma membrane in a manner independent of either $Na^+$ or $Ca^{2+}$ gradients (Busch et al., 1996; Gorboulev et al., 1997; Brosseau and Ramotar, 2019). OCT1 not only mediates the delivery of many cationic drugs and endogenous substrates into hepatocytes from the hepatic sinuses but also the release of organic cations from hepatocytes into the hepatic sinuses (Jonker and Schinkel, 2004; Koepsell et al., 2007; Nies et al., 2009). Consistent with its tissue expression patterns, OCT1 is also involved in the transport of certain substances in other organs. For example, it can regulate the secretion and absorption of organic cations in the small intestine (Koepsell, 1998), the reabsorption of ultrafiltration cations in the kidney (Koepsell et al., 1999), and the absorption of some drugs in the lung (Lips et al., 2005). Furthermore, OCT1 has been reported to promote organic

**TABLE 1 |** List of substrates and inhibitors of OCT1. The related information is cited from Drugbank https://www.drugbank.ca/categories/DBCAT004550, https://www.drugbank.ca/categories/DBCAT004549 and the references of this review.

| Drug category | Substrates | Inhibitors |
|---|---|---|
| Alkaloids | Coptisine, jatrorrhizine, epiberberine and berberrubine, nitidine chloride, monocrotaline, retrorsine | Nuciferine, berberine, retrorsine, anisodine, monocrotaline |
| Alpha-2A adrenergic receptor agonists | Uanfacine | Guanfacine |
| Alpha-blockers | Prazosin | Prazosin, phenoxybenzamine |
| Anesthetics | | Cocaine, lidocaine |
| Antiarrhythic drugs | Verapamil | Procainamide, verapamil, disopyramide, quinidine, dronedarone, propafenone |
| Antibiotics | Amoxicillin | Levofloxacin, trimethoprim, moxifloxacin |
| Anticancer drugs | Cytarabine, nintedanib, oxaliplatin, picoplatin | Rucaparib, dacomitinib, gilteritinib, palbociclib, nintedanib, irinotecan, erlotinib, nilotinib, dasatinib, mitoxantrone, paclitaxel, tamoxifen, amsacrine |
| Anticoagulant drugs | Nafamostat | |
| Anticonvulsant drugs | Lamotrigine | Lamotrigine |
| Antidepressant drugs | Fluoxetine | Desipramine, fluoxetine, imipramine, amitriptyline, trimipramine, citalopram, fluvoxamine, maprotiline, nomifensine, paroxetine, reboxetine, nefazodone, imipramine |
| Antifungal drugs | | Ketoconazole, itraconazole, clotrimazole, isavuconazole, griseofulvin |
| Antihistamine agents | Chlorpheniramine maleate, diphenhydramine | Chlorpheniramine, dexchlorpheniramine maleate, diphenhydramine |
| Antihypertensive drugs | Amiloride | Reserpine, doxazosin, amiloride, diltiazem, clonidine |
| Antimalarial drugs | Quinine, proguanil | Quinine |
| Antimuscarinic drugs | | Atropine |
| Antiparasitic drugs | | Pyrimethamine |
| Antiparkinson drugs | Pramipexole, amantadine | Amantadine |
| Antiplatelet drugs | | Clopidogrel |
| Antiprotozoal drugs | Pentamidine, furamidine | Pentamidine, furamidine, eflornithine |
| Antipsychotic drugs | Sulpiride, amisulpride, haloperidol | Quetiapine, chlorpromazine, clozapine, levomepromazine, remoxipride |
| Antituberculosis drugs | Ethambutol, isoniazid, prothionamide, *para*-aminosalicylic acid | Pyrazinamide |
| Antitussive drugs | | Carbetapentane |
| Antiviral drugs | Ganciclovir, acyclovir, amantadine, lamivudine, peramivir | Ganciclovir, acyclovir, saquinavir, nelfinavir, indinavir, ritonavir, darunavir, efavirenz, nevirapine, daclatasvir |
| Beta-2 adrenergic agonist | Fenoterol, formoterol, salmeterol | Formoterol, salmeterol |
| Nonselective beta adrenergic receptor blocker | Nadolol | Carvedilol, bucindolol |
| Bronchodilators | Ipratropium, salbutamol | Ipratropium, metaproterenol, salbutamol |
| Diuretics | | Spironolactone |
| Endogenous compounds | Histamine, dopamine, choline, epinephrine, norepinephrine, spermine, spermidine, serotonin, noradrenaline | Prostaglandin, choline, guanidine |
| Experimental compounds | Acetylcholine, choline salicylate, rhodamine, tropane alkaloids, cycloguanil, 4-(4-(dimethylamino)styryl)-N-methylpyridinium, synephrine | Nicotine, choline salicylate, tropane alkaloids, N1-methylnicotinamide, creatinine, corticosterone |
| Flavonoids | Quercetin | |
| Histamine H3 receptor antagonists | | Pitolisant |
| Hormone drugs | | Progesterone, estradiol acetate, estradiol benzoate, estradiol cypionate, estradiol dienanthate, estradiol valerate, osilodrostat |
| Hypoglycemic drugs | Metformin, phenformin, buformin | Phenformin, linagliptin, repaglinide, rosiglitazone, sitagliptin |
| H2 receptor antagonists | Cimetidine, ranitidine, famotidine | Cimetidine, ranitidine, famotidine |
| Immunosuppressants | | Cyclosporine |
| Janus kinase inhibitors (JAK inhibitors) | | Peficitinib |
| Muscarinic antagonists | Trospium chloride, oxybutynin | Oxybutynin |
| Neuromuscular blockers | Pancuronium, tubocurarine, rocuronium | Pancuronium, tubocurarine, rocuronium |
| Opioids | Methylnaltrexone, morphine, hydromorphone, norlevorphanol, norfentanyl, noroxycodone, meptazinol, 3-methoxymorphinan, oxymorphone, dextrorphan | Dextromethorphan, dextrorphan, levorphanol, levomethorphan, dextromethorphan, meptazinol, sufentanil, tapentadol, pethidine, norlevorphanol, tilidine, fentanyl, N-desmethyltramadol, morphine, nortilidine, tramadol |
| Selective serotonin receptor agonists | Sumatriptan | |
| Serotonin 5-HT3 receptor antagonists | Ondansetron, tropisetron | Ondansetron |

(Continued on following page)

**TABLE 1 |** (*Continued*) List of substrates and inhibitors of OCT1. The related information is cited from Drugbank https://www.drugbank.ca/categories/DBCAT004550, https://www.drugbank.ca/categories/DBCAT004549 and the references of this review.

| Drug category | Substrates | Inhibitors |
|---|---|---|
| Uricosuric drugs | | Probenecid |
| Vitamins | Thiamine | |
| Serotonin (5-HT)1F receptor agonists | | Lasmiditan |

cation crossing of the blood-brain barrier (BBB) (Lin et al., 2010), mediate the uptake of endogenous substrates into olfactory and respiratory mucosae (Chemuturi and Donovan, 2007) and the antiviral drugs into human immune cells (Minuesa et al., 2008; Jung et al., 2013).

## 4 ALTERATION OF ORGANIC CATION TRANSPORTER 1 BY LIVER DISEASES

The liver predominantly expresses OCT1 and is the major organ responsible for drug metabolism in human body (Nishimura and Naito, 2005). A growing body of evidence suggests that the expression and function of OCT1 changes in liver diseases, which could affect drug disposition in the body, not only by increasing the possibility of DDIs but also by enhancing the complexity of drug treatment (Schaeffeler et al., 2011; Lautem et al., 2013; Li et al., 2019). Compared to that in normal rat liver tissues, rOCT1 mRNA expression was decreased in the presence of cholestasis (Cherrington et al., 2004). Interestingly, in the early stage of liver fibrosis associated with hepatitis C virus (HCV) infection, the hOCT1 mRNA expression was significantly increased (Ogasawara et al., 2010); however it decreases during the aggravation of fibrosis (Hanada et al., 2012). In addition, the alteration in human OCT1 expression in miscellaneous tumor cells, such as hepatocellular carcinoma (HCC) cells and cholangiocellular carcinoma (CGC), has also been reported. Compared with adjacent normal liver tissue, the expression of OCT1 was significantly down-regulated in primary liver cancers originating from epithelial cells such as HCC, CGC, and hepatoblastoma (Herraez et al., 2013; Lautem et al., 2013; Namisaki et al., 2014). In HCC and CGC, the reduced expression of OCT1 was associated with advanced tumor stages and poor patient survival (Heise et al., 2012; Lautem et al., 2013). The decreased expression appears to be caused by DNA methylation in the promoter of the *SLC22A1* gene (Schaeffeler et al., 2011).

## 5 ORGANIC CATION TRANSPORTER 1 SUBSTRATES AND INHIBITORS

OCT1 works to regulate the cellular uptake of substrates. The substrates of OCT1 are usually organic cations with one or two positive charges, or weak bases with positive charges at physiological pH (Koepsell et al., 2003). Some uncharged compounds such as cimetidine can also be transported under alkaline conditions. The molecular weight of non-substrate inhibitors for OCT1 is in general larger than those of substrates. Sometimes, multiple inhibitor molecules can bind to the transporter protein simultaneously (Koepsell et al., 2003; Nies et al., 2011b; Shu, 2011; Koepsell, 2020). Most, if not all, of the substrates and inhibitors of OCT1 reported in the literature are summarized in **Table 1**.

## 6 INTERACTION OF ORGANIC CATION TRANSPORTER 1 WITH CLINICAL MEDICATION (FIGURE 2)

Many drugs are present as cations at physiological pH. As the most abundant organic cation transporter in the human liver, OCT1 mediates the transport of many organic cationic drugs across the hepatocyte membrane and may play an important role in regulation of metabolism of many drugs (Koepsell et al., 2007; Shu, 2011). Two or more therapeutic drugs that are OCT1 substrates may be administered simultaneously or subsequently in clinical applications. Because the expression level of OCT1 is relatively constant, competition between these substrates can happen for their cellular transport *via* OCT1. Likewise, some endogenous substrates can also compete for the uptake of xenobiotic drug substrates (Brosseau and Ramotar, 2019). In addition to the competitive inhibition of one substrate by another, many compounds such as lidocaine, prazosin, cocaine and dasatinib, which are not substrates of OCT1, can inhibit the uptake of OCT1 substrates (Brosseau and Ramotar, 2019). The inhibitors of OCT1, like those of other transporter proteins, are generally classified as competitive and non-competitive, depending on how the compounds interact with the binding site at the transporter protein and subsequently the dissociation manner (Belzer et al., 2013; Chen E. C. et al., 2017; Boxberger et al., 2018). However, both competitive and non-competitive inhibition may result in DDIs of clinical significance.

There is abundant evidence supporting a role of OCT1 in DDIs pre-clinically; however, only a few DDIs between OCT1 inhibitors/substrates have been reported in human subjects. Notably, even for these clinical DDIs, a contribution from other transporters or mechanism may not be excluded. For example, metformin is the victim drug in all of these clinical DDIs (**Table 2**). However, metformin is a substrate not only for OCT1 but also for OCT2, OCT3, plasma membrane monoamine transporter (PMAT), serotonin reuptake transporter (SERT), and others (Graham et al., 2011; Gong et al., 2012). In particular,

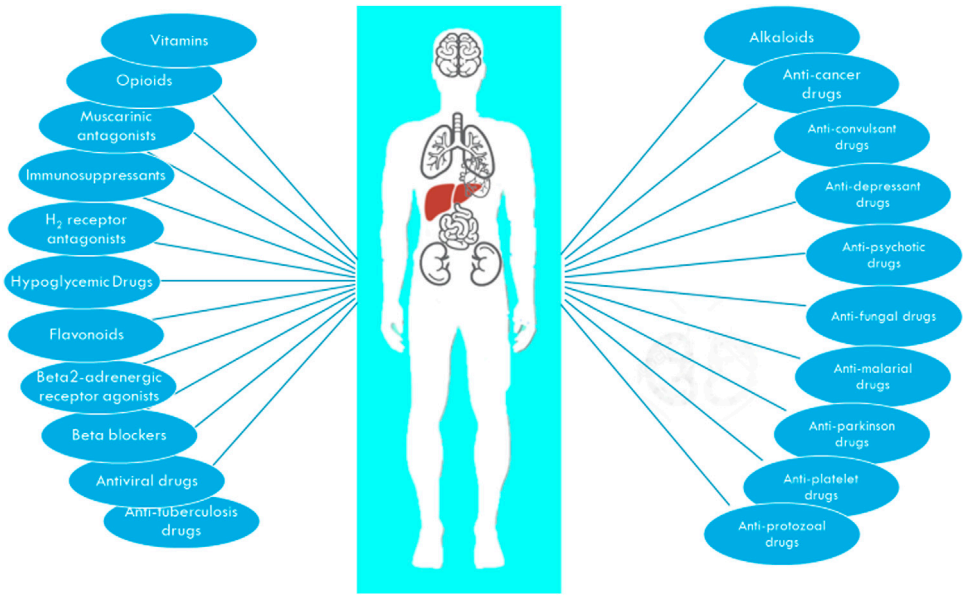


**FIGURE 2 |** The major drug classes interacting with OCT1.

**TABLE 2 |** Clinically relevant DDIs between OCT1 substrates/inhibitors with metformin. Note: Although OCT1 is assumed to play a role in these DDIs, the contribution from other transporters or mechanism cannot be ruled out.

| Perpetrator drug | Victim drug | Effects |
|---|---|---|
| Isavuconazole | Metformin | The AUC and $C_{max}$ of metformin were significantly higher with ivaconazole treatment Yamazaki et al. (2017) |
| Daclatasvir | Metformin | The number of adverse events increased in subjects that received both daclatasvir and metformin as compared to those receiving metformin alone Smolders et al. (2017) |
| Peficitinib | Metformin | The AUC, $C_{max}$, and renal clearance ($CL_R$) of metformin were reduced by peficitinib treatment, which is likely due to inhibition OCT1 and MATE1/2-K by peficitinib Shibata et al. (2020) |
| Opioids | Metformin | Opioids reduced the effect of metformin on the abundance of gut bifidobacterium, which is likely due to OCT1 inhibition by opioids Barengolts et al. (2018) |
| Verapamil | Metformin | Verapamil treatment reduced metformin's ability to lower blood glucose, which is likely due to hepatic OCT1 inhibition by verapamil Cho et al. (2014) |
| Sitagliptin | Metformin | In subjects who did not reach the maximal goal of HbA1c with a sub-maximal dose of metformin, the addition of sitagliptin improved the glycemic response and glycated hemoglobin goals for metformin treatment Frias et al. (2019) |
| Rifampin | Metformin | Rifampin can up-regulate the expression of OCT1 in peripheral blood cells, increase the concentration of metformin in the blood and enhance the hypoglycemic effect of metformin Cho et al. (2011) |
| OCT1 inhibitors | Metformin | Concomitant use of medications, known to inhibit OCT1 activity, was associated with gastrointestinal side effects and intolerance of metformin Dujic et al. (2015) |

OCT2 plays an important role in the renal elimination of metformin (Kimura et al., 2005). Although the role of OCT1 in metformin disposition and efficacy in human subjects is well supported by genetic evidence (Mofo Mato et al., 2018), the perpetrator drugs could have affected the activities of other metformin transporters, which might also contribute to the observed DDIs.

The International Transporter Consortium, in collaboration with US FDA, has issued recommendations on transporter function assessment during drug development (Giacomini et al., 2010; Zamek-Gliszczynski et al., 2012; Brouwer et al., 2013; Hillgren et al., 2013; Tweedie et al., 2013). However, although the assessment of OCT1 is suggested by the European Medicines Agency (EMA), it has not been included in the industry guidance on DDI studies by FDA. According to the FDA guidance, an inhibitory $K_i$ value of more than one-tenth of $C_{max}$ has been suggested for the perpetrator drug to cause a clinically relevant interaction with another victim drug that is transported by the same transporter. It is expected that many drugs may significantly inhibit the activity of OCT1 at their clinical plasma concentrations. Specific DDIs mediated by OCT1 for major classes of drugs are reviewed below. Notably, the majority of these DDIs are either speculated from cellular findings or only evident at the preclinical level of animal studies. Therefore, additional clinical studies are highly needed to ascertain the role of OCT1 in various DDIs.

## 6.1 Alkaloids

Protoberberine alkaloids belong to isoquinoline alkaloids and are mainly found in the plants of *Fumariaceae*, *Berberidaceae* and *Papaveraceae* families, which include berberrubine, coptisine, jatrorrhizine, palmatine, epiberberine and corydaline. Proberberine alkaloids have been reported to possess a potent inhibitory effect on human OCT1/2/3 (Li et al., 2016). Moreover, coptisine, jatrorrhizine, epiberberine were found to be high-affinity substrates of OCTs, while berberrubine was a selective substrate for human OCT1 and OCT2, but not OCT3. The findings have provided useful information to understand the pharmacological effects of alkaloids or traditional herb medicines containing those alkaloids. In particular, the results suggest potential DDIs mediated by OCT1 between alkaloids and clinical used drugs.

Specifically, berberine, a quaternary ammonium alkaloid isolated from several plants, is the main effective component of rhizoma coptidis. Berberine has been reported to inhibit OCT1/2-mediated uptake of metformin in HEK293-OCT1 cells in a concentration dependent manner (Kwon et al., 2015). After intravenous administration of metformin with berberine, the initial blood concentration and Area Under Curve (AUC) of metformin were increased in rats, but the clearance rate and distribution volume of metformin were decreased. However, there was no change in the plasma concentration of berberine after administration with metformin. Shi *et al.* also reported a pharmacokinetic interaction between metformin and berberine (Shi et al., 2019). In their study, metformin and berberine were dosed by oral gavage. The plasma concentration and the AUC of metformin were decreased in rats that received berberine co-treatment compared to those that received metformin alone. Metformin was believed to be absorbed in the small intestine *via* OCT1 (Zhou et al., 2007; Graham et al., 2011; Han et al., 2015). rOCT1 inhibition by berberine may have contributed to the pharmacokinetic changes of oral metformin in rats.

Additional evidence suggests that the hepatic uptake of jatrorrhizine is mediated by a transport system belonging to the OATP and OCT families. In particular, prazosin, an OCT1 inhibitor, could potently inhibit OCT1-mediated uptake of jatrorrhizine in HEK-OCT1 cells. There are likely DDIs between the herbs containing jatrorrhizine and a substrate or inhibitor of OCT1 at the hepatobiliary disposition (Liang et al., 2020).

Nuciferine, one of the main active components of *Nelumbo nucifera Gaertn*, is considered as a promising agent for the treatment of obesity-related diseases. Li *et al.* characterized nuciferine as an inhibitor of OCT1 (Li et al., 2018). They found that nuciferine could reduce the concentration of metformin in the liver through mOct1 inhibition in mice. In addition, it could weaken the hypoglycemic effect of metformin. However, the effects of nuciferine on the hepatic concentration and hypoglycemic effect of metformin were present only for a period of time after nuciferine administration, suggesting that intermittent administration of nuciferine and metformin, if necessary, might prevent the DDI mediated by OCT1.

Nitidine chloride (NC) is a quaternary ammonium alkaloid with numerous pharmacological effects such as anticancer activity. However, NC also has hepatocellular toxicity. Li *et al.* reported that NC was a high affinity substrate of human OCT3 and OCT1 (Li et al., 2014b). The two transporters were believed to mediate the uptake of NC into hepatocytes and subsequently cause hepatotoxicity. Quinidine, an OCT1 inhibitor, could significantly reduce the hepatic uptake of NC and NC-induced toxicity in cultured primary rat hepatocytes. This study suggests OCT inhibition as a strategy to prevent clinical hepatotoxicity associated with NC use.

Monocrotaline (MCT) is a pyrrolizidine alkaloid and it has pneumotoxic and hepatotoxic effects in animals (Copple et al., 2002). Tu *et al.* demonstrated that MCT is a substrate and inhibitor of OCT1 and has a high affinity to the transporter (Tu et al., 2013). In MDCK-hOCT1 cells, OCT1 was found to play a vital role in the uptake and toxicity of MCT, and the inhibitor of OCT1, quinidine, could significantly inhibit the uptake of MCT, thereby reduce the MCT-induced toxicity.

## 6.2 Anti-Cancer Drugs

Oxaliplatin is an anti-cancer chemotherapeutic drug. Oxaliplatin has been characterized as an excellent substrate of human OCT1 and OCT2. The results by Zhang *et al.* have indicated that the cytotoxicity of oxaliplatin and its cellular accumulation could be inhibited by the OCT1 inhibitor disopyramide in MDCK-hOCT1 cells (Zhang et al., 2006). Furthermore, Buss *et al.* reported that pre- and co-incubation with atropine, an inhibitor of OCT1, significantly reduced oxaliplatin accumulation in drug-sensitive cells but not in drug-resistant cells (Buss et al., 2018). One possible mechanism is the alteration of transporter localization in the drug-resistant cells. The data suggest an association between OCT1 expression and oxaliplatin resistance.

Picoplatin is a third-generation platinum drug. It is very effective in the treatment of drug-resistant or refractory lung cancer. The chemical structures of picoplatin and oxaliplatin are similar. More *et al.* reported that the monoaqua complex of picoplatin (but not the diaqua complex) was a substrate of OCT1 (More et al., 2010). As similar for oxaliplatin in MDCK-hOCT1 cells, disopyramide reduced the cytotoxicity of picoplatin in lung cancer cell lines and the accumulation of platinum in HEK-hOCT1 cells. These studies have provided a foundation to delineate the role of OCT transporters in platinum-based chemotherapy and the related toxicity.

Mitoxantrone is an anthraquinone drug which is used to treat prostate cancer and leukemia. It has been demonstrated as an inhibitor of OCT1 (Gupta et al., 2012). In addition, Li *et al.* have shown that mitoxantrone could reduce the apical (AP) to basolateral flux of peramivir in Caco-2 cells (Chen J. et al., 2017). The reason might be that mitoxantrone could inhibit the activity of OCT1 which is expressed in the AP membrane of Caco-2 cells and plays a role in the influx of solutes in enterocytes. Thus, MCT administration may lead to a reduction in peramivir absorption.

Sorafenib, a multi-tyrosine kinase inhibitor (TKI), is considered as an effective targeting therapy for advanced liver cancer (Keating, 2017). OCT1 plays a role in the uptake of sorafenib into cells. The results by Al-Abdulla *et al.* indicated that sorafenib uptake was enhanced in the cells expressing

hOCT1, which could be inhibited by the OCT inhibitor quinine (Al-Abdulla et al., 2019). Co-exposure with quinine suppressed not only hOCT1-mediated uptake of sorafenib but also sorafenib-induced cytotoxicity. In HCC patients treated with sorafenib, the protein expression of OCT1 at the plasma membrane was significantly associated with a beneficial response to sorafenib treatment. Interestingly, using the total healthy liver mRNA, there was no such association found (Geier et al., 2017). However, the relevance of OCT1 expression to sorafenib response remains controversial. Chen *et al.* recently reported that sorafenib was not a substrate of OCT1, and that the transporter was unlikely to participate in sorafenib disposition and influence its therapeutic effects in HCC (Chen et al., 2020).

Pazopanib is also a tyrosine kinase inhibitor. OCT1 has been reported to be responsible for the uptake of pazopanib in hepatocytes (Ellawatty et al., 2018). In addition, pazopanib is a potential inhibitor of OCT1 at clinically relevant concentrations. The unbound plasma concentration of pazopanib is slightly higher than the $IC_{50}$ value of the pazopanib inhibiting OCT1-mediated uptake of metformin, suggesting a clinically relevant interaction between pazopanib and other drugs mediated by OCT1.

There are additional TKIs that have been reported as OCT1 inhibitors. Minematsu *et al.* demonstrated that erlotinib and nilotinib were potent inhibitors of OCT1 at clinically relevant concentrations (Minematsu and Giacomini, 2011). The two drugs could significantly inhibit metformin uptake mediated by OCT1 in HEK-hOCT1 cells. At a concentration similar to the clinically achievable unbound plasma concentrations, the two TKIs could inhibit the uptake of oxaliplatin. These data implicate clinical DDIs between TKIs and other OCT1 substrates or inhibitors.

OCT1 also interacts with additional anti-cancer drugs. For example, rucaparib is a potent small-molecule inhibitor of poly(ADP-ribose) polymerase enzymes that are important in cancer development and metastasis (Colombo et al., 2018). The results by Liao et al. indicated that rucaparib could potently inhibit human OCT1/2-mediated metformin uptake in cells (Liao et al., 2020). Because inhibition of OCT1/2 could decrease the uptake of metformin in the liver and its elimination in the kidney, and might reduce its hepatic anti-hyperglycemic action, there is a possible undesirable interaction between rucaparib and metformin in diabetic cancer patients who are treated by these two drugs.

## 6.3 Anti-Convulsant Drugs

Lamotrigine, an anti-epileptic medication, is also used to delay mood episodes in adults with bipolar disorder. Dickens *et al.* have reported that lamotrigine is a substrate and inhibitor of OCT1 and its transport into human brain endothelial cells can be mediated via OCT1 (Dickens et al., 2012). In addition, the anti-psychotic quetiapine, an inhibitor of OCT1, could inhibit the uptake of lamotrigine in the hOCT1-transfected cells. Importantly, the *in vitro* $IC_{50}$ value for the inhibition was slightly lower than the steady state $C_{max}$ in patients treated with quetiapine. Therefore, the concentration required to inhibit OCT1 in the patient is achievable after treatment with quetiapine. Although the effect of lamotrigine on cellular transport of quetiapine is uncertain, the potential DDI in patients between the two drugs should be considered.

## 6.4 Anti-Depressant and Anti-Psychotic Drugs

The BBB is an important physiological barrier between the central nervous system and the blood circulation. The antidepressants and antipsychotics must cross the BBB into the central nervous system to function. OCT transporters have been reported to be expressed in the BBB and could mediate the uptake of these drug (Amphoux et al., 2006) (Lin et al., 2010). Dos Santos Pereira *et al.* and Takano *et al.* reported that amisulpride and sulpiride were substrates of OCT1 (Dos Santos Pereira et al., 2014; Takano et al., 2017). Sekhar et al. also reported that amisulpride and haloperidol were transported by OCT1 (Sekhar et al., 2019). In addition, Kang *et al.* demonstrated that the neurotoxic pyridinium metabolites of haloperidol were substrates of OCT1, and pretreatment with OCT1 inhibitors verapamil, cimetidine, phenoxybenzylamine, and corticosterone could significantly inhibit the accumulations of these metabolites in Caco-2 cells (Kang et al., 2006). However, because certain drugs such as amisulpride can be a substrate of multiple transporters in different cells, sometimes OCT1-mediated DDIs involved these drugs may not be evident. For example, an inhibitor of OCT1, did not change the uptake rate of amisulpride in hCMEC/D3 cells but could inhibit the uptake of sulpiride, leading to a reduction of intracellular sulpiride accumulation (Dos Santos Pereira et al., 2014). Amantadine could increase the accumulation of amisulpride in bEnd.3 cells, but it had no effect in hCMEC/D3 cells. In contrast, prazosin could reduce the uptake of amisulpride in hCMEC/D3 cells but not in bEnd.3 cells. The accumulation of amisulpride was not affected by haloperidol in either cell line (Sekhar et al., 2017). Conversely, the uptake of haloperidol could be significantly reduced by amantadine, prazosin and amisulpride in Caco-2 cells (Kang et al., 2006).

Some other antidepressants and antipsychotics may have a potential inhibitory effect on OCT1 activity (Ahlin et al., 2008). Haenisch *et al.* reported that at the concentrations relevant to their clinical plasma levels, a wide range of pharmacologically different antidepressants and antipsychotics could inhibit the activity of human OCT1 by more than 20%, thereby likely interfering with the pharmacokinetics of OCT1 substrates in the liver, kidney and brain (Haenisch et al., 2012).

## 6.5 Anti-Fungal Drugs

Ketoconazole and itraconazole are antifungal medications, and they are generally regarded as clinically importantly CYP3A4/5 inhibitors (Varhe et al., 1994; Greenblatt, 2016). Recently, Vermeer *et al.* reported that ketoconazole and itraconazole are inhibitors of OCT1. The two drugs could inhibit the uptake of quinidine *in vitro* (Vermeer et al., 2016). However, they are not OCT1 substrates as the data indicated that they were not transported into the liver by hepatic OCT1 (Higgins et al., 2014).

Isavuconazole, a novel triazole antifungal prodrug, is used to treat invasive mucormycosis and aspergillosis (Maertens et al.,

2016; Marty et al., 2016). In a clinical study, Yamazaki *et al.* has provided data in support of isavuconazole as an inhibitor of OCT1 (Yamazaki et al., 2017). Isavuconazole treatment could significantly alter the pharmacokinetics of metformin, such as the increase of its AUC and $C_{max}$. Of note, isavuconazole PK was unaffected by metformin treatment.

## 6.6 Anti-Malarial Drugs

It has been reported that anti-malarials such as amodiaquine, primaquine, proguanil, pyrimethamine can significantly reduce the cellular activity of OCT1 (van der Velden et al., 2017). Moreover, proguanil and cycloguanil are found to be the substrates of OCT1 and other organic cation transporters including OCT2, MATE1 and MATE2-K. Because the endemic of malaria, HIV/AIDS and tuberculosis is always overlapped geographically, the incidence of co-infection among patients is high. Multiple drugs are required for the treatment of co-infection. As described above, the interaction with OCT1 is also common with anti-viral and anti-tuberculosis drugs. OCT1-mediated DDIs are expected in concurrent therapy for the co-infection.

## 6.7 Anti-Parkinson Drugs

Pramipexole is a dopamine receptor agonist, which is used to treat the symptoms of Parkinson disease. The drug has been reported as a substrate for rat OCT1 (Ishiguro et al., 2005). However, a study by Diao *et al.* indicated that pramipexole was not a substrate for human OCT1 (Diao et al., 2010). Instead, pramipexole was identified as a substrate of human OCT2 and OCT3. It is likely that the absorption of pramipexole in human intestine may be mediated by OCT3 and possibly OCT2. In addition, OCT2 and OCT3 may function to transport pramipexole in renal elimination and brain distribution, respectively.

## 6.8 Anti-Platelet Drugs

Clopidogrel (CP) is a widely used anti-platelet drug. It is either metabolized by cytochrome P450s into active metabolites in the liver or hydrolyzed by esterase to clopidogrel carboxylate (CPC). A study by Li *et al.* indicated that CP could strongly inhibit the uptake of lamivudine and amantadine mediated by human OCT1 in MDCK-hOCT1 cells (Li et al., 2014a). CPC could also significantly reduce the uptake of lamivudine in these cells but only had slight inhibition on the uptake of amantadine. The likelihood of clinical DDIs between CP and amantadine is expected to be low. On the other hand, although CP itself inhibits the uptake of OCT1 substrates such as metformin, lamivudine and amantadine, in consideration of the short duration of CP in the liver and a low plasma concentration, Li *et al.* thought that the DDI mediated by OCT1 between CP and those substrate drugs may not be serious *in vivo*. Future clinical observation is needed to confirm this postulation.

## 6.9 Anti-Protozoal Drugs

Pentamidine and furamidine are used to prevent severe lung infection in AIDS patients. They belong to a class of drugs called antiprotozoals. Ming *et al.* have shown that pentamidine and furamidine are good substrates of hOCT1. Ranitidine, a known OCT1 inhibitor, could significantly reduce the cytotoxicity of pentamidine and furamidine in CHO-hOCT1 cells (Ming et al., 2009). In addition, Sekhar *et al.* reported that pentamidine was a substrate for OCT1 transporter at the BBB (Sekhar et al., 2017). The OCT1 inhibitor amantadine could decrease the accumulation of pentamidine in hCMEC/D3 and bEnd.3 cell lines. However, another OCT1 inhibitor prazosin decreased pentamidine accumulation only in hCMEC/D3 cells, but not in bEnd.3 cells. Those OCT1 inhibitors may be non-specific, and other transporters might contribute to the cellular uptake of pentamidine as well. The significance of OCT1 in mediating a DDI between antiprotozoals and other drugs has yet to be confirmed.

## 6.10 Anti-Tuberculosis Drugs

The anti-tuberculosis (anti-TB) drugs are divided into two categories according to use frequency and efficacy: first-line and second-line anti-TB drugs. Among the approved drugs, the first-line essential agents that form the core of treatment regimens are rifampin (RIF), isoniazid (INH), and ethambutol (EMB) (Sotgiu et al., 2015).

Te Brake *et al.* reported that EMB is a substrate of OCT1. Moxifloxacin, which was characterized as a potent inhibitor of OCT1, could significantly inhibit the cellular transport of EMB (Te Brake et al., 2016). Later, Parvez *et al.* confirmed that EMB, amoxicillin, INH and prothionamide were novel substrates of OCT1 and as expected, the OCT1 inhibitor verapamil could strongly reduce their cellular uptake (Parvez et al., 2018). In addition, they found that the DDI indices of OCT1-mediated uptake of EMB and prothionamide were similar to that of verapamil, suggesting a strong *in vivo* potential of DDIs for these drugs with others.

Moreover, the DDI analysis by Pan *et al.* indicated that EMB has a strong potential for DDIs mediated by human OCT1 and OCT3 which are expressed in intestinal epithelial cells and hepatocytes. These DDIs may result in an altered absorption, distribution and excretion of the cationic drugs which are co-administered with EMB (Pan et al., 2013). For example, TB patients with coexisting diabetes or HIV might develop significant DDIs when co-treated with EMB and an OCT1/OCT3 substrate (e.g., lamivudine or metformin).

In addition, Parvez *et al.* reported that pyrazinamide, levofloxacin, and RIF could significantly inhibit OCT1-mediated metformin uptake in HEK-OCT1 cells (Parvez et al., 2016). With a static model-based approach to assess the correlation between the inhibitory potential of anti-TB drugs and the prognosis, they predicted a strong possibility of DDIs for these drugs interacting with other OCT1 substrate drugs *in vivo* on affecting anti-TB efficacy.

*Para*-aminosalicylic acid (PAS) is a second-line anti-TB drug used to treat multidrug resistant tuberculosis. The results by Parvez *et al.* indicated that PAS is a substrate of several transporters including OCT1 (Parvez et al., 2017). While they demonstrated that metformin effectively inhibited PAS uptake *via* OCT1, their estimated DDI index did not support the existence of clinical DDIs. They also found that omeprazole,

lansoprazole, cimetidine, verapamil and quinidine could decrease the levels of OCT1-mediated PAS uptake *in vitro*. In contrast to that between PAS and metformin, the estimated DDI index values for the interaction between PAS and these OCT1 inhibitors were greatly higher than the cutoff and suggested possible clinical DDIs. The data are useful for future studies in patients to understand PAS disposition and clinical efficacy.

## 6.11 Antiviral Drugs

Many antiviral drugs have shown a binding affinity to OCT1 as substrates or inhibitors. Lamivudine, which is used to treat hepatitis B and HIV infection, belongs to a class of medications called nucleoside reverse transcriptase inhibitors (NRTIs). Human OCTs are characterized as important determinants of intracellular and plasma concentrations of lamivudine because they transport lamivudine and express in not only the organs of lamivudine disposition, such as liver and kidney, but also immune cells and excretory tissues that are critical to lamivudine action (Minuesa et al., 2009). Zalcitabine, another NRTI, has been demonstrated as a highly efficient substrate of OCT1 and OCT2 as well (Jung et al., 2008). Interestingly, the NRTIs abacavir and azidothymidine (zidovudine), the protease inhibitors nelfinavir, ritonavir, saquinavir, indinavir, and the anti-infective drugs pentamidine, trimethoprim are all high affinity inhibitors of OCT1 and OCT2. The concomitant administration of lamivudine and these potent OCT inhibitors is common in the regimen of highly active antiretroviral therapy (HAART). The DDIs may be of great significance in clinical practice, particularly for the pharmacokinetics, of lamivudine (Zhang et al., 2000; Jung et al., 2008; Minuesa et al., 2009; Jung et al., 2013; Arimany-Nardi et al., 2016). Consistently, Jung *et al.* documented that the addition of OCT1 and OCT2 inhibitors such as ritonavir and nelfinavir could reduce the accumulation of lamivudine in the CD4 cells of HIV-infected patients (Jung et al., 2013).

Efavirenz is antiviral drug in another class of medications called non-nucleoside reverse transcriptase inhibitors. It decreases the amount of HIV in the blood. Efavirenz has been demonstrated as an inhibitor of OCT1 by using hOCT1-overexpressing MDCK and KCL2 cells. The drug could inhibit the cellular transport and intracellular accumulation of lamivudine (Moss et al., 2015; Ceckova et al., 2018). The possible DDIs should be considered when co-administering efavirenz to HIV patients with other drugs.

Daclatasvir is used in combination with other medications to treat hepatitis C infection. Daclatasvir is a reversible and time-dependent inhibitor of OCT1 and OCT2 in cellular studies (Gandhi et al., 2018). However, Smolders *et al.* demonstrated that daclatasvir did not affect PK and PD parameters of the OCT1 substrate metformin in healthy subjects (Smolders et al., 2017). Interestingly, when daclatasvir was combined with metformin, the number of adverse events increased in human subjects. It has been suggested to monitor the adverse events during the treatment of type 2 diabetes mellitus (T2DM) patients with HCV infection under the combination treatment of daclatasvir and metformin.

In addition, Takeda *et al.* found that human OAT1 and hOCT1 are responsible for the renal transport of acyclovir and ganciclovir (Takeda et al., 2002). Caution should be taken when these antiviral drugs are used in conjunction with other drugs that share the same transporters for urinary tract excretion. Concomitant administration of these drugs may cause an increase in their plasma concentrations, leading to adverse drug reactions.

## 6.12 Beta-Adrenergic Receptor Blockers

Nadolol is a beta blocker that can be used alone or in combination with other drugs to treat high blood pressure. It is also used to prevent angina. Misaka *et al.* reported that nadolol was a substrate of human OCT1 and that OCT1-mediated nadolol uptake could be inhibited by cimetidine and trimethoprim *in vitro* (Misaka et al., 2016). In addition, carvedilol, another beta blocker, could inhibit metformin uptake mediated by human OCT1 and mouse OCT1 (Guo et al., 2018). These data will contribute to future human studies on OCT1-mediated DDIs involved beta blockers.

## 6.13 Beta2-Adrenergic Receptor Agonists

Beta-2-adrenergic agonists are first line agents in the treatment of asthma and other pulmonary disorders, such as chronic obstructive pulmonary disease. In a study by Salomon *et al.*, β2- adrenergic agonists such as salbutamol sulfate, formoterol fumarate, and salmeterol xinafoate were found to be substrates and inhibitors of OCT1 in human respiratory epithelial cells (Salomon et al., 2015). They demonstrated that the cellular uptake was mediated by hOCT1 in a time- and concentration-dependent manner for salbutamol, which was sensitive to inhibition by the OCT1 inhibitor verapamil. There was expression of hOCT1 and other organic cation transporters in human pulmonary epithelial cells. Therefore, OCT1 may be involved in the pulmonary disposition of beta2-adrenergic receptor agonists. Certain non-steroidal anti-inflammatory drugs (NSAIDs) were found to effectively inhibit the activity of OCT1 in leukemic cells (Wang et al., 2012). Mamlouk *et al.* found that the uptake of salbutamol was decreased in the presence of NSAIDs and proposed that NSAIDs could inhibit the absorption of salbutamol across the bronchial epithelium via the effects on OCT transporters (Mamlouk et al., 2013). In consideration of the highly polymorphic *SLC22A1* gene and a wide spectrum of substrates and inhibitors for this transporter protein, the DDIs mediated by OCT1 between drugs of this class and others may be clinically important.

## 6.14 Flavonoids

Flavonoids, such as phloretin and quercetin are secondary plant metabolites that can be found in different vegetables and fruits. Some flavonoids have been reported to possess health protective effects against cancer and cardiovascular diseases. There are studies indicating that quercetin is not a potent OCT1 inhibitor (Mandery et al., 2012; Glaeser et al., 2014). However, quercetin was characterized as a substrate of OCT1. In HEK293-hOCT1 cells, the uptake of quercetin could be significantly reduced by the OCT1 inhibitors such as amipamine, quinidine, and trimethoprim. There is also evidence in support

of flavonoids as OCT1 inhibitors. Mimura *et al.* reported that hOCT1-mediated atenolol transport could be inhibited by rhestin and quercetin, which are the main components of apple juice, as well as several other flavonoids (Mimura et al., 2015). In a cellular study by Taur *et al.*, quercetin could inhibit the activity of the OCT system and reduce the intracellular accumulation of the substrate tetraethylammonium in LLC-PK1 cells (Taur and Rodriguez-Proteau, 2008). The flavonoids, such as quercetin, have the potential to alter the disposition profile of certain therapeutics by which cellular transport is mediated by cation transporters including OCT1.

## 6.15 Hypoglycemic Drugs

Diabetic patients frequently have to be treated with more than one drug. Among the anti-diabetic drugs, metformin is the most widely studied in relation to OCT1 function (Inzucchi et al., 2012). Previous reports mainly focus on the effect of metformin on the disposition of other drugs. However, recent studies have shown that some drugs used in combination with metformin in the clinical treatment of diabetes can also affect the disposal process of metformin through OCT1 (Dawed et al., 2019) (**Table 2**). In addition, studies have shown that the intestinal OCT1 and concomitant medications play a vital role in the gastrointestinal adverse effects of metformin (Dujic et al., 2016). When metformin is used in combination with proton pump inhibitors (PPIs), tricyclic antidepressants (TCAs) or codeine, the likelihood of metformin intolerance is greatly increased (Stage et al., 2016).

Naringenin is a colorless flavorless flavanone. Mata Mofo *et al.* reported that naringenin could up-regulate the expression of human OCT1, thereby improving the symptoms associated with diabetes (e.g., weight gain, heavy drinking, metabolic acidosis) (Mato Mofo et al., 2020). The diabetic patients treated with metformin may thus take grapefruit juice of which the predominant flavanone is naringenin. Stage *et al.* analyzed 32 drugs which may inhibit metformin transporters to assess the risk of early discontinuation of metformin (Stage et al., 2016). The odds ratio for early discontinuation of metformin was only found to be associated with codeine use. The results indicated that co-administration of codeine may be associated with a risk of early discontinuation of metformin.

Although deletion of *Slc22a1* gene in mice did not cause any apparent physiological defects, OCT1 can transport various endogenous metabolites, suggesting a physiological role by OCT1 activity in drug action (Chen et al., 2014). In addition to the transport of metformin, OCT1 may be a target for metformin (Chen et al., 2014; Chen et al., 2015). Metformin can competitively inhibit OCT1-mediated thiamine uptake in cells, resulting in reduced intestinal and systemic plasma thiamine levels, as well as liver thiamine levels. Modulation of thiamine levels *via* OCT1 by metformin might be critically important in its beneficial effects in treatment of diabetes, obesity, hepatic steatosis and cancer.

In recent years, gut microbiota has been linked to diabetes and other metabolic disorders. Metformin has an effect on the balance of gut microbiota. A study by Barengolts *et al.* found that the interaction between opioids and metformin had a significant effect on the abundance of bifidobacteria in the gut (Barengolts et al., 2018). Metformin treatment was associated with a decrease in the abundance of gut bifidobacterium in opioid users. In contrast, in the opioid non-users, metformin treatment was associated with an increase in the abundance of gut bifidobacteria. While the exact mechanism remains unclear, the authors hypothesized that opioids were inhibitors of OCT1, leading to a higher level of metformin in the blood and/or tissues which contributes to the observation. Response to metformin can be affected by other OCT1 inhibitors. Cho *et al.* indicated that verapamil could reduce metformin's ability to lower blood glucose, but did not affect its pharmacokinetics (Cho et al., 2014). One of the reasons is that verapamil likely act as a potent competitive OCT1 inhibitor, preventing metformin uptake into the liver. Interaction between verapamil and metformin in patients with hypertension and T2DM may thus affect their efficacy and safety. In addition, OCT1 inhibitors were regarded as important players in metformin gastrointestinal side effects experienced by up to 20–30% of patients (Dujic et al., 2015). The DDI between metformin and an OCT1 inhibitor could become even complex in individuals with *SLC22A1* genetic polymorphisms (Dujic et al., 2016).

Common genetic variation of the *SLC22A1* gene could reduce the transport of substrates such as metformin in the liver (Shu et al., 2007; Ahlin et al., 2011). Compared with fully functional hOCT1- reference (NM_003057), the polymorphic hOCT1 proteins such as M420del and R61C were more susceptible to the inhibition by inhibitors. Specifically, the uptake of metformin via hOCT1- M420del was subjected to more inhibition by clinically relevant concentrations of verapamil, as compared to the hOCT1- reference. The enhanced sensitivity to drug inhibition toward OCT1 variants may lead to an increased risk of DDIs in individuals with these variants.

There are additional reports on DDIs between metformin and clinical used drugs. Frias *et al.* reported that in subjects who did not reach the maximal goal of HbA1c with a sub-maximal dose of metformin, the addition of sitagliptin improved the glycemic response and glycated hemoglobin goals, while the safety and tolerability were similar with metformin treatment alone (Frias et al., 2019). A possible mechanism is that the inhibition of OCT1 by sitagliptin could reduce the phosphorylation of AMPK, the first step in metformin's action (Choi et al., 2010). In addition, Cho *et al.* found that rifampin can up-regulate the expression of *SLC22A1* gene in peripheral blood cells, increase the concentration of metformin in the blood and enhance the hypoglycemic effect of metformin (Cho et al., 2011). Rifampin could also increase renal tubule secretion of metformin. In patients with T2DM and tuberculosis, the interaction between metformin and rifampicin may thus affect drug safety and efficacy. On the other hand, because the most toxic side effect of metformin, lactic acidosis, is a dose-dependent effect, reducing the dose of metformin may reduce the risk of lactic acidosis.

*In vitro* evidence has also suggested that OCT1 may be able to mediate an interaction of metformin with other clinical drugs or diet supplements. For example, green tea and its most abundant catechin epigallocatechin gallate (EGCG) could inhibit the

transport of metformin mediated by hOCT1 in cellular studies (Knop et al., 2015; Albassam and Markowitz, 2017). Interestingly, the inhibitory effect by green tea even exceeded that by EGCG. Bachmakov *et al.* also reported that the anti-diabetic repaglinide and rosiglitazone could significantly inhibit hOCT1-mediated metformin uptake in cells (Bachmakov et al., 2008).

Of note, DDIs *in vitro* may not necessarily translate into a clinical DDI. Recent *in vitro* studies have found that PPIs may interfere with the effectiveness of metformin (Nies et al., 2011a). However, Flory *et al.* has shown that the use of PPIs did not impair the effectiveness of metformin and that PPIs themselves had no significant clinical impact on glycemic control (Flory et al., 2015). Metformin was at least as effective in reducing glycosylated hemoglobin in patients with chronic PPIs treatment as in patients without PPIs treatment. Peficitinib, a pan-Janus kinase inhibitor, is used to treat rheumatoid arthritis (Takeuchi et al., 2016). Peficitinib has been shown to inhibit the uptake of metformin in hOCT1-overexpressing cells (Shibata et al., 2020). However, in clinical studies, the AUC, $C_{max}$ and $CL_R$ of metformin were only slightly reduced by peficitinib treatment in healthy male subjects. As metformin is a relatively safe and generally well tolerated by patients, the interaction between peficitinib and metformin may not be clinically important and metformin dose adjustment may be not required. However, further clinical studies in patients are always needed to confirm the assumption based on *in vitro* findings and those from healthy human subjects.

## 6.16 $H_2$ Receptor Antagonists

Cimetidine, ranitidine and famotidine belong to a class of drugs called $H_2$-receptor antagonists. These drugs have been reported as the substrates for OCTs but are used primarily as the inhibitors of OCTs in many studies (Barendt and Wright, 2002; Bourdet et al., 2005). Meyer *et al.* found that as a substrate or competitive inhibitor of OCT1, ranitidine could inhibit hOCT1-mediated uptake of morphine and metformin at clinically relevant concentrations (Meyer et al., 2017). In addition, the uptake of ranitidine was also affected by common genetic polymorphisms of *SLC22A1* gene. However, although co-medication of ranitidine significantly reduced the rate of renal clearance of trospium chloride, the oral absorption and distribution did not change in healthy subjects (Abebe et al., 2020). Because of potential effects by disease status and genetic polymorphisms on transporter function, the clinically relevant impact of ranitidine on the pharmacokinetics of trospium chloride and other drugs in patients remain to be further delineated.

## 6.17 Immunosuppressants

Cyclosporine A (CsA) is a large lipophilic cyclic polypeptide. It can prevent organ rejection after transplant and is used to treat rheumatoid arthritis and psoriasis. In a cellular study, CsA was identified as an inhibitor of hOCT1 (Panfen et al., 2019). In particular, the inhibitory potency of CsA against hOCT1-mediated metformin uptake was 50-fold higher with CsA pre-incubation as compared to co-incubation. Interestingly, the difference in inhibitory potency between pre-incubation and co-inhibition with CsA seemed to be substrate-dependent. The $IC_{50}$ shift ranged from >1.2- to 50.2-fold with different substrates. While it would be interesting to understand the mechanism underlying the shift of hOCT1 inhibition by CsA with different incubation conditions, the potent and persistent inhibitory effect on hOCT1 after exposure to CsA implies hOCT1-mediated DDIs with other drugs in patients.

## 6.18 Muscarinic Antagonists

Trospium chloride (TC) is a muscarinic antagonist that is used to treat overactive bladder and symptoms of urinary frequency, urgency and incontinence (Wenge et al., 2011). TC is not completely absorbed from the gut. While it is widely distributed after absorption, it does not significantly pass the BBB (Bexten et al., 2015). TC can be eliminated from the kidney, liver, and intestine. It has been characterized as a substrate of several transporters including OCT1, P-glycoprotein, and OATP1A2. In cell studies, TC was taken up by human bladder urothelial cells through a mechanism that is susceptible to the inhibition by verapamil, an inhibitor toward several transporters. Although OCT1 may contribute to the disposition of TC, currently there is no evidence in support of any serious OCT1-mediated DDIs for this drug.

## 6.19 Opioids

Morphine, an opioid receptor agonist, has been determined as a substrate of OCT1 (Balyan et al., 2017). Zhu *et al.* has shown that both OCT1 and OCT2 can mediate the cellular uptake of morphine (Zhu et al., 2018). Moreover, irinotecan could alter the distribution of morphine *in vivo* in mice by inhibiting mouse OCT1 activity. In addition, cellular hOCT1-mediated uptake of morphine was found to be inhibited by a variety of inhibitors, including irinotecan, verapamil, ondansetron, imipramine, codeine, amitriptyline, tropisetron, fluoxetine, and clomipramine, at the concentrations relevant to those at the portal vein in patients receiving these inhibitors (Tzvetkov et al., 2013). Although the plasma concentrations of these drugs are too low to inhibit the activity of OCT1, these drugs may still have a potential to cause DDIs with morphine because their oral administration may result in a higher concentration in the hepatic portal vein. However, morphine and codeine by themselves may have very moderate inhibitory effects on OCT1-mediated drug uptake, due to their low portal vein concentrations following oral administration. Considering that those patients requiring morphine for pain relief commonly receive concomitant medications, clinicians should be aware that the therapeutic and/or toxic effects of morphine may be altered by the co-administrated inhibitors and/or substrates of OCT1, such as irinotecan.

## 6.20 Vitamins

Thiamine, also known as vitamin B1, is found in foods such as cereals, whole grains, beans, meat, nuts and peas. It plays an important role in the breakdown of carbohydrates from foods into intermediate metabolites needed by the body. Thiamine has been identified as a substrate of OCT1 (Kato et al., 2015). However, multiple transporters may mediate the hepatocellular uptake of thiamine. The hOCT1-mediated uptake of thiamine may be only physiologically relevant at high concentrations,

whereas other transporters are responsible for thiamine uptake into the liver at typical blood concentrations (Jensen et al., 2020). In the intestine, while the absorption of thiamine has been reported to be mediated by thiamine transporters ThTr1 and/or ThTr2, there is also contribution by OCT transporters, most likely by OCT1 and/or OCT3 (Lemos et al., 2012). The findings of thiamine as an OCT1 inhibitor have implicated an interaction mediated by OCT1 between nutrients and drugs, especially in patients who have been chronically treated with certain drugs and under a special diet. For example, as discussed above, there is potential OCT1-mediated interaction between thiamine and metformin in T2DM patients (Chen et al., 2014).

## 7 CLOSING REMARKS

In recent years, more and more attention has been paid to OCTs in the fields of clinical pharmacology and pharmaceutical research. Among these OCTs, OCT1 is widely distributed in different tissues with an extremely high level in the liver. A broad spectrum of substrates and inhibitors has been characterized for this transporter. Increasing evidence has indicated that OCT1 might be an important mediator for DDIs of clinical significance. However, the confirmed DDIs mediated by OCT1 in human subjects remain limited. A major reason is that an effective and convenient tool to probe OCT1 activity in humans has yet to be discovered and validated. OCT1 is highly polymorphic, with multiple common variants leading to functional alteration. The effort to study the DDIs of OCT1 substrates and inhibitors in the patients with different OCT1 genotypes may yield important clinical evidence in the near future. Current effort in characterizing the interaction of OCT1 with an increasing number of compounds will bring us valid probe drugs to assess OCT1 function in patients and lead to appreciation of its clinical importance in drug disposition and response. Our understanding of OCT1-mediated DDIs will eventually have an impact on optimization of pharmacotherapy in order to improve drug efficacy and avoid unnecessary DDIs.

## AUTHOR CONTRIBUTIONS

SZ: Manuscript draft; YS and SZ: manuscript revision; All authors have read and approved the final version of the manuscript.

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# Influence of YES1 Kinase and Tyrosine Phosphorylation on the Activity of OCT1

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Organic cation transporter 1 (OCT1) is a transporter that regulates the hepatic uptake and subsequent elimination of diverse cationic compounds. Although OCT1 has been involved in drug-drug interactions and causes pharmacokinetic variability of many prescription drugs, details of the molecular mechanisms that regulate the activity of OCT1 remain incompletely understood. Based on an unbiased phospho-proteomics screen, we identified OCT1 as a tyrosine-phosphorylated transporter, and functional validation studies using genetic and pharmacological approaches revealed that OCT1 is highly sensitive to small molecules that target the protein kinase YES1, such as dasatinib. In addition, we found that dasatinib can inhibit hepatic OCT1 function in mice as evidenced from its ability to modulate levels of isobutyryl L-carnitine, a hepatic OCT1 biomarker identified from a targeted metabolomics analysis. These findings provide novel insight into the post-translational regulation of OCT1 and suggest that caution is warranted with polypharmacy regimes involving the combined use of OCT1 substrates and kinase inhibitors that target YES1.

**Keywords: organic cation transporter 1, YES1 kinase, tyrosine kinase inhibitors, drug-transporter interactions, post-translational modification**

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## INTRODUCTION

In the last two decades, considerable advances have been made toward understanding the pharmacological role of cationic transporters belonging to the SLC22A subfamily. The advent of heterologous overexpression systems and genetically-engineered murine models has substantiated that the members of this subfamily facilitate the cellular uptake of a large number of structurally diverse endogenous metabolites and an increasingly large number of cationic xenobiotics. Organic cation transporter 1 (OCT1, *SLC22A1*) is the most abundant cationic transporter expressed on the sinusoidal membrane of hepatocytes (Chen et al., 2014), and is a rate-limiting step in the sodium-independent uptake and elimination of many xenobiotic substrates (Koepsell et al., 2007; Shu et al., 2007; Huang et al., 2020).

The *in vivo* contribution of OCT1 to the hepatic elimination of xenobiotics was first conclusively demonstrated for the prototypical organic cation, tetraethylammonium (TEA), in mice harboring a genetic deletion of OCT1 (Jonker et al., 2001; Jonker et al., 2003). Many subsequent studies have focused on the biguanide analog metformin, a first-line medication for the treatment of type 2 diabetes. These studies have led to the recognition that the glucose-lowering effects of metformin are

partially dependent on OCT1 (Chae et al., 2016; Heckman-Stoddard et al., 2016), and that OCT1 deficiency is associated with diminished metformin uptake in hepatocytes (Shu et al., 2007; Higgins et al., 2012). More recently, OCT1 has also been identified as a critical determinant of the therapeutic efficacy of fenoterol (Tzvetkov et al., 2018), morphine (Fukuda et al., 2013), sumatriptan (Matthaei et al., 2016), thiamine (Chen et al., 2014), tramadol (Tzvetkov et al., 2011), and tropisetron (Tzvetkov et al., 2012).

Due to its predominant role in determining the efficacy of many clinically-important drugs, multiple regulatory aspects of OCT1 have been widely studied. For example, polymorphic variants in OCT1 (Gomez and Ingelman-Sundberg, 2009) have been linked to the pharmacokinetics and glycemic response in diabetic patients receiving metformin (Giacomini et al., 2012), and epigenetic mechanisms have been identified that can functionally modulate OCT1 and can profoundly affect therapeutic outcomes of substrate drugs (Schaeffeler et al., 2011). Although post-translational modification *via* phosphorylation has been reported to influence the function of transporters (Mehrens et al., 2000; Czuba et al., 2018), surprisingly, this has not been extensively studied as a regulatory mechanism of OCT1. We previously reported that the related transporter OCT2 (*SLC22A2*) is sensitive to inhibition by several FDA-approved tyrosine kinase inhibitors (TKIs) through a mechanism that involves YES1-mediated tyrosine phosphorylation (Sprowl et al., 2016). Since OCT1 and OCT2 share structural features, a high degree of sequence homology, and have overlapping substrate recognition sites and conserved tyrosine motifs (Tanaka and Herr, 1990; Gorboulev et al., 1997), we hypothesized that the activity of OCT1 is also dependent on kinase-mediated tyrosine phosphorylation. In the current study, we tested this hypothesis by employing phospho-proteomics screens, genetic strategies, pharmacological approaches, and metabolomics analyses in heterologous models overexpressing mouse or human OCT1, as well as OCT1-deficient mice.

# MATERIALS AND METHODS

## Cell Culture and Reagents

Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HEK293 cells stably transfected with mouse OCT1 (mOCT1) or human OCT1 (hOCT1) were cultured in Dulbecco's Modified Eagle Media (DMEM) media supplemented with 10% fetal bovine serum (FBS) and grown at 37°C in a humidified incubator containing 5% $CO_2$. Radiolabeled [$^{14}$C] TEA and [$^{14}$C] metformin were obtained from American Radiochemicals (St. Louis, MO). Cellular uptake assays were performed 48 h following transient transfection by Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA). ON-TARGETplus Human YES1 siRNA was obtained from Dharmacon (Lafayette, CO). RNA extraction kits were obtained from Omega Bio-tek (Norcross, GA). Reference standards of decynium22, a positive control inhibitor, as well as the TKIs bosutinib, dasatinib, gilteritinib, ibrutinib, lapatinib, sunitinib, vandetanib, and CH6953755 were obtained from MedChemExpress (Monmouth Junction, NJ).

## Cellular Accumulation Studies

Uptake assays were performed with radiolabeled TEA (2 μM) or metformin (5 μM) as described previously (Sprowl et al., 2013; Pabla et al., 2015) in the presence or absence of TKIs. The results were normalized to uptake values in cells stably transfected with an empty vector treated with vehicle alone (**Supplementary Figure S1**). Prior to cellular accumulation experiments, cells were grown to 90% confluence on poly-lysine coated multi-well plates. For uptake studies, cells were rinsed with warm PBS and incubated in the presence of a vehicle or inhibitor, prepared in serum and phenol red-free DMEM media for 15 min. Subsequently, media was removed followed by the addition of radiolabeled TEA and metformin along with inhibitor, and cellular uptake was measured after a 15-min co-incubation period. Total radioactivity originating from TEA and metformin was determined using liquid scintillation counting after lysing the cells with 1 N NaOH, a neutralizing step with 2 M HCl. A Pierce protein assay (Thermo Fisher Scientific, Waltham, MA) was used to normalize radioactivity readings to account for variation in cell number between samples.

## Site-Directed Mutagenesis

The YES1 plasmid with pCMV6-Entry (C-terminal FLAG-tagged) backbone was obtained from Origene (Rockville, MD). Mutants in OCT1 and YES1 were generated using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Mutagenesis primers were designed using QuikChange Primer Design software and generated according to the manufacturer's instructions. Successful mutagenesis was confirmed by Sanger sequencing and constructs used for transient transfection experiments.

## siRNA-Mediated Knockdown

HEK293 cells overexpressing hOCT1 were plated at a density of $1.25 \times 10^5$ per well in a 12-wells plate and incubated overnight at 37°C with 5% $CO_2$. The next day, cells were transfected with 50 nM siRNA targeting YES1, positive control siRNA, and negative control siRNA (Dharmacon, Lafayette, CO) according to manufacturer protocols. After 48 h of exposure to siRNA, OCT1 function was evaluated with TEA or metformin as described above.

## RT-qPCR

Total RNA was extracted from cells treated with siRNA by E.Z.N.A. Total RNA Kit I (Omega Bio-tek), and reverse transcribed to cDNA by qScript XLT cDNA SuperMix (QuantaBio, Beverly, MA). Primer sequences included YES1 (Hs00736972_m1) and human GAPDH (Hs02758991_g1), and quantitative RT-PCR was performed using TaqManTM Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA). The Ct values of the YES1 gene were subtracted from the mean of GAPDH (ΔCt). All samples were analyzed in triplicate, and the mean value of ΔCt was calculated.

## Protein Analysis

Cell treated with non-targeting siRNA and YES1 siRNA were lyzed using sonication. Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to determine protein concentrations. Next, an equal amount of protein was separated on a Bis-Tris 4–12% SDS-polyacrylamide gel with MOPS buffer according to the instructions from manufacturer (Life Technologies, Grand Island, NY) and transferred to PVDF membranes. Western blot analysis was performed using antibodies against YES1 (Product # 3201S), vinculin (Product # 13901S), and HRP-conjugated secondary anti-rabbit (Product # 7074) obtained from Cell Signaling Technology (Danvers, MA). Proteins were visualized by chemiluminescence using the SignalFire ECL Reagent (Cell Signaling Technology, Danvers, MA) or SuperSignal West Femto Maximum Sensitivity Substrate (Invitrogen, Carlsbad, CA) using film.

## Proteomics and Metabolomics Studies

In order to evaluate the tyrosine-phosphorylation landscape of ADME proteins in FVB mice, the genetic background strain used in our transporter-deficient *in vivo* models, tissue samples were subjected to a PhosphoScan analysis (Cell Signaling, Danvers, MA). This analysis provides purification and characterization of tyrosine phosphorylation sites in cellular proteins when paired with liquid chromatography tandem mass-spectrometry (LC-MS/MS) technology. The assay comprises enhanced phospho-tyrosine-containing peptides using P-Tyr-100, a mouse anti-phospho-tyrosine antibody paired with protein G agarose beads. Following protease-mediated digestion, immune-affinity purify-cation of peptides, and MS analysis on phospho-peptides, spectra were assessed using Sequest 3G and the Sorcerer 2 platform (Sage-N Research, Milpitas, CA).

For metabolomics studies, plasma and tissue samples were collected from wild-type mice and OCT1/OCT2 (OCT1/2)-deficient mice (Taconic, Petersburgh, NY). Tissue samples were washed with ice-cold 0.9% saline, and snap-frozen using liquid nitrogen. Further preparation of plasma and tissue samples for metabolomics analysis was done using LC-MS/MS, as previously described (Huang et al., 2018).

## Animal Experiments

For all *in vivo* studies, plasma and tissue samples were collected from both males and females wild-type mice, OCT1/2-deficient mice, and mice additionally deficient for MATE1 (OCT1/2/MATE1), following an established protocol (Leblanc et al., 2018). Mice were maintained under pathogen-free conditions at the Ohio State University Laboratory Animal Resources, and all *in vivo* experiments were approved by University Animal Care and Use Committee (protocol number: 2015A00000101-R1). Mice were accommodated in a temperature-, and light-controlled environment with access to water and food. OCT1/2/MATE1-deficient mice was obtained by crossing male OCT1/2-knockout mice with female MATE1-knockout mice to generate heterozygous breeders. The MATE1-deficient mice used to generate this model were kindly provided by Dr. Yan Shu (University of Maryland, Baltimore, MD), and backcrossed onto an FVB background. Next, heterozygous males and females were used to obtain OCT1/2/MATE1-knockout mice. Genetic deletion of OCT1/2 and MATE1 was confirmed by performing RT-PCR analysis.

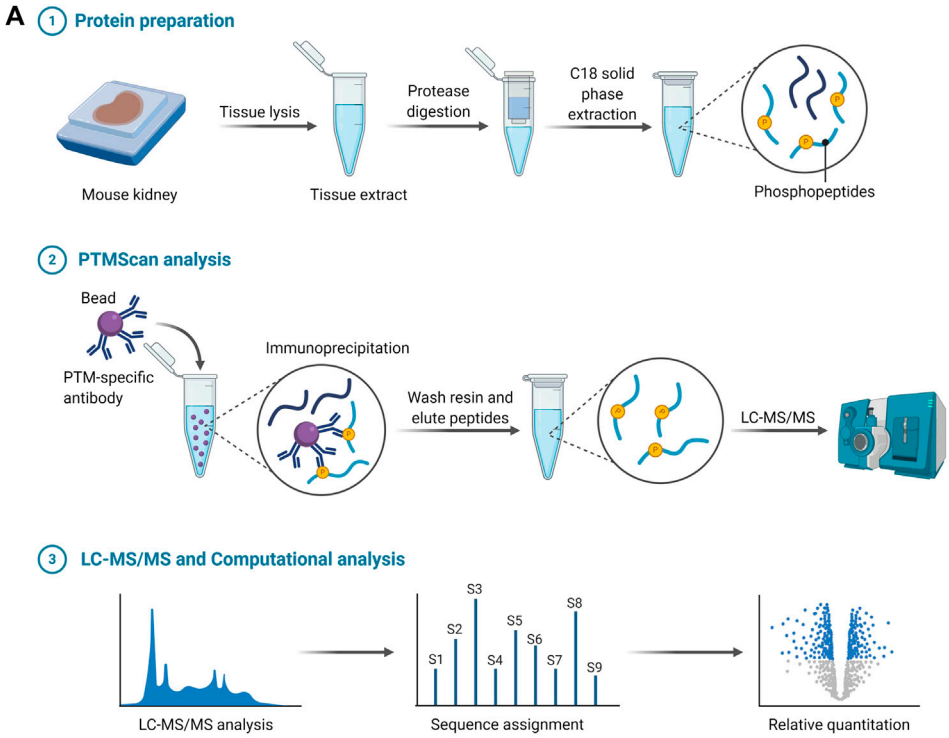
Dasatinib was dissolved in 80 mM citric acid (pH 3.1) and administered *via* oral gavage at a dose of 15 mg/kg. For studies involving TEA, dasatinib was given orally 30 min before the intravenous administration of [$^{14}$C] TEA (0.2 mg/kg) *via* the caudal vein. Concentrations of total TEA-derived radioactivity in plasma and homogenized liver samples were measured by liquid scintillation counting.

## Quantification of Isobutyryl L-Carnitine (IBC)

A Vanquish UHPLC paired with a Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific) was used to perform LC-MS/MS analysis of IBC and the internal standard, isobutyryl L-carnitine-d3 (Cayman Chemical, Ann Arbor, MI). Chromatographic separation of analytes was achieved on an Accucore aQ column (150 mm × 2.1 mm, dp = 2.6 μm) with a C18 AQUASIL guard cartridge (2.1 mm × 10 mm, dp = 3 μm). The temperature of the column and autosampler was retained at 40 and 4°C, respectively. The mobile phase contains solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile-methanol, 50:50 v:v). The gradient elution was 5.0 min at a flow rate of 0.4 ml/min, and conditions were as follows: 0–0.5 min, 0% B; 0.5–2.3 min, 30% B; 2.3–3.8 min, 30–95% B; 3.8–4.2 min, 95% B; 4.2–5.0 min, 0% B. The extracted samples (5 μl) were injected for analysis, and following parameters were established for the mass spectrometer: 40 Arb, 12 Arb, 3.3 Arb, 350, and 375°C for sheath gas, aux gas, sweep gas, ion transfer tube, and vaporizer temperature, respectively. The ion source was managed by heated ESI in positive ion mode with ion spray voltage at 3,500 V. Argon was used as a collision gas at a pressure of 1.5 mTorr. Precursor molecular ions and product ions were recorded for confirmation and detection of IBC (232.144 > 85.083) and the internal standard (236.056 > 85.056). Assay validation studies demonstrated that the within-day precision and between-day precision ranged from 0 to 6.16%, and the accuracy ranged from 92.8 to 105%. The lower limit of quantification for IBC was 0.1 ng/ml.

## Statistical Analysis

All data are presented as mean ± SEM, either as the experimental readings or after normalization to baseline values, and then expressed as a percentage. All experiments were conducted in triplicate unless specified, and were performed on at least two independent occasions. Comparisons between two groups were analyzed by unpaired two-sided Student's *t*-test with Welch's correction while one-way ANOVA with Dunnett's post-hoc test was performed for comparing more than two groups. Statistical analyses were conducted using GraphPad Prism version 8.1.2 (GraphPad Software, San Diego, CA), and $p < 0.05$ was considered as the cutoff for statistical significance.




**B**

| Transporter | Name |
|---|---|
| SLC22A1 | Organic Cation Transporter 1 |
| SLC25A3 | Phosphate carrier protein |
| SLC25A4 | ADP/ATP translocase 1 |
| SLC25A5 | ADP/ATP translocase 2 |
| SLC25A31 | ADP/ATP translocase 4 |
| SLC27A2 | Very long-chain acyl-CoA synthetase |

**C**

| Enzyme | Name |
|---|---|
| COMT | Catechol O-methyltransferase |
| CYB5R1 | NADH-cytochrome B5 reductase 1 |
| CYP4B1 | Cytochrome P450 4B1 |
| GPX1 | Glutathione peroxidase 1 |
| INMT | Indolethylamine N-methyltransferase |
| UGT3A2 | UDP-glucuronosyltransferase 3A2 precursor |

**FIGURE 1 |** Phosphotyrosine proteomics screen. **(A)** Schematic diagram depicting the PhosphoScan analysis from wild-type mouse kidney samples. Identified SLC transporters **(B)** and enzymes **(C)** that are tyrosine-phosphorylated from the phosphotyrosine proteomics screen.

# RESULTS

## Conserved Tyrosine Phosphorylation of OCT1

In order to initially demonstrate that OCT1 is tyrosine phosphorylated, in a manner similar to that reported previously for OCT2 (Sprowl et al., 2016), an unbiased MS-based proteomics analysis was performed to identify all tyrosine-phosphorylated proteins, membrane-localized or intracellular, from murine tissues (**Figure 1A**; **Supplementary Table S1**). A total of 802 redundant phosphorylated peptide assignments to 438 non-redundant phosphorylated peptides for the phospho-tyrosine motif antibody were identified, applying a 5% false-positive rate to filter the results. The hits included multiple transporters (**Figure 1B**), including OCT1, but also several ion channels and enzymes (**Figure 1C**). These preliminary findings thus verified our hypothesis, suggest that tyrosine-phosphorylation may be a much more widespread regulatory mechanism of ADME proteins than held previously, and that these proteins are potentially sensitive to off-targeted de-regulation by clinically-used TKIs.

We previously reported that several TKIs can modulate OCT2 function through inhibition of the protein kinase YES1, and that tyrosine-to-phenylalanine (Y-F) OCT2 mutants at three sites (241, 362, and 377) considerably diminished OCT2 function without affecting OCT2 expression in plasma membrane (Sprowl et al., 2016). In addition, OCT2 has a proline-rich (PXXPR) sequence, which is known to attach the Src Homology 3 (SH3) domain present in YES1 kinase, and mutations in this proline-rich SH3 binding domain decreased OCT2 function and tyrosine-phosphorylation. Interestingly, all these OCT2 domains, including the functionally most relevant 362 residue, are uniquely conserved in phylogenetically-linked transporters, such as

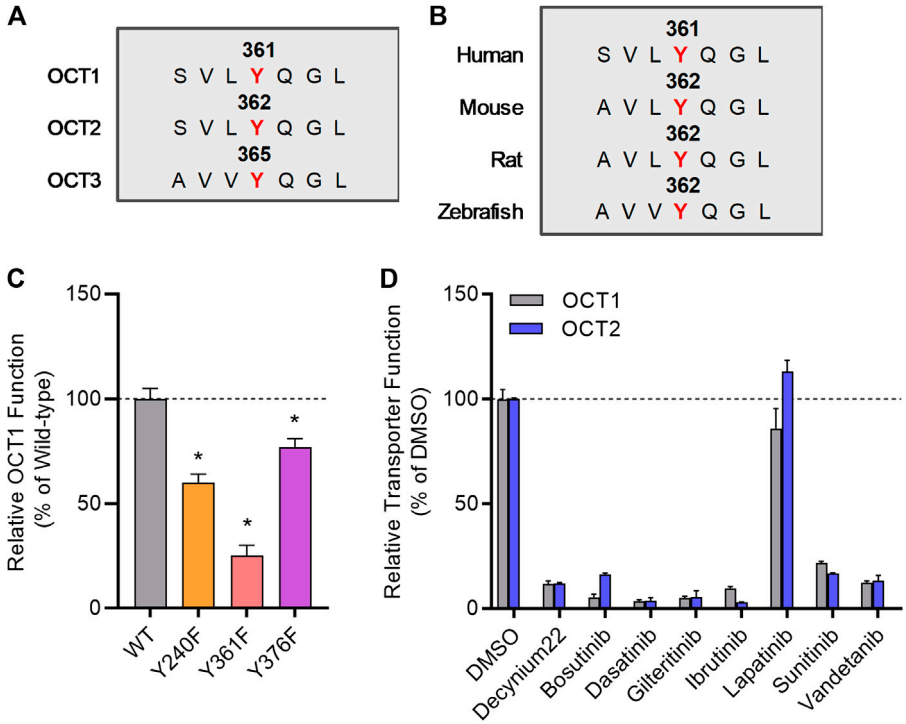



**FIGURE 2 |** Inhibition of organic cation transporters by TKIs. **(A)** The protein sequence of hOCT1, hOCT2, and hOCT3 was aligned by a multiple sequence alignment program (MAFFT). **(B)** OCT1 protein sequence from indicated organisms was aligned by a multiple sequence alignment program (MAFFT). **(C)** HEK293 cells were transiently transfected with wild-type (WT), Y240F, Y361F, and Y376F mutant plasmids, uptake assays were performed using [$^{14}$C] TEA (2 μM) for 15 min. Cellular accumulation of [$^{14}$C] TEA was determined by liquid scintillation counter, and the graph represents relative uptake values compared to wild-type after normalization of protein levels. **(D)** Relative transporter function in HEK293 cells stably transfected with hOCT1 was evaluated by a substrate drug TEA in the presence of FDA-approved TKIs (10 μM) previously found to inhibit OCT2. Lapatinib was included as a negative-control TKI, and decynium22 as a non-TKI positive control inhibitor. The graph represents relative transport activity of indicated substrate drug compared to DMSO. *$p$ < 0.05 vs. wild-type control. All values represent mean ± SEM.

OCT1, and across model organisms (**Figures 2A,B**). In addition, a naturally-occurring single nucleotide variant in the OCT1 gene, causing a P283L change, is known to reduce OCT1 function and alter metformin transport in humans (Mato et al., 2018), and this site is located in the proline-rich SH3 binding sequence of OCT1.

To investigate directly if the regulation of OCT2 by phosphorylation is conserved in OCT1, we performed functional assays after mutagenesis of relevant sites, and found that OCT1 mutants lacking the putative phosphorylation sites in OCT1 at residues 240, 361, and 376, corresponding to the 241, 362, and 377 sites in OCT2, had significantly reduced transport function (**Figure 2C**). Moreover, we found that distinct OCT2-inhibiting TKIs, including bosutinib, dasatinib, gilteritinib, ibrutinib, sunitinib, and vandetanib, but not the negative-control TKI lapatinib, also inhibit OCT1 function (**Figure 2D**). These results support the possible existence of a common inhibitory mechanism by which TKIs can modulate the function of OCT1 and OCT2, a conclusion, that is consistent with the notion that the OCT1- and OCT2-inhibitory properties of the studied TKIs are strongly correlated. Interestingly, compared to OCT1 and OCT2, a highly distinct TKI-mediated inhibitory profile was observed for the related transporter OCT3 (**Supplementary Figure S2**), with some TKIs (e.g., dasatinib, sunitinib) potently inhibiting all three transporters and some (e.g., bosutinib, gilteritinib, ibrutinib) having no influence on OCT3 function.

## TKI-Based Inhibition of OCT1 *In Vitro*

Dose-response experiments with select TKIs (**Table 1**) indicated that dasatinib, gilteritinib, ibrutinib, and vandetanib potently inhibited OCT1 function in a species-independent manner (**Supplementary Figure S3**), and regardless of the test substrate at concentrations that are clinically achievable at the recommended daily doses. Among the tested TKIs, dasatinib was found to be the most potent inhibitor against both mOCT1 ($IC_{50}$, 1.09 μM) and hOCT1 ($IC_{50}$, 0.56 μM) (**Figure 3A**), and was selected for further mechanistic studies. In line with previous observations for OCT2-inhibitory TKIs (Minematsu and Giacomini, 2011; Sprowl et al., 2016), inhibition of mOCT1 and hOCT1 by TKIs was independent of the substrate concentration, and a Dixon plot of the reciprocal velocity against the TKI concentration to derive inhibition constants indicated that the mechanism of inhibition is non-competitive (**Figures 3B,C**). This conclusion is consistent with our previous observation that TKs such as dasatinib are not themselves transported substrate of OCT1 (Furmanski et al., 2013).

**TABLE 1 |** Features of TKIs used in the experiments.

| TKI | Indication(s) | Primary target(s) | YES1 $K_d$ (nM) | OCT1 $IC_{50}$ (μM) | OCT1 inhibition | OCT2 inhibition |
|---|---|---|---|---|---|---|
| Dasatinib | CML, GIST | BCR/ABL, SRC | 0.3 | 0.56–1.09 | Yes | Yes |
| Gilteritinib | AML | FLT3, AXL | 445 | 0.01–0.02 | Yes | Yes |
| Ibrutinib | CLL, MCL | BTK | 27 | 0.89–1.18 | Yes | Yes |
| Lapatinib | Breast cancer | HER2, EGFR | >10,000 | — | No | No |
| Vandetanib | Thyroid cancer | EGFR, VEGFR | 120 | 1.35–9.05 | Yes | Yes |

*CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; MCL, mantle cell lymphoma; AML, acute myeloid leukemia.*

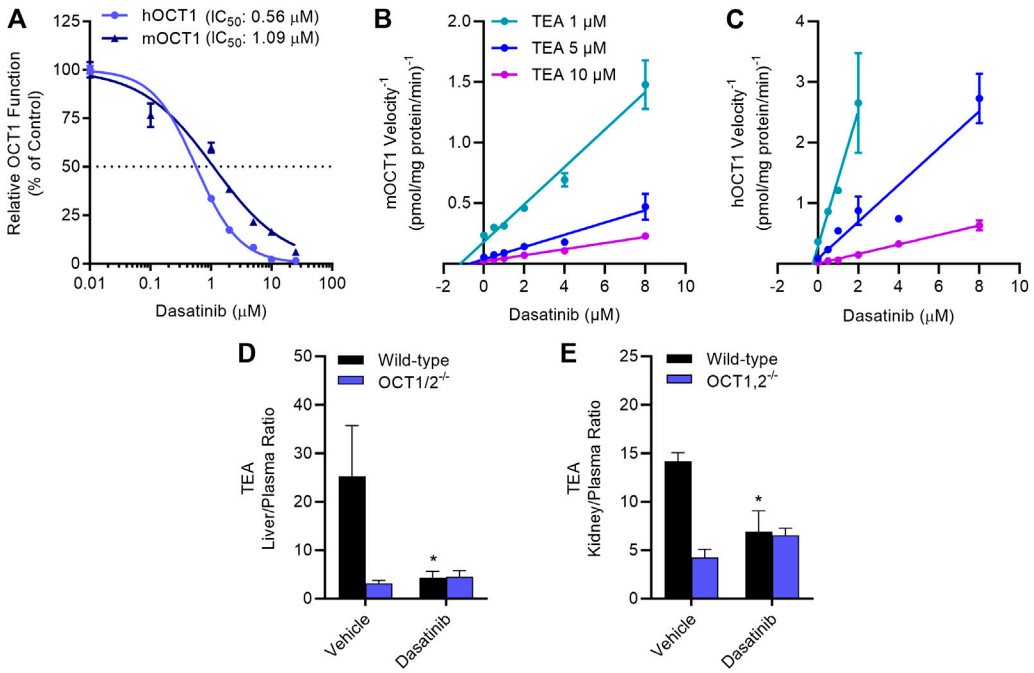



**FIGURE 3 |** TKI-mediated inhibition of OCT1 function. **(A)** Uptake of [$^{14}$C] TEA (2 μM) was measured in HEK293 cells overexpressing hOCT1 and mOCT1 after pre-incubation with dasatinib at various concentrations (0.1–25 μM) for 15 min, followed by the co-incubation with TEA for 15 min. Data represent the mean ± SEM and are expressed as a percentage over control. **(B,C)** Dixon plot showing varying concentrations of [$^{14}$C] TEA (1, 5, and 10 μM) uptake assay in the presence of dasatinib (0.1–8 μM) in HEK293 cells overexpressing hOCT1 and mOCT1, data expressed as 1/velocity. In a Dixon plot, the point of intersection of the lines represent the negative inhibition constant (-Ki); this analysis revealed dasatinib-mediated inhibition constants of 0.18 μM for hOCT1 and 0.87 μM for mOCT1 ($n = 3$ per group). **(D,E)** Wild-type and OCT1/2-deficient male mice ($n = 5$) were treated with either vehicle or dasatinib (15 mg/kg) 30 min before an intravenous administration of [$^{14}$C] TEA (0.2 mg/kg). Liver **(D)** and kidney **(E)** samples were collected at 5 min after TEA treatment, and graphed as tissue-to-plasma ratios. *$p < 0.05$ vs. vehicle control. All values represent mean ± SEM.

## TKI-Mediated Modulation of OCT1 *In Vivo*

The notion that the OCT1-inhibitory properties of dasatinib are species-independent is consistent with and recapitulates several prior observations (Shu et al., 2007; Sprowl et al., 2016; Floerl et al., 2020; Meyer et al., 2020), and suggests that mice can serve as a suitably predictive model for humans. To directly assess the influence of dasatinib on the function of OCT1 *in vivo*, the pharmacokinetic profile of TEA was examined in wild-type mice and OCT1/2-deficient mice receiving a single oral dose of dasatinib, given 30 min before the administration of TEA. We found that the hepatic uptake of TEA, as determined from the liver-to-plasma concentration ratio, was dramatically reduced in the OCT1/2-deficient mice, and that the genotype could be phenocopied by a single dose of dasatinib (**Figure 3D**). Similar observations were made in the murine kidney (**Figure 3E**), an organ that expresses both OCT1 and OCT2 (Holle et al., 2011).

In order to provide further evidence that the ability of dasatinib to modulate TEA disposition is causally related to modulation of hepatic OCT1, we next performed an LC-MS/MS-based targeted metabolomics study in samples from wild-type mice and OCT1/2-deficient mice that was designed to identify a liver-specific endogenous biomarker of OCT1. This study revealed that among 121 metabolites examined, the hepatic concentration of several compounds, including isobutyryl-l-carnitine (IBC), was substantially

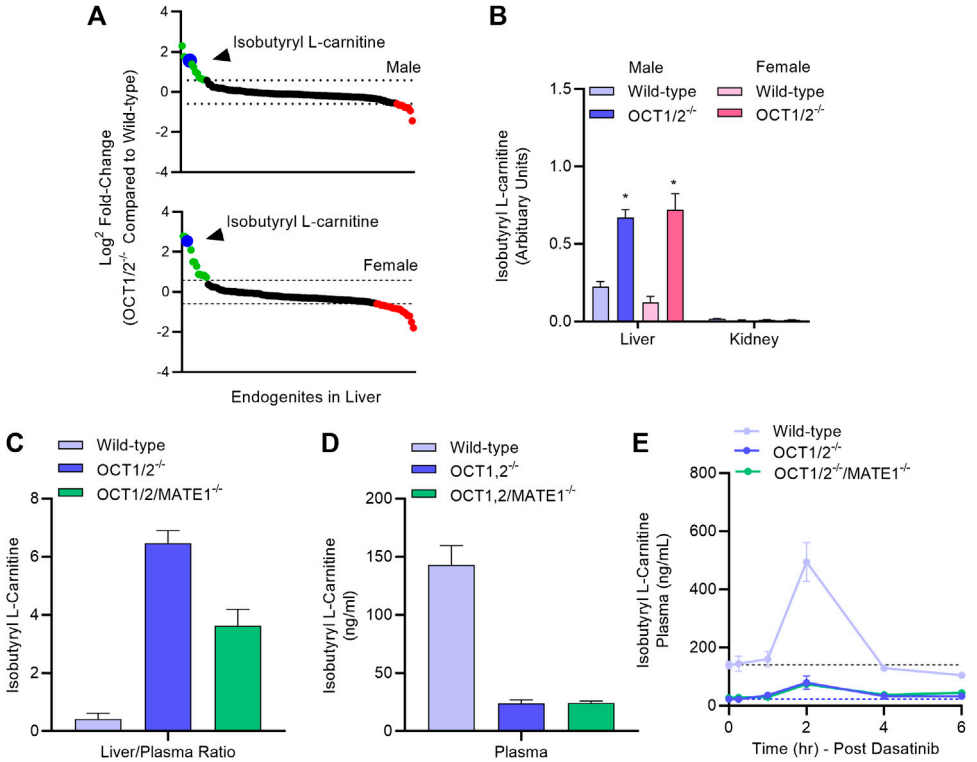



**FIGURE 4 |** Targeted metabolomics and endogenous OCT1 biomarker identification. **(A)** Differentially quantitated endogenous metabolites ("endogenites") in the liver of male and female wild-type mice and OCT1/2-deficient mice. Endogenites highlighted in green and red were significantly increased and decreased, respectively in livers of OCT1/2-deficient mice. The blue symbol represents isobutyryl L-carnitine (IBC). **(B)** Liver and kidney concentrations of IBC in wild-type and OCT1/2-deficient mice ($n = 5$). **(C,D)** Liver-to-plasma ratio and plasma level of IBC at baseline in wild-type mice, OCT1/2-deficient mice, and OCT1/2/MATE1-deficient mice ($n = 5$). **(E)** Plasma concentration-time profile of IBC in wild-type mice, OCT1/2-deficient mice, and OCT1/2/MATE1-deficient mice ($n = 5$) after a single oral dose of dasatinib (15 mg/kg). *$p < 0.05$ vs. wild-type. All values represent mean ± SEM.

elevated in OCT1/2-deficient mice compared to wild-type mice (**Figure 4A**; **Supplementary Table S2**), in both male and female animals. We also observed that reduced hepatic levels of IBC in wild-type mice were accompanied by significantly elevated levels in plasma (**Figure 4B**), that IBC levels in the kidney were negligible (**Figure 4B**) regardless of mouse genotype, and that additional deficiency of MATE1 (**Figure 4C**), which forms a functional unit with OCT1 in the liver and with OCT2 in the kidney, did not influence the results. These findings suggest that IBC, a natural four-carbon acylcarnitine involved in fatty acid oxidation and organic acid metabolism, serves as a *bona fide* biomarker for hepatic OCT1 function, a conclusion, that is in line with a recent clinical report (Luo et al., 2020). We next evaluated the impact of dasatinib on concentrations of IBC and found that administration of the TKI resulted in a transient, statistically significant increase in the plasma levels of IBC in wild-type mice, but not in OCT1/2-deficient mice or OCT1/2/MATE1-deficient mice (**Figure 4D**). Taken together, these data indicate that dasatinib, given at a dose that affects the liver uptake of TEA, causes significant inhibition of hepatic OCT1 function.

## Kinase-Mediated Regulation of OCT1 Function

The existence of tyrosine motifs that are conserved between OCT1 and OCT2, and the similarity in sensitivity to inhibition by TKIs between these two transporters raises the possibility that the tyrosine phosphorylation and activity of OCT1 are regulated by YES1, as described for OCT2 (Sprowl et al., 2016). In support of this hypothesis, we found that pre-treatment of OCT1-expressing cells with the selective YES1 inhibitor, CH6953755, causes substantial inhibition of hOCT1-mediated transport of TEA ($IC_{50}$, 2.76 μM) and metformin ($IC_{50}$, 2.31 μM) (**Figure 5A**; **Supplementary Figures 4A,B**). This degree of inhibition by CH6953755 was also observed in models overexpressing mOCT1 (**Supplementary Figure S4C**) or hOCT2 (**Supplementary Figure S4D**). The connection of TKI-mediated OCT1 inhibition with the function of YES1 was further substantiated by the observed relationship between potency of target engagement by the studied TKIs, as determined by the affinity constant ($K_d$) (Klaeger et al., 2017; KINOMEscan data—HMS LINCS Project, 2020), and their ability to modulate OCT1-mediated transport (**Supplementary Figure S4E**).

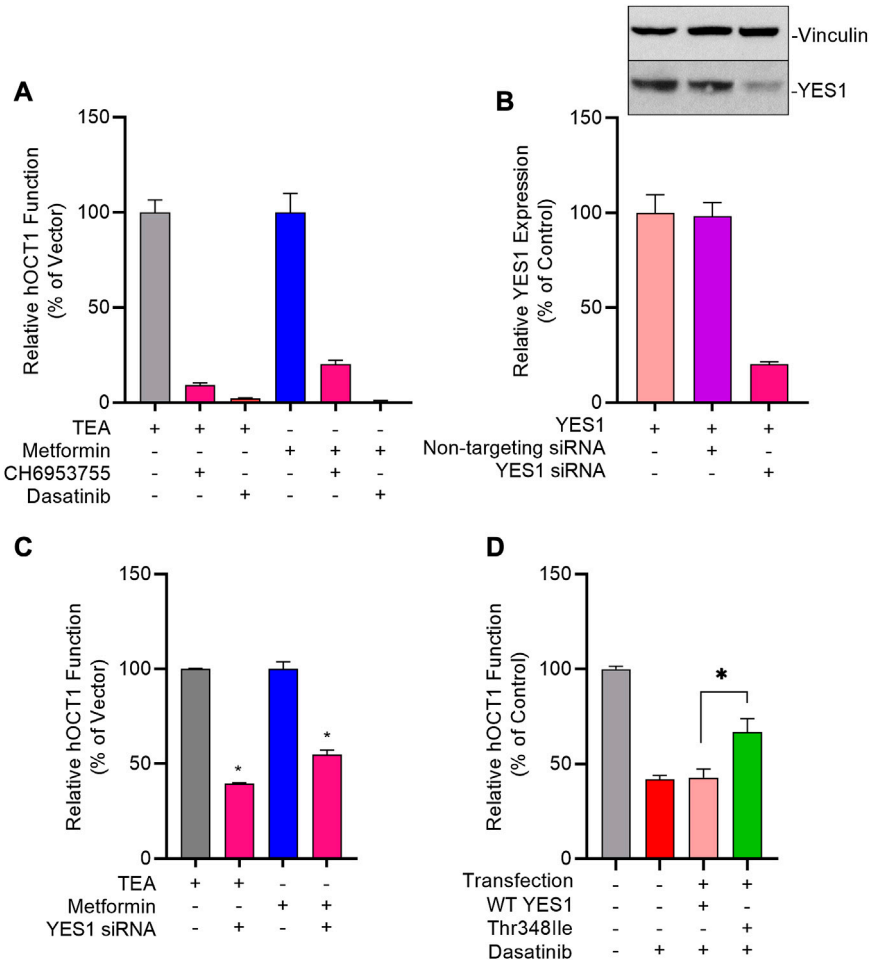



**FIGURE 5 |** Genetic and pharmacological inhibition of YES1 kinase impairs OCT1 activity. **(A)** HEK293 cells stably transfected with vector control (VC) and hOCT1 were pre-incubated with CH6953755 or dasatinib (10 μM) for 15 min followed by the co-incubation with [$^{14}$C] TEA (2 μM) or [$^{14}$C] metformin (5 μM). Data represents relative uptake values compared to VC control after normalization of protein levels. **(B)** Expression of YES1 protein (top) and gene (bottom) in hOCT1-expressing HEK293 cells 48 h after transfection with non-targeting siRNA or YES1 siRNA. **(C)** Influence of YES1 silencing by siRNA on hOCT1 function was measured in HEK293 cells using uptake assays with [$^{14}$C] TEA or [$^{14}$C] metformin. **(D)** Influence of YES1 mutants on dasatinib-mediated inhibition of hOCT1 function in HEK293 cells following transient transfection of constructs carrying either wild-type YES1 or the YES1 Thr348Ile gatekeeper mutant. After 48 h, cells were pre-treated with dasatinib (1 μM) for 15 min, followed by uptake assay using [$^{14}$C] TEA. *$p < 0.05$ vs. control. All values represent mean ± SEM.

To demonstrate causality of this relationship, we found that even partial downregulation of YES1 expression by siRNA in HEK293 cells (**Figure 5B**) was already associated with a statistically significant loss of OCT1 transport function (**Figure 5C**). To unambiguously identify YES1 as the TKI-sensitive protein kinase that phosphorylates OCT1, we next carried out a screen utilizing a chemical genetics approach in which HEK293 cells expressing hOCT1 are transfected with either the wild-type or TKI-resistant (T348I gatekeeper) mutant of YES (Du et al., 2009; Li et al., 2010), followed by dasatinib treatment and OCT1 uptake assays (**Supplementary Figure S5**). These studies revealed that the TKI-resistant YES1 mutant was able to rescue OCT1 inhibition by dasatinib, whereas cells carrying the YES1 wild-type construct retained sensitivity to dasatinib-mediated OCT1 inhibition (**Figure 5D**).

## DISCUSSION

In the present study, we identified OCT1 as a tyrosine-phosphorylated transporter from a phospho-proteomics screen, and demonstrated through functional validation studies using genetic and pharmacological approaches that OCT1 is highly sensitive to small molecules in the class of TKIs that target the protein kinase YES1, such as dasatinib. In addition, we found that dasatinib can inhibit hepatic OCT1 function in mice as evidenced from its ability to modulate levels of the prototypical substrates TEA and metformin as well as the OCT1 endogenous biomarker, isobutyryl L-carnitine. These findings provide novel insight into the posttranslational regulation of OCT1 and suggest that caution is warranted with polypharmacy regimes involving the use of OCT1 substrates in combination with TKIs that target YES1 (Minematsu and Giacomini, 2011; Lautem et al., 2013;

Jensen et al., 2020). This is particularly relevant in view of the fact that more than one-third of approved prescription drugs are positively charged at neutral pH, and that the membrane transport of many of these agents relies on facilitated carriers such as OCT1.

Previous studies have indicated that OCT1 expression is regulated at different levels, including transcriptionally, by intracellular trafficking, and through alteration of functional properties. Among these mechanisms, transcriptional regulation by hepatic nuclear factors (HNF1 and HNF4α) has been well documented. This work has suggested that HNF1 ties to an evolutionary conserved region within intron 1 (O'Brien et al., 2013), whereas HNF4α is involved in bile acid-dependent regulation of OCT1 in the liver *via* activation by the bile acid-inducible transcriptional repressor (Saborowski et al., 2006). In addition, OCT1 expression can be regulated by hepatic growth factor (Le Vee et al., 2009), and activity of the OCT1 promoter is affected by methylation (Shu et al., 2007; Schaeffeler et al., 2011; Mato et al., 2018).

In contrast to this knowledge on transcriptional mechanisms, details of short-term posttranslational regulation of OCT1 activity have remained incompletely understood. It was previously reported that substrate transport of OCT1 is reduced by activation of protein kinase A and by inhibition of calmodulin, CaM-dependent kinase II, or p56lck tyrosine kinase (Ciarimboli et al., 2005). Our current findings add to this prior knowledge and demonstrate that many ADME proteins, including multi-specific drug-transporters such as OCT1, are directly regulated through tyrosine-phosphorylation by a mechanism that involves the kinase YES1 in a manner, that is analogous to that previously reported for OCT2 (Sprowl et al., 2016). Our study also indicates that disruption of this phosphorylation process by YES1 by several clinically-used TKIs can result in dramatically impaired OCT1 function. Furthermore, our study suggests that phospho-proteomic analysis should be considered during the drug development process to predict potential drug-drug interactions and to avoid unwanted consequences when potent inhibitors of YES1 kinase are administered together with agents that undergo OCT1-dependent hepatic transport (Tzvetkov et al., 2013; Matthaei et al., 2016).

During the course of our investigation, we identified several FDA-approved TKIs as previously unrecognized, potent inhibitors of OCT1, including dasatinib, ibrutinib, sunitinib, and vandetanib. In addition, we confirmed the OCT1-inhibitory potential of several other TKIs, such as bosutinib and gilteritinib, which are listed as OCT1 inhibitors in their prescribing information. It should be pointed out that the mechanism by which these agents impede OCT1 transport function is not distinctly illustrated in the prescribing information of most TKIs (e.g., reversible *vs*. irreversible; non-competitive vs. competitive). The presence or absence of either pre- and co-incubation of TKIs with probe substrates could influence on the inhibitory potential toward transporters, and lead to false-negative results. For example, addition of dasatinib in pre-incubation conditions potently inhibits OCT2 function in experimental studies (Sprowl et al., 2016), whereas co-incubation designs, based on an *a priori* presumed competitive mechanism of inhibition, dasatinib was identified as only a weak inhibitor of OCT2, that is unlikely to have *in vivo* relevance (Minematsu and Giacomini, 2011). Because of the discrepancies in published reports and prescribing information, we have previously argued that a reliable and reproducible approach needs to be implemented to explicitly determine TKI-transporter interactions with a statistically meaningful and unbiased manner is essential in order to evade contradictory results, and should ultimately be applied for the design of subsequent *in vivo* validation studies (Huang et al., 2020). In addition, variations among different laboratory settings, including selection of the test substrate(s) (Sandoval et al., 2018), demand that choosing appropriate model substrates should become an essential component in conducting *in vitro* cationic-type transport assays to generate useful and translationally-relevant predictions.

The lack of regulatory guidelines on the experimental design and clarification of *in vitro* studies to determine an inhibitory potential of drugs with transporters has likely influenced many of the reported inconsistencies. Since TKI agents are most frequently prescribed as a chronic treatment (e.g., once or twice daily) along with numerous other medications, it is anticipated that researchers will be vigilant regarding the potential transporter-mediated drug-drug interactions of TKIs as a perpetrators in order to achieve new mechanistic insights and to enhance the safety of currently used polypharmacy regimens. One approach explored in our current study to demonstrate direct *in vivo* modulation of hepatic OCT1 function following the administration of dasatinib is through the identification of novel biomarkers that could ultimately be utilized to guide the selection of optimal doses and schedules of potential perpetrators to be used in conjunction with OCT1 substrates. This was accomplished by probing for novel endogenous metabolites of OCT1 that reflect hepatic transport function and that can be detected in the circulation, by conducting targeted MS-based metabolomic analyses. This analysis was performed using plasma and liver specimens from wild-type mice and OCT1/2-deficient mice, and ultimately resulted in the identification of various structurally named molecules of possible significance, including isobutyryl-l-carnitine (IBC). Interestingly, while we were completing the current study, Luo et al. reported that IBC is also a potentially useful endogenous biomarker to predict OCT1-mediated drug-drug interactions in humans (Luo et al., 2020). These collective findings are largely congruent with prior studies by Kim et al. on the transport of carnitines in liver-specific OCT1-knockout mice (Kim et al., 2017). This work suggests that levels of certain short-chain acylcarnitines are increased in livers of OCT1-deficient mice but unchanged in plasma, and also that OCT1 serves to efflux carnitines out of cultured hepatocytes but not to take them up. This is consistent with the prior observation that acylcarnitines are not taken up by cells engineered to overexpress OCT1 (Lancaster et al., 2010). In our metabolomics data, we did not observe apparent changes in the levels of IBC in the kidney, where OCT2 is most highly expressed, and we found that additional deficiency of MATE1 had no influence on the results. Although the baseline differences of IBC in plasma between wild-type mice and the various OCT1-deficient strains suggests that levels are predominantly influenced by OCT1-mediated hepatic efflux, we found that dasatinib administration to wild-type mice was actually associated with an increase in the levels of IBC in

plasma. Although this observation seems counterintuitive, it should be noted that OCT1 can serve as a bi-directional hepatic transporter to either mediate the electrogenic cellular influx or alternatively to mediate efflux of organic cations under *trans*-zero conditions, depending on the substrate concentration gradient. Regardless of the mechanistic basis, the recorded discrepancy with the recently published human study (Luo et al., 2020) suggests that further investigation into the use of IBC as an OCT1 biomarker in the context of transport inhibitors is warranted.

In conclusion, we identified a novel regulatory mechanism for OCT1 function that involves tyrosine phosphorylation by the kinase YES1, and, that is highly sensitive to inhibition by multiple TKIs, including dasatinib. OCT1 is highly expressed in hepatocytes and plays a crucial role in the elimination and pharmacological activity of many prescription drugs, and this makes OCT1 uniquely vulnerable to phosphorylation-mediated interactions with TKIs.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/metabolights/MTBLS2433; https://datadryad.org/stash/share/iV95lgOy_VGJLpUReX4ukg77I7vcJueCEwJ0S5p4a0Y.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Ohio State University Animal Care and Use Committee (Protocol No.: 2015A00000101-R1).

## AUTHOR CONTRIBUTIONS

SH and AS conceived this study. MU, AS, and SH designed the experiments. MU, EE, and KH performed the *in vivo* studies. MU, DG, KK, and AG conducted the *in vitro* experiments. ZH and YJ performed *in vivo* biomarker studies. MU, AS, and SH wrote the manuscript. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.644342/full#supplementary-material.

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REVIEW
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# Organic Cation Transporter 1 an Intestinal Uptake Transporter: Fact or Fiction?

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Intestinal transporter proteins are known to affect the pharmacokinetics and in turn the efficacy and safety of many orally administered drugs in a clinically relevant manner. This knowledge is especially well-established for intestinal ATP-binding cassette transporters such as P-gp and BCRP. In contrast to this, information about intestinal uptake carriers is much more limited although many hydrophilic or ionic drugs are not expected to undergo passive diffusion but probably require specific uptake transporters. A transporter which is controversially discussed with respect to its expression, localization and function in the human intestine is the organic cation transporter 1 (OCT1). This review article provides an up-to-date summary on the available data from expression analysis as well as functional studies *in vitro*, animal findings and clinical observations. The current evidence suggests that OCT1 is expressed in the human intestine in small amounts (on gene and protein levels), while its cellular localization in the apical or basolateral membrane of the enterocytes remains to be finally defined, but functional data point to a secretory function of the transporter at the basolateral membrane. Thus, OCT1 should not be considered as a classical uptake transporter in the intestine but rather as an intestinal elimination pathway for cationic compounds from the systemic circulation.

**Keywords: organic cation transporter 1, intestine, human, gene expression, protein abundance, localization**

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## INTRODUCTION

The intestinal epithelium is by far more than a simple passive diffusion barrier as assumed in earlier days. On the contrary, enterocytes are equipped with many physiologically highly relevant transporter proteins that mediate on the one hand a selective and specific absorption of important nutrients and endogenous compounds including peptides via the peptide transporter (PEPT)1 (SLC15A1), glucose via the sodium dependent glucose transporter 1 (SGLT1, SLC5A1), fatty acids via the monocarboxylate transporter 1 (MCT1, SLC16A1), cholesterol and phytosterols via ABCG5/G8, bile acids via the apical sodium-dependent (ASBT, SLC10A1), and vitamins via the sodium-dependent multivitamin transporter (SMVT, SLC5A6) (**Figure 1**) (Drozdzik et al., 2014; Estudante et al., 2016; Müller et al., 2017; Drozdzik et al., 2019).

On the other hand, intestinal transporters are recognized as significant determinants of intestinal absorption of many drugs and thus as important factors influencing their efficacy and safety (Giacomini et al., 2010; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2018). In this regard, especially ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2) and the multidrug resistance-associated protein 2 (MRP2,

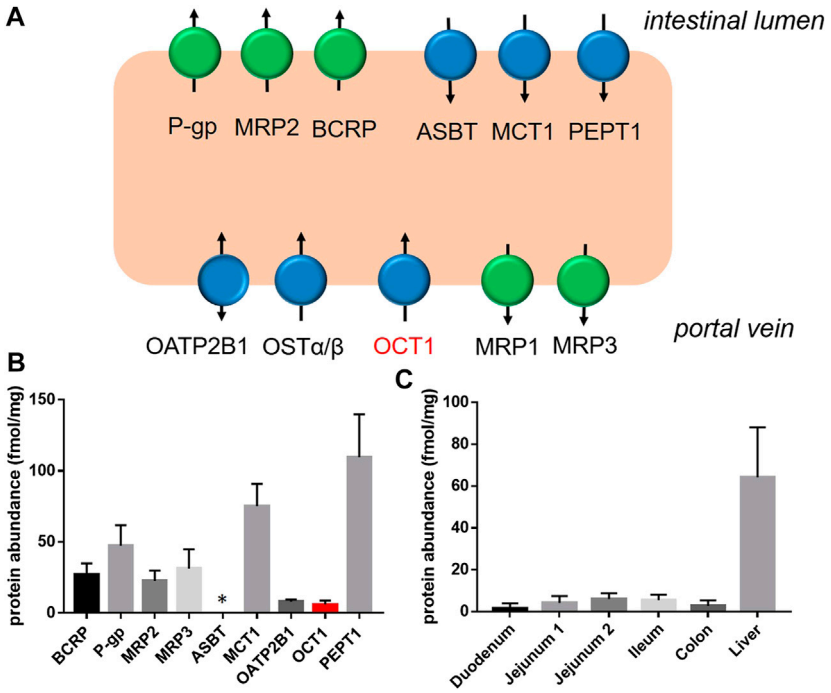


**FIGURE 1 |** Intestinal drug transporters: **(A)**, Schematic overview of clinically relevant transporters in human enterocytes (blue symbols, SLC transporters; green, ABC transporters); **(B)**, Protein abundance of clinically relevant transporters in the human jejunum, and **(C)**, of OCT1 in different intestinal segments and the liver as observed in nine organ donors using the targeted proteomics approach (Drozdzik et al., 2019).

ABCC2) have been extensively investigated. Related to this, inhibition of those transporters resulted in significantly increased absorption of respective transporter substrates (Westphal et al., 2000a; Schwarz et al., 2000; Rengelshausen et al., 2003; Oswald et al., 2006a), whereas induction strikingly reduced their systemic exposure (Greiner et al., 1999; Westphal et al., 2000b; Oswald et al., 2006b). Differences in the longitudinal expression of ABC transporters along the intestine, such as P-gp, were identified as the potential reason for the phenomenon of regio-selective drug absorption ("absorption window"), as observed when comparing different oral dosage forms or by using intestinal perfusion catheter techniques (Gramatté et al., 1996; Weitschies et al., 2005; Tubic et al., 2006; Drozdzik et al., 2014).

In contrast to this, our knowledge is much more limited when it comes to intestinal drug uptake carriers of the SLC family. Although this family contains some 450 members (Hediger et al., 2013), only few SLC transporters have been associated and investigated in terms of their involvement in drug absorption. This is somewhat surprising considering the fact that many drugs are highly polar and permanently or temporarily charged in the rather acidic environment of the upper small intestine (due to their basicity), which are not expected to be absorbed by passive diffusion (Di et al., 2020). Frequently discussed intestinal drug transporters are PEPT1, the organic anion transporting peptides (OATPs) OATP1A2 and OATP2B1 as well as the polyspecific organic cation transporter OCT1. Of the aforementioned carriers, the knowledge on the peptide transporter PEPT1 is the most reliable and consistent. Accordingly, PEPT1 is highly abundant at the apical membrane of human enterocytes along the entire small intestine but not in the colon, and acts as a low affinity-high capacity uptake carrier for peptide-like drugs including beta-lactam antibiotics (e.g., amoxicillin, cefadroxil), angiotensin converting enzyme inhibitors (e.g., enalapril, benazepril) and antiviral drugs (e.g., valacyclovir, valganciclovir) (Brandsch, 2013; Drozdzik et al., 2019). This transporter is even used for innovative drug delivery strategies, in which the oral absorption of several drugs is substantially increased by administering prodrugs being recognized by PEPT1 (e.g., valacyclovir, cefuroxime axetil, oseltamivir) (Kramer, 2011; Brandsch, 2013). OATP1A2 was assumed to be involved in the intestinal absorption of several compounds (e.g., talinolol, nadolol) and responsible for several profound interactions with juices and green tea (Schwarz et al., 2005; Glaeser et al., 2007; Misaka et al., 2014). While gene expression studies were able to detect it along the entire human intestine (Nishimura and Naito, 2005; Glaeser et al., 2007) protein expression could only be verified in one study by immunohistochemistry (Glaeser et al., 2007). In contrast, several more recent studies were not able to detect protein expression by targeted proteomics which leads to the conclusion that OATP1A2 may not be considered as an intestinal transporter (Hilgendorf et al., 2007; Meier et al.,

**TABLE 1 |** Overview of clinically relevant drugs described to be substrates of human OCT1 and their physicochemical properties as obtained from DrugBank (https://go.drugbank.com). If available, experimental data have been preferred over predicated data (*permanent cations, no pKa available).

| Substrate | Drug class | Molecular mass (Da) | logP/pKa | Other transporters/Enzymes involved | Reference |
|---|---|---|---|---|---|
| Acyclovir | Antiviral drug | 225.2 | −1.76/2.5 and 9.4 | Alcohol and aldehyde dehydrogenase, OAT1, OAT3, MATE1, MATE2K | Ito et al. (2014) |
| Amantadine | NMDA receptor antagonist (morbus Parkinson and influenza a drug) | 151.2 | 2.4/10.7 | OCT2 | Kristufek et al. (2002) |
| Amiloride | Diuretic | 229.6 | −0.3/8.7 | OCT2, OCTN1 | Koepsell (2020) |
| Amisulpride | Antipsychotic drug | 369.5 | 1.1/9.4 | | dos Santos Pereira et al. (2014) |
| Atenolol | ß1-adrenoreceptor blocker | 266.3 | 0.16/9.6 | CYP2D6 (minor), OATP1A2 | Mimura et al. (2015) |
| Atropine | Anticholinergic drug | 289.4 | 1.8/9.4 | | Müller et al. (2005); Koepsell (2020) |
| Butylscopolamine | Anticholinergic drug, spasmolytic | 360.4 | −1.9/* | | Koepsell (2020) |
| Cimetidine | Histamine H2 receptor antagonist | 252.3 | 0.4/6.9 | FMO1, FMO3, P-gp | Urakami et al. (1998) |
| Codeine | Analgetic, antitussive drug | 299.4 | 1.4/8.2 | CYP2D6, UGT2B4, UGT2B7, P-gp (metabolite) | Meyer et al. (2020) |
| Diphenhydramine | Histamine H1 receptor antagonist | 255.3 | 3.3/9.0 | CYP2D6, CYP2C9, CYP2C19 | Müller et al. (2005) |
| Etilefrine | α-Adrenoceptor agonist (antihypotensive drug) | 181.2 | 0.23/9.7 | | Jensen et al. (2020) |
| Fenoterol | ß2-sympathicomimetic, antiasthmatic | 303.3 | 1.4/9.6 | | Tzvetkov et al. (2018) |
| Formoterol | ß2-sympathicomimetic, antiasthmatic | 344.4 | 2.2/9.8 | CYP2D6, CYP2C9/19, UGTs | Jensen et al. (2020) |
| Fluoxetine | Serotonin reuptake inhibitor (antidepressant) | 309.3 | 4.1/9.8 | CYP2D6, CYP2C9, CYP3A4 | Koepsell (2020) |
| Ipratropium | Anticholinergic drug, bronchospasmolytic | 332.5 | 0.04/* | OCTN1/2 | Hendrickx et al. (2013); Chen et al. (2017) |
| Ketamine | NMDA receptor antagonist (anesthetic) | 237.7 | 3.1/7.5 | CYP2B6, CYP3A4, CYP2C9, P-gp | Keiser et al. (2018) |
| Metformin | Antidiabetic drug | 129.2 | −2.6/12.4 | OCT2, OCT3, MATE1/2 K | Hendrickx et al. (2013) |
| Metoclopramide | Antiemetic drug | 299.8 | 2.7/9.3 | CYP2D6, CYP3A4, P-gp | Hendrickx et al. (2013) |
| Morphine | Analgetic | 285.3 | 0.9/8.2 | UGT2B7, P-gp | Tzvetkov et al. (2013) |
| Oxaliplatin | Antineoplastic | 397.3 | −0.5/* | OCT2, OCT3, SLC31A1 | Zhang et al. (2006) |
| Oxybutynin | Anticholinergic drug (overactive bladder) | 357.5 | 4.3/8.0 | CYP3A4 | Koepsell (2020) |
| Procainamide | Antiarrhythmic | 235.3 | 0.9/9.3 | CYP2D6, OCT2, OCT3, OCTN1/2, MATE1/2 K | Koepsell (2020) |
| Proguanil | Antimalarial drug | 253.7 | 2.5/10.4 | CYP2D6, CYP2C9, CYP2C19 | Matthaei et al. (2019) |
| Ranitidine | Histamine H2 receptor antagonist | 314.1 | 0.2/8.2 | CYP1A2, CYP2D6, CYP3A4 (all minor), OCT2, P-gp | Meyer et al. (2017) |
| Salbutamol | ß2-sympathicomimetic, antiasthmatic | 239.3 | 1.4/10.3 | | Jensen et al. (2020) |
| Sulpiride | Antipsychotic drug | 341.4 | 0.6/9.1 | | dos Santos Pereira et al. (2014) |
| Sumatriptan | Anti-migraine | 295.4 | 0.9/4.9 | MAO-A, OATP1A2, P-gp | Matthaei et al. (2016) |
| Terazosin | α-Adrenoceptorantagonist | 387.2 | 1.1/7.2 | Hepatic CYPs | Hendrickx et al. (2013) |
| Tiotropium | Anticholinergic drug, bronchospasmolytic | 392.5 | −1.8/* | CYP2D6, CYP3A4 (all minor), OCTN1/2 | Hendrickx et al. (2013) |
| Triamterene | Diuretic | 253.3 | 1.0/3.1 | CYP1A2 | Koepsell (2020) |
| Trimethoprim | Antibiotic | 290.3 | 0.9/7.1 | CYP2C9, CYP3A4, CYP1A2 | Hendrickx et al. (2013) |
| Trospium | Anticholinergic drug (overactive bladder) | 392.5 | −0.5/* | OATP1A2, P-gp | Abebe et al. (2020) |

2007; Drozdzik et al., 2014; Miyauchi et al., 2016; Vaessen et al., 2017; Drozdzik et al., 2019). On the contrary, OATP2B1 is homogenously abundant along the human intestine (Drozdzik et al., 2014). As this carrier was shown to be a potent transporter of statins and other drugs *in vitro*, an important *in vivo* role in intestinal drug absorption was hypothesized (Oswald, 2019). However, the enterocyte localization of OATP2B1 remains still uncertain. While Kobayashi et al. found it in the apical membrane using immunohistochemistry analysis (Kobayashi et al., 2003), Keiser et al. via targeted proteomics approach revealed its basolateral membrane expression, which was also confirmed by functional data from vectorial transport studies across human and porcine jejunum in the Ussing chamber (Keiser et al., 2017).

The same controversy exists for the intestinal expression of OCT1, which is predominately (if not exclusively) expressed at the sinusoidal membrane of human hepatocytes where it mediates the hepatic uptake of many drugs (Drozdzik et al., 2019; Hilgendorf et al., 2007; Nishimura and Naito, 2005). In this regard, OCT1 considers especially small (<300–400 Da), hydrophilic and cationic compounds (**Table 1**). An additional feature of its substrates is, in most cases, a considerable basicity (pKa~ 8–10) which results in a domination of the positively charged moiety of the drug at physiological conditions (pH 7.4 in the systemic circulation and pH 3–5 in the small intestine). OCT1 was convincingly demonstrated to be involved in the pharmacokinetics of several frequently used drugs as concluded in most cases from *in vitro* and pharmacogenetic

**TABLE 2 |** Overview of available data on mRNA expression, protein abundance and localization of OCT1 in the human intestine (+, gene/protein expression was shown; n.d., not detectable; -, not investigated). Data are ranked in chronological order (publication date).

| | Small intestine | | |
|---|---|---|---|
| References | mRNA | Protein (method) | Localization (method) |
| Gründemann et al. (1994) | + | – | – |
| Nishimura and Naito (2005) | + | – | – |
| Terada et al. (2005) | + | – | – |
| Müller et al. (2005) | – | + (immunohistochemsity) | Lateral (immunohistochemsity) |
| Englund et al. (2006) | + | – | – |
| Seithel et al. (2006) | + | – | – |
| Hilgendorf et al. (2007) | + | – | – |
| Meier et al. (2007) | + | – | – |
| Han et al. (2013) | – | + (immunohistochemsity) | Apical (immunohistochemsity) |
| Gröer et al. (2013) | + | + (proteomics) | – |
| Drozdzik et al. (2014) | + | + (proteomics) | – |
| Miyauchi et al. (2016) | – | < LLOQ (proteomics) | – |
| Vaessen et al. (2017) | – | + (proteomics) | – |
| Drozdzik et al. (2019) | + | + (proteomics) | – |

studies (Zamek-Gliszczynski et al., 2018). Associated to this, SLC22A1 genetic loss-of-function polymorphisms were associated with diminished hepatic drug uptake, which in turn increased the systemic drug exposure of OCT1 substrates like sumatriptan, fenoterol, tramadol or morphine (Tzvetkov et al., 2011; Tzvetkov et al., 2013; Matthaei et al., 2016; Stamer et al., 2016; Tzvetkov et al., 2018; Matthaei et al., 2019). In this regard, the frequently prescribed antidiabetic drug metformin is almost exclusively eliminated via the kidney which shows expression of OCT2/3 but no OCT1 (Shu et al., 2008; Tzvetkov et al., 2009; Zamek-Gliszczynski et al., 2018). Thus, in contrast to earlier assumptions, the pharmacokinetics of metformin is not expected to be significantly affected by OCT1 (see also chapter: "Evidence from clinical studies") (Zamek-Gliszczynski et al., 2018). However, conclusions on intestinal OCT1 can only be derived indirectly from those studies. The same is true as for the evidence from clinical drug-drug interaction (DDI) studies in humans. Finally, available *in vitro*, *ex vivo* and *in vivo* models are only partly appropriate to allow conclusive deductions on the function of OCT1 in the human intestine and so far published data have to be interpreted with caution. The following paragraphs will summarize the current knowledge about the expression and localization of intestinal OCT1 as well as available *in vitro*, *ex vivo* and animal study findings. Finally, the evidence from clinical observations will be recapitulated in relation to the potential role of intestinal OCT1 in human drug absorption.

## EVIDENCE FROM EXPRESSION STUDIES

According to former studies on human OCT1, the transporter was reported to be localized in the basolateral membrane of epithelial cells in kidney, intestine as well as the liver (Jonker et al., 2001; Jonker and Schinkel, 2004; Nies et al., 2009). Thus, it was assumed to be involved in the intestinal excretion, hepatic uptake and renal elimination of endogenous compounds and drugs, although more recent studies have clearly demonstrated that OCT1 was not expressed in the kidney (Prasad et al., 2016; Cheung et al., 2019; Oswald et al., 2019).

In contrast to the well-established role of OCT1 in the hepatic disposition of drugs, its role in the intestine remains still unclear. This can be explained by the limited and in part controversial data on its expression there. Several studies unambiguously demonstrated mRNA expression of OCT1 in human intestinal tissue although the expression levels were much lower than that in the liver (**Table 2**). More recent mass spectrometry-based studies could also verify its protein abundance (Drozdzik et al., 2014; Miyauchi et al., 2016; Vaessen et al., 2017; Zamek-Gliszczynski et al., 2018; Drozdzik et al., 2019). In each case, the protein abundance was low compared to other important intestinal transporters such as P-gp or PEPT1. However, in this regard, the method of sample preparation seems to affect the relative and absolute expression data considerably (Wegler et al., 2017). A comparative analysis revealed that the frequently used protocol of analyzing transporter proteins in the crude membrane fraction may overestimate the amount of intestinal OCT1 compared to whole tissue lysates (Drozdzik et al., 2014; Drozdzik et al., 2019) (**Figure 2**). Accordingly, analysis of transporter proteins in enriched membranes overestimated the relative expression of OCT1 by 3–9-fold (compared to other transporters). This is most likely due to substantial intracellular sequestration of the transporter and indicates another source of intra-lab variability of targeted proteomics data on drug transporters.

With respect to the cellular localization of OCT1, immunohistochemistry analysis by Müller et al. demonstrated lateral localization (Müller et al., 2005). In contrast to this, immunostaining and functional studies by the Thakker lab provided convincing evidence that OCT1 is may be present in the apical membrane of mouse and human enterocytes (Han et al., 2013). In addition, these observations have been also confirmed in Caco-2 cells (see next Chapter). As a consequence of those contradictory findings, recent summaries or review articles have indicated OCT1 either as an apical or basolateral transporter (Estudante et al., 2013; Hillgren et al.,

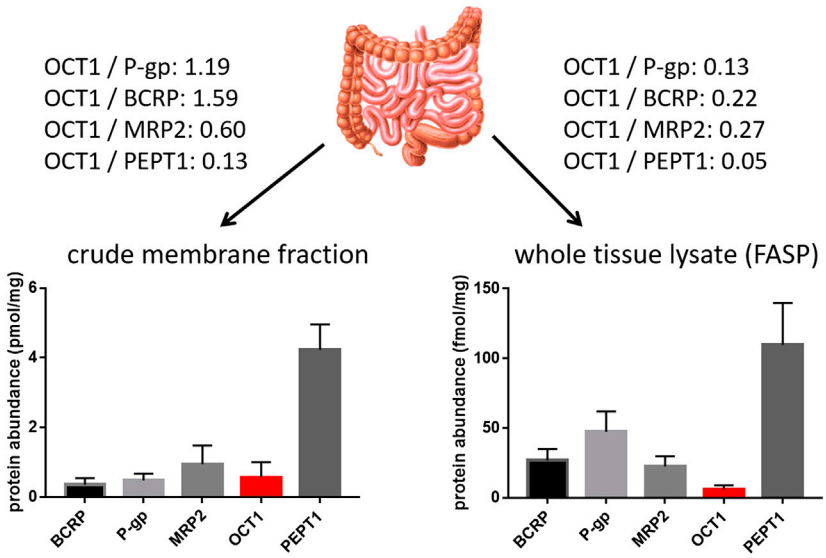


**FIGURE 2 |** Impact of sample preparation on the observed protein abundance of relevant intestinal transporter proteins in the human jejunum. Data on the left diagram were observed from six organ donors after isolation and targeted proteomics analysis of the crude membrane fration (Drozdzik et al., 2014), while data on the right diagram were observed from nine organ donors after sample preparation using the FASP (filter aided sample preparation) protocol and targeted proteomics analysis of the resulting whole tissue lysates. Relative expression ratios of OCT1 to the other transporters are given.

2013; Proctor et al., 2016; Müller et al., 2017). From the functional perspective, this uncertainty cannot answer the question whether OCT1 is directly involved in the intestinal uptake/intestinal absorption (in the case of an apical localization) or rather in the elimination of its substrates from the systemic circulation (in the case of a basolateral localization). In this regard, additional data on the function of OCT1 from *in vitro* and *ex vivo* studies or animal and clinical findings may provide more robust evidence on the expression and function of intestinal OCT1.

## EVIDENCE FROM FUNCTIONAL STUDIES *IN VITRO*, *EX VIVO* AND ANIMAL STUDIES

Stably transfected cell lines overexpressing OCT1 (e.g., MDCKII, HEK293 or CHO cells) are well established *in vitro* models for the identification of substrates and inhibitors of OCT1 and other SLC transporters (Tzvetkov et al., 2011; Brouwer et al., 2013; Tzvetkov et al., 2018). However, due to their artificial character, e.g., strong overexpression of OCT1, different background of other transporters, species origin and cell type (different species for MDCKII, CHO and cell type for MDCKII, HEK293 or CHO cells), lack of polarization (HEK293, CHO) and inability to form tight monolayers (HEK293, CHO), those cell models cannot be used for predictive studies on intestinal OCT1 function. A substantial step forward in this direction are Caco-2 cells that originate from human colorectal adenocarcinoma cells. Among advantages of Caco-2 cells is their morphologic resemblance to the intestinal epithelium due to ability of formation of a confluent monolayer of polarized cells with microvilli on the apical side as well as connecting tight junctions (Hidalgo et al., 1989).

Caco-2 cell monolayers have been frequently used to study transepithelial transport of several OCT1 substrates (Lee et al., 2002; Watanabe et al., 2002; Kuwayama et al., 2008; Proctor et al., 2008; Horie et al., 2011; Elsby et al., 2017). Considering their polarity and biorelevant localization of intestinal transporters, studies on the vectorial basolateral-to-apical (B-A) compared to the apical-to-basolateral (A-B) transport in the absence and presence of OCT inhibitors allow assumptions on the cellular localization of intestinal OCT1. Associated to this, a higher B-A (i.e., secretory transport) compared to the opposite direction was observed for sulpiride, ranitidine, famotidine and 3,4-methylenedioxymethamphetamine indicating basolateral OCT1 working in concert with apical P-gp which considers most OCT1 substrates (Lee et al., 2002; Watanabe et al., 2002; Kuwayama et al., 2008). For metformin, the B-A transport was numerically higher than in the A-B direction but failed to reach statistical significance (Elsby et al., 2017).

On the contrary, Horie et al. observed a markedly higher cellular uptake of OCT1 substrates, i.e., tetraethylammonium (TEA), quinidine and metformin, after the apical side cells exposure in comparison with the basolateral side (Horie et al., 2011).

However, this study does not represent a typical bidirectional transport study and possesses a substantial experimental bias. Considering that in vectorial transport studies, Caco-2 cell monolayers are grown on a porous filter membrane (e.g., Transwell® inserts) it becomes clear that the apical membrane is freely accessible to a drug, whereas the basolateral membrane is partly shielded by the artificial filter membrane. Thus, substantial differences in freely assessable membrane are neglected and it remains uncertain whether the substrates may stick to the filter membrane. In each case, polar OCT1 substrates are not expected

to diffuse freely across the lipophilic membrane. This limitation would have been canceled out in comparative A-B and B-A transport studies. However, these control experiments have not been performed. The interpretation of those experiments is moreover complicated by the fact that the apical membrane forms brush border membrane-like microvilli resulting in a substantially higher surface area compared to the basolateral membrane which is so far not considered when calculating intestinal effective permeability (Peff) values. Due to these limitations, accumulation studies in Caco-2 cells are not expected to provide valid conclusions on the cellular localization of OCT1. Because Han et al. used the same methodical approach for transport studies of TEA and pentamidine, the derived conclusions on apical OCT1 remain questionable (Han et al., 2013). In this study, a markedly lower uptake of pentamidine and TEA into Caco-2 cells were observed from the basolateral membrane compared to the apical side; the apical absorption was significantly reduced in the presence of quinidine and mitoxantrone (Han et al., 2013).

Taken together, the available data from bidirectional transport studies of OCT1 substrates across Caco-2 cell monolayers provide evidence for basolateral OCT1 cellular localization.

However, it should be noted that the expression of OCT1 in Caco-2 cells seems to be low and highly variable as also described for many other transporters (Hayeshi et al., 2008). While several studies where able to detect OCT1 mRNA expression (Schwarz et al., 2000; Müller et al., 2005; Seithel et al., 2006; Hilgendorf et al., 2007; Hayeshi et al., 2008; Horie et al., 2011; Brück et al., 2017), protein levels could only be verified by few studies (Han et al., 2013; Vaessen et al., 2017), while other targeted-based studies failed to detect OCT1 protein (Uchida et al., 2015; Ölander et al., 2016; Brück et al., 2017).

Given the already mentioned complexity and uncertainties in the expression and functional interplay of intestinal and hepatic OCT1, animal studies in rodents appear to be a promising approach to extrapolate findings to humans. However, general differences between rodents and human should be critically considered, e.g., different expression levels of transporters, differences in blood flow, bile flow and enzymatic activity (Cao et al., 2006; Glaeser and Fromm, 2008).

In rodents, OCT1 was also shown to be strongly expressed in the liver, kidney and small intestine. Here, OCT1 was located in the sinusoidal membrane of hepatocytes, in the basolateral membrane of enterocytes, and the basolateral membrane of epithelial cells of proximal tubules (Meyer-Wentrup et al., 1998; Karbach et al., 2000; Chen et al., 2001). The amino acid identity between the human and mouse/rat OCT1 orthologs is 78%. A very recent study by Meyer et al. has comprehensively demonstrated that the difference of about 22% in amino acid sequence could result in tremendous differences in the intrinsic clearance between human and mouse OCT1 (e.g., 4.7-fold higher for mouse Oct1 in metformin uptake) (Meyer et al., 2020) and thereby highlighted the limited transferability of findings from rodent pharmacokinetic models to humans. Nevertheless, although a direct transfer of data observed in animal studies is not possible, general insights into the expression and function of OCT1 are possible.

In this regard, Shu et al. demonstrated in Oct1-deficient mice that the hepatic uptake of metformin was dramatically reduced which resulted in completely abolished glucose-lowering effects of the drug (Shu et al., 2007). However, metformin concentration-time profiles in blood were not different between wild-type and knockout animals (Shu et al., 2007; Shu et al., 2008) (see also paragraph: "Evidence from clinical studies"). Considering that metformin was orally administered in this study and Oct1-knockout mice did not show any changes in their serum exposure, intestinal OCT1 seems not to be principally involved in metformin absorption, which points to a rather basolateral localization of OCT1 as demonstrated by previous immunostaining analysis (Chen et al., 2001). This assumption is also confirmed by a pharmacokinetic study with OCT1 model substrates TEA and MPP+ (1-methyl-4-phenylpyridinium) in Oct1-knockout mice. After intravenous administration of the probe compounds, not only their hepatic accumulation was reduced (4–6-fold) but also their uptake into the intestinal tissue was nearly halved (Jonker et al., 2001). Comparable findings have been observed for metformin; after i. v. administration of the drug, hepatic accumulation was more than 30 times lower in Oct1-knockout mice than that in wild-type animals, while basolateral uptake from blood into the tissue of duodenum, jejunum and ileum was 3–7-fold lower, which suggests a role of OCT1 in intestinal metformin excretion (Wang et al., 2002). In Oct1/2 double knockout mice, intravenously administered sulpiride resulted in significantly higher serum exposure but lower accumulation of the drug in hepatic, renal and small intestinal tissue (Takano et al., 2017).

Similarly, the hepatic exposure and the duodenal content of sumatriptan, fenoterol, ondansetron, and tropisetron after their intravenous administration was lower in Oct1-knockout mice than that in their wild-type counterparts (Morse et al., 2020). Furthermore, this study compared the pharmacokinetics of the above-mentioned drugs after oral and intravenous administration in wild-type and Oct1-knockout mice. After oral administration, maximum serum levels ($C_{max}$) and serum AUC of all investigated drugs were found to be markedly elevated in OCT1-knockout animals; oral bioavailability was not different or even increased. Assuming OCT1 as apically localized and therefore acting as an intestinal uptake transporter, one would expect a significantly decreased oral bioavailability in knockout mice. The study indicates that OCT1 is rather involved in transport from blood into deeper compartments than in uptake from intestinal lumen to blood. Consequently, OCT1 deficiency in knockout mice was associated with increased serum exposure (decreased serum clearance) and with a decreased volume of distribution of the respective substrates. This again suggests that OCT1 is most likely expressed in the basolateral membrane of the enterocytes.

In contrast to this, again the already mentioned study by Han et al., 2013 hypothesized an apical localization of OCT1 as concluded from uptake studies into mouse intestine. However, very similar to the discussed Caco-2 experiments, no vectorial transport study was performed but a rather simple accumulation experiment after either apical or basolateral exposure to the OCT1 substrate pentamidine (Han et al., 2013). While the

uptake from the basolateral membrane tended to be higher compared to the apical membrane, only the uptake from the apical side was reduced in the presence of quinidine and desipramine, both inhibitors of OCT1.

Ideally, confirmative transport studies should be performed as bidirectional transport studies using animal or human tissue mounted in the Ussing chamber, which represents so far the gold standard experiment to provide reliable and biorelevant information on the intestinal drug metabolism and transport (Kisser et al., 2017). Although the mentioned method would be suitable to provide further insights into the localization of OCT1, it was so far exclusively used for absorptive studies. However, reliable conclusions can only be derived from bidirectional transport studies (A-B vs. B-A) (Kim et al., 2005; Arnold and Kalia, 2020).

## EVIDENCE FROM CLINICAL STUDIES

Investigative approaches to estimate the function of intestinal OCT1 comprise pharmacogenetic studies and DDI studies with orally administered OCT1 substrates. The evidence from clinical studies showing that OCT1 might be a clinically relevant intestinal drugs uptake carrier is limited. This can be attributed to the following aspects which counteract reliable conclusions on the distinct role of OCT1 in the human intestine: first, substrates of OCT1 are partly subjected to extensive metabolism (e.g., morphine, codeine, sumatriptan, tramadol); second, multiple other transporters can be involved in the pharmacokinetics of a certain OCT1 substrate (e.g., OCT2, MATE1/2K, P-gp); third, OCT1 is not inducible by prototypical inducers of enzymes and transporters such as rifampin which disqualifies meaningful inductive studies; fourth, likewise, there is a lack of specific inhibitors that can be used *in vivo*; and fifth, the expression and function of intestinal and hepatic OCT1 results in opposite clinical effects in case of transporter inhibition. Hence, inhibition of intestinal OCT1 (assuming its apical localization) is expected to result in decreased oral drug absorption and systemic exposure, whereas inhibition of hepatic uptake will cause increased plasma levels of OCT1 substrates. Thus, a substantial overlap is expected which may mask the certain effect of intestinal OCT1. In the case of intestinal OCT1 on the basolateral membrane of the enterocyte, transporter inhibition may cause to some extent additionally increased serum levels caused by reduced direct intestinal excretion of the drug.

In addition, renal OCT2 and MATE1/2 K may contribute also to clinical DDI studies because they accept many OCT1 substrates (Koepsell et al., 2007; Koepsell, 2015; Koepsell, 2020), i.e., inhibition of renal cation transporters will result in increased drug exposure as seen for inhibition of hepatic uptake by OCT1.

Considering that there are no specific clinical inhibitors of OCT1 available, the use of pharmacogenetic studies in carriers of OCT1 null alleles (~9% in Caucasians) is expected to provide additional evidence (Zamek-Gliszczynski et al., 2018).

A prominent example of the difficulties in the interpretation of human clinical studies on OCT1 is the antidiabetic drug metformin. In this regard, the first pharmacogenetic study in a small number of healthy volunteers ($N = 20$) indicated that OCT1 significantly affected the serum exposure and efficacy of metformin. In detail, carriers of SCL22A1 loss-of-function alleles showed a reduced response to the drug (Shu et al., 2007) which was explained by the decreased uptake of metformin to its predominate site of action, namely the liver, as concluded from the significantly elevated serum exposure of metformin in carriers of the genetic variants (Shu et al., 2008). However, the extent of increase in metformin serum AUC was rather little (~20%). A subsequent and more comprehensive pharmacogenetic study in 103 healthy males could not find any significant changes in the serum pharmacokinetics between carriers of the SLC22A1 wild-type or loss-of-function alleles (Tzvetkov et al., 2009). On the contrary, it was found that the renal clearance of metformin was significantly affected by the number of low-activity OCT1 alleles. Thus, the authors concluded that renal OCT1 might be an important carrier in renal elimination of the drug. Although the authors confirmed their hypothesis by providing immunohistochemical staining of human kidneys, which demonstrated OCT1 expression in the luminal (apical) membrane of proximal and distal tubules, more recent targeted proteomics failed to detect substantial levels renal OCT1 (Prasad et al., 2016; Cheung et al., 2019; Oswald et al., 2019).

Finally, Cho et al. observed that the unspecific OCT inhibitor verapamil did not change the serum pharmacokinetics but significantly decreased the glucose-lowering effect of metformin in 12 healthy volunteers (Cho et al., 2014). This finding was also confirmed by an independent group (Christensen et al., 2015). Consequently, one can summarize that the serum pharmacokinetics of metformin is not significantly affected by OCT1 because this frequently prescribed antidiabetic drug is almost exclusively eliminated via the kidney, which does not express OCT1 but OCT2/3 and MATE1/2K (Shu et al., 2008; Tzvetkov et al., 2009; Cho et al., 2014; Zamek-Gliszczynski et al., 2018). In line with this conclusion, metformin was also shown to be accepted by other cation transporters including OCT2, MATE1, and MATE2-K, which contribute to the pharmacokinetics and DDI studies of the drug (Wang et al., 2008; Kusuhara et al., 2011; Ito et al., 2012; Yoon et al., 2013; Cho et al., 2014; Dujic et al., 2015). Accordingly, inhibition of OCT1-mediated hepatic uptake by co-administered drugs are expected to reduce hepatic drug levels and in turn its antihyperglycemic effects with no considerable changes in systemic metformin concentrations (Cho et al., 2014; Sundelin et al., 2017). In the same manner, the observed impact of genetic polymorphisms of SLC22A1 gene on antidiabetic effects of metformin could be explained by differences in tissue exposure to the drug (Shikata et al., 2007; Shu et al., 2007; Cho et al., 2014), which seems to be also affected by sinusoidal efflux transporters (Zamek-Gliszczynski et al., 2013). However, it should be noted that this finding could not be verified in a larger cohort study in 3,450 type 2 diabetes patients on the level of glycated hemoglobin (HbA1c) (Zhou et al.,

2009). Moreover, the impact of genetic variants of OCT1 on the metformin response were shown to be population specific (Mofo Mato et al., 2018). This complex example nicely demonstrates that it can be challenging to conclude from pharmacokinetic data alone on the distinct relevance of OCT1. Taken together, DDI studies with metformin should include a pharmacodynamic measure but the drug may not be a suitable drug to conclude on the function of intestinal OCT1 (Zamek-Gliszczynski et al., 2018).

In contrast to metformin, it was demonstrated for several other OCT1 substrates, that OCT1-mediated hepatic uptake is the rate-determining step in their hepatic processing, and thus are expected to be more suitable markers to provide deeper insights into the role of OCT1 for systemic drug exposure; i.e., being probe substrates for clinical DDI studies. An example is the beta2-adrenergic receptor agonist fenoterol, a narrow therapeutic index drug, for which it was demonstrated that SLC22A1 homozygous carriers of loss-of-function alleles possessed about 2-fold higher systemic drug exposure at significantly increased heart rate and blood glucose but significantly lowered serum potassium levels, all of which are pharmacodynamic side effects of the drug (Tzvetkov et al., 2018). However, fenoterol was administered in this study via intravenous infusion, which hampers conclusions on intestinal OCT1 function. Considering furthermore, that fenoterol is regularly administered via inhalation for the treatment of asthma and COPD, it is not surprising that human DDI studies on OCT1 are, unfortunately, not available.

Another even more frequently used example is the opioid analgetic drug tramadol, which active metabolite O-desmethyl tramadol is a substrate of OCT1. Similarily to fenoterol, oral administration of tramadol resulted in about 2-fold greater metabolite exposure in healthy volunteers carrying loss-of-function SLC22A1 polymorphisms, resulting in significantly prolonged miosis, i.e., a characteristic opioid effect (Tzvetkov et al., 2011). Moreover, these prolonged opioid effects resulted in decreased self-administration of the drug in patients suffering from postoperative pain in clinical practice (Stamer et al., 2016). As a considerable limitation, tramadol undergoes extensive metabolism by CYP2D6 which represents a substantial confounder in DDIs studies on OCT1. An example for this aspect might be the observed decrease of the analgesic efficacy of tramadol in the presence of ondansetron (De Witte et al., 2001; Vale et al., 2011). As both drugs are substrates of CYP2D6 and OCT1, the distinct contribution of OCT1 remains uncertain (Tzvetkov et al., 2012). Thus, the function of intestinal OCT1 can not be directly anticipated from DDI studies with tramadol because of interferences of the perpetrator drug with the hepatic oxidative metabolism.

Under consideration of the first examples and the respective limitations, an OCT1 substrate which might be suitable to provide further insights into the expression and function of intestinal OCT1 requires the following features: first, oral administration (oral dosage form available); second, no or only minor metabolism; third, no or only minor passive diffusion; and fourth, no other transporters influencing its pharmacokinetics in a significant manner.

Applying these criteria to the substrates summarized in **Table 1**, they would disqualify at first glance acyclovir, codeine, diphenhydramine, formoterol, fluoxetine, ipratropium, ketamine, morphine, oxaliplatin, oxybutynin, procainamide, proguanil, salbutamol, terazosin, tiotropium, triamterene, and trimethoprim. On the other side, drugs such as amantadine, amiloride, amisulpride, atenolol, butylscopolamine, etilefrine, ranitidine, sulpiride, sumatriptan, and trospium may be suitable to derive conclusions on intestinal OCT1.

Because potent inducers of OCT1 are not available, only pharmacogenetic and DDI studies with orally administered unspecific inhibitors of OCT transporters can be used to provide further insights into intestinal OCT1. **Table 3** summarizes appropriate inhibitors that are expected to be suitable candidates in clinical studies. As discussed elsewhere, there is no doubt that there is a tremendous variability in the published IC50 values even when using the same probe substrate (e.g., MPP+), which makes it challenging to estimate clinically relevant DDIs (Nies et al., 2011; Koepsell, 2015). This uncertainty is further amplified by the partly unknown concentrations *in vivo*; e.g., in portal vein (up to 100-fold higher compared to the systemic blood concentration) relevant for OCT1-mediated uptake into the liver or in the intestinal lumen affecting interaction with intestinal uptake carriers (assumption so far: dose/250 ml, although the intestine is known to contain much less volume of water (50–100 ml) (Schiller et al., 2005)). However, only for very few of the mentioned OCT1 substrates, confirmative clinical pharmacogenetic or DDI studies have been performed.

A well investigated drug in this regard is the antimigraine drug sumatriptan, which systemic exposure was over 2-fold increased after oral administration in carriers of SLC22A1 loss-of-function alleles (Matthaei et al., 2016). Although sumatriptan is subjected to extensive first pass metabolism (bioavailability, ~15%) by monoamine oxidase A (MAO-A), this metabolic pathway might be only a confounder in very few DDI studies, because known potent inhibitors are rather less frequently prescribed drugs including moclobemide, tranylcypromine, linezolid, selegiline, and zonisamide. Despite the fact that significant DDIs studies with known unspecific inhibitors of OCTs (**Table 3**) cannot be found, the described pharmacogenetic data on sumatriptan do not support the hypothesis of apically expressed OCT1 in the human intestine. Otherwise, carriers of loss-of-function alleles should exhibit lower instead of higher drug exposure as observed by Matthaei and colleagues (Matthaei et al., 2016).

Similarily to sumatriptan, morphine is also in most cases orally administered and was shown to be a substrate of OCT1 (Tzvetkov et al., 2013). However, due to its pronounced lipophilicity (logP, 0.9) and its moderate basicity (pKa, 8.2), considerable diffusion from the systemic circulation can be assumed (ionization degree at pH 7.4, 86.3%), which may counteract reliable conclusions on the quantitative contribution of hepatic OCT1. Moreover, extensive glucuronidation via UGT2B7, which is predominately expressed in the human liver (Drozdzik et al., 2018), further limits application of morphine as an OCT1 probe drug. In contrast to this, in the intestinal lumen (pH 3–5), over 99.99% of morphine is expected to be ionized and would

**TABLE 3 |** Overview of clinically relevant drugs that are orally administered and potent inhibitors of OCT1.

| Drug/compound | Class | Inhibitory effect | References |
|---|---|---|---|
| Amitriptyline | Non-selective NSRI | $IC_{50}$ = 4.4 μM | Tzvetkov et al. (2013) |
| Cimetidine | $H_2$-receptor antagonist | $IC_{50}$ = 60 μM | Koepsell (2020) |
| Citalopram | SSRI | $IC_{50}$ = 2.8 μM | Koepsell et al. (2007) |
| Clonidine | α-adrenoceptor antagonist | $IC_{50}$ = 0.6–6.5 μM | Koepsell et al. (2007) |
| Desipramine | Non-selective NSRI | $IC_{50}$ = 5.4 μM | Koepsell et al. (2007) |
| Diphenhydramine | $H_1$-receptor antagonist | $IC_{50}$ = 3.4 μM | Müller et al. (2005) |
| Fluoxetine | SSRI | $IC_{50}$ = 6.0 μM | Tzvetkov et al. (2013) |
| Imipramine | Non-selective NSRI | $IC_{50}$ = 6.2 μM | Tzvetkov et al. (2013) |
| Memantine | NMDA receptor antagonist | $IC_{50}$ = 3.7 μM | Busch et al. (1998) |
| Metoclopramide | $D_2$/5-$HT_3$ receptor anatgonist | $IC_{50}$ = 16–95 μM | Koepsell (2020) |
| Morphine | Opioid receptor agonist | $IC_{50}$ = 4.2–28 μM | Koepsell (2020) |
| Ondansetron | 5-$HT_3$ receptor antagonist | $IC_{50}$ = 1.2 μM | Tzvetkov et al. (2013) |
| Oxybutynin | Muscarinic receptor antagonist | $IC_{50}$ = 20 μM | Koepsell (2020) |
| Prazosin | α-adrenoceptor antagonist | $IC_{50}$ = 1.8 μM | Hayer-Zillgen et al. (2002) |
| Quinidine | $Na^+$channel blocker (antiarrhythmic) | $IC_{50}$ = 18 μM | Koepsell et al. (2007) |
| Quinine | Antimalaria drug | $IC_{50}$ = 13–23 μM | Koepsell et al. (2007) |
| Ranitidine | $H_1$-receptor antagonist | $IC_{50}$ = 28 μM | Müller et al. (2005) |
| Ritonavir | HIV protease inhibitor | $IC_{50}$ = 5.2 μM | Zhang et al. (2000) |
| Trospium | Muscarinic receptor antagonist | $IC_{50}$ = 5.3–18 μM | Koepsell (2020) |
| Verapamil | $Ca^{2+}$channel blocker | $IC_{50}$ = 1.6–2.9 μM | Koepsell et al. (2007), Tzvetkov et al. (2013) |

*$IC_{50}$, half maximal inhibitory concentration; NMDA, N-methyl-D-aspartate; NSRI, norepinephrine and serotonin reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.*

necessarily require an uptake transporter such as OCT1. From this perspective, oral morphine might be a suitable drug to derive conclusion on intestinal OCT1. Associated to this, Nielson et al. could not find any changes in the pharmacokinetics or pharmacodynamic effects of orally administered morphine in 37 healthy volunteers related to common genetic variants of SLC22A1, ABCB1, and UGT2B7 (Nielsen et al., 2017). In line with those findings, there are no DDI studies with orally administered morphine and the aforementioned inhibitors of OCT1 (**Table 3**) compromising the oral opioid absorption. In contrast to this, neither Cmax nor serum AUC of oral controlled release morphine were significantly different in combination with oral metoclopramide (MCP). Only morphine tmax occurred significantly earlier in the MCP group as explained by the known prokinetic effect of MCP resulting in accelerated gastric emptying (Manara et al., 1988). The simultaneous oral intake of morphine and the antiarrhythmic drug quinidine resulted even in a 1.9-fold and 1.6-fold higher $C_{max}$ and AUC of morphine and significantly increased opioid effects (Kharasch et al., 2003). A similar outcome has been reported for the oral combination of morphine and ranitidine. Here, AUC0-90min of morphine was 1.5-fold increased in the presence of ranitidine (Aasmundstad and Størset, 1998). While those effects can be attributed to inhibition of intestinal P-gp (morphine is a P-gp substrate, while quinidine and ranitidine are inhibitors of OCT1 and P-gp), one can conclude from the pharmacogenetic and DDI studies again that OCT1 may not be localized in the apical membrane of the human enterocytes.

Interestingly, significant associations between SLC22A1 loss-of-function variants and the pharmacokinetics of morphine (i.e., morphine clearance was significantly reduced) and higher frequency of side effects have been observed in children after intravenous administration (Fukuda et al., 2013; Venkatasubramanian et al., 2014; Balyan et al., 2017; Hahn et al., 2019). Furthermore, Tzvetkov et al. found gene dose-dependent changes in the pharmacokinetics of morphine in healthy volunteers after oral administration of the prodrug codeine, which is bioactivated in the liver via CYP2D6 to morphine (Tzvetkov et al., 2013; Drozdzik et al., 2018). However, although those studies demonstrated that the pharmacokinetics of morphine is significantly affected by OCT1 (despite the aforementioned limitations), they did not allow any conclusions on the function of intestinal OCT1, since morphine was in both scenarios administered to the systemic circulation, either directly by intravenous administration or indirectly by using a prodrug, which has to be bioactivated in the liver.

Additional arguments against OCT1 at the apical membrane in the human intestine provide interaction studies of atenolol with cimetidine, metoclopramide with ranitidine and metformin with trospium (Houtzagers et al., 1982; Leucuţa et al., 2004; Oefelein et al., 2013). In all studies, serum levels of the victim drugs were not changed or only marginally elevated (MCP). However, DDI studies with cimetidine have to be interpreted with caution as this drug inhibits also the renal secretion of many drugs in proximal tubules by blocking OCT2-mediated uptake at the basolateral membrane and/or inhibition of efflux at the apical membrane mediated by MATE1, MATE2-K, OCTN1, and/or OCTN2 (Koepsell, 2020).

Finally, the poorly absorbable bladder spasmolytic trospium (intestinal absorption and oral bioavailability about 10%) might be a good candidate to conclude on the function of intestinal OCT1 because this drug is given orally, is not metabolized and is not subjected to significant hepatic uptake but undergoes almost exclusively renal excretion (Doroshyenko et al., 2005). In this regard, interaction studies with oral ranitidine and metformin are available (Oefelein et al., 2013; Abebe et al., 2020). In both studies, trospium serum AUC and

**TABLE 4 |** Overview of clinical drug-drug interactions which may allow conclusions on intestinal OCT1.

| Substrate (victim drug) | Perpetrator (inhibitor) | PK change | References |
|---|---|---|---|
| Atenolol (100 mg, oral) | Cimetidine (1,000 mg, oral) | AUC and cmax unchanged | Houtzagers et al. (1982) |
| Metformin (500 mg, BID, oral) | Trospium (60 mg, QID, oral) | AUC and cmax unchanged | Oefelein et al. (2013) |
| Metoclopramide (20 mg, oral) | Ranitidine (150 mg, oral) | AUC↑, +13% ($p < 0.05$); Cmax↑, +12% (N.S.) | Leucuța et al. (2004) |
| Morphine (20 mg, oral) | Metoclopramide (10 mg, oral) | AUC and cmax unchanged | Manara et al. (1988) |
| Morphine (30 mg, oral) | Quinidine (600 mg, oral) | AUC↑, 1.6-fold; Cmax↑, 1.9-fold | Kharasch et al. (2003) |
| Morphine (10 mg, oral) | Ranitidine (150 mg, oral) | $AUC_{0-90\ min}$, ↑1.5-fold | Aasmundstad und Størset (1998) |
| Trospium (60 mg, QID, oral) | Metformin (500 mg, BID, oral) | AUC↑, +29% (N.S.); Cmax↑, +34% (N.S.) | Oefelein et al. (2013) |
| Trospium (30 mg, oral) | Ranitidine (300 mg, oral) | AUC and cmax unchanged | Abebe et al.(2020) |

*AUC, area under the concentration-time curve; BID, twice daily; CL, clearance; Cmax, maximum serum concentration; Css, trough serum concentrations at steady-state; d, days; MD, multiple doses; QID, four times daily; SID, once daily; SD, single dose; t1/2, elimination half-life.*

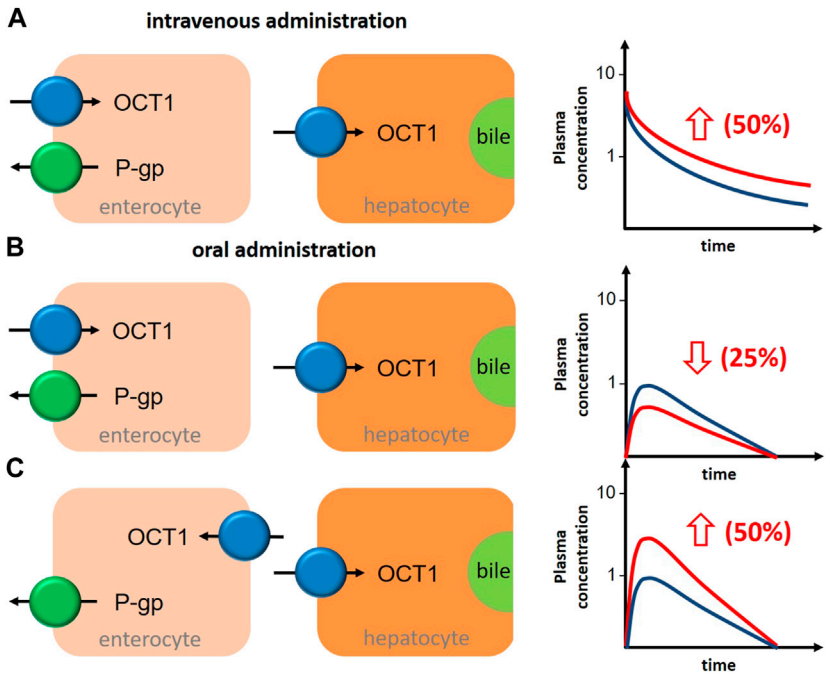


**FIGURE 3 |** Schematic illustration about the impact of OCT1 inhibition in human liver and intestine on the bioavailability of the victim drug in depencence on the localization of intestinal OCT1 and the route of drug administration. **(A)**, after intravenous administration of an OCT1 substrate, inhibition of hepatic OCT1 will increase systemic drug exposure by 50%. **(B)**, after oral administration of an OCT1 substrate, inhibition of intestinal (apical localization) and hepatic OCT1 will decrease oral bioavailability by 25%. **(C)**, after oral administration of an OCT1 substrate, inhibition of intestinal (basolateral localization) and hepatic OCT1 will increase oral bioavailability by 50%. General assumptions for all estimations: intestinal and hepatic uptake of the drug are 50% and mediated by OCT1; OCT1 inhibition results in 50% reduction in the intestinal absorption (fa) and/or hepatic uptake (blue graph, OCT1 substrate without inhibitor; red graph, OCT1 substrate with inhibitor).

Cmax were not significantly different in the presence of the inhibitor of OCT1. In the interaction study with metformin, the systemic exposure of trospium was numerically even slightly increased. Also these studies indicate that OCT1 might not be present in the apical but rather in the basolateral membrane of the human enterocytes.

However, as mentioned earlier, the interpretation of clinical interaction studies with OCT1 substrates and inhibitors as summarized in **Table 4** is complicated by the interference of intestinal and hepatic uptake function of OCT1 resulting theoretically in opposite effects–assuming OCT1 in the apical membrane of the enterocytes contributing significantly to oral drug absorption (**Figure 3**).

Assuming oversimplified that intestinal and hepatic uptake would contribute equally to the bioavailability of a certain OCT1 substrate, simultaneous inhibition of intestinal and hepatic OCT1 would result in only slightly changed systemic exposure of the victim drug (**Figure 3B**). However, this is mostly not the case as the intestinal absorption of most OCT1 substrates is limited (suggesting a rate-determining intestinal transporter) and their hepatic extraction and biliary excretion is even lower (predominate renal excretion of about 80–90%). Based on the

**TABLE 5 |** Estimated impact on oral bioavailability (F) of OCT1 substrates caused by inhibition of intestinal and/or hepatic OCT1 and observed clinical data.

| Scenario | Atenolol | Metoclopramide | Metformin | Trospium |
|---|---|---|---|---|
| No inhibition of intestinal and hepatic OCT1 | 0.425 | 0.714 | 0.48 | 0.095 |
| Predicted inhibition of intestinal OCT1 (assuming apical localization) | 0.213 (↓50%) | 0.357 (↓50%) | 0.24 (↓50%) | 0.048 (↓50%) |
| Predicted inhibition of hepatic OCT1 only (i.v. administration or basolateral intestinal OCT1) | 0.463 (↑9%) | 0.777 (↑9%) | 0.54 (↑13%) | 0.098 (↑3%) |
| Predicted inhibition of intestinal (apical) and hepatic OCT1 (oral administration) | 0.231 (↓46%) | 0.389 (↓46%) | 0.27 (↓44%) | 0.049 (↓49%) |
| Observed interaction data | unchanged AUC Houtzagers et al. (1982) | AUC↑, 13% Leucuţa et al. (2004) | unchanged AUC Oefelein et al. (2013) | unchanged AUC Abebe et al. (2020) |

*Used data for estimations: Atenolol (fa, 0.5; fh, 0.85), Metoclopramide (fa, 0.84; fh, 0.85); Metformin (fa, 0.6; fh, 0.8) and Trospium (fa, 0.1; fh, 0.95). In the case of inhibition, 50% reduction of intestinal absorption or hepatic extraction was assumed. As data on fh were not available, they have been indirectly estimated from excretion pathways (fh ~ renal excretion after i.v. administration).*

simple equation on oral bioavailability F = fa*fg*fh, where (fa) is the absorbed dose fraction (fg) is the fraction of drug escaping first-pass gut wall metabolism that enters the portal blood, and (fh) is the fraction of drug escaping hepatic metabolism and biliary secretion entering the systemic circulation (Huang et al., 2009), and assuming that (fg) is not relevant for a confirmative OCT1 probe drug (fg = 1), systemic drug exposure is a function of intestinal absorption and hepatic extraction. Applying this very simple conception to the discussed interference of intestinal and hepatic OCT1 transport, it becomes clear that the contribution of intestinal OCT1 (assuming its apical localization) is expected to dominate the entire process (**Table 5**); i.e., interaction studies with OCT1 inhibitors should result in pronounced reduction of serum exposure to OCT1 substrates. As none of the available studies showed this result, there are no arguments from pharmacogenetic and DDI studies to assume an apical localization of OCT1 in the human enterocytes but rather its presence in the opposite membrane. As mentioned earlies, this oversimplification omits the potential simultaneous inhibition of renal OCT2/3 and MATE1/2K transporters by unspecific inhibitors of OCT1.

## SUMMARY AND CONCLUSION

There is no doubt that hepatic OCT1 can influence the pharmacokinetics and in turn the efficacy and safety of several drugs in a significant manner (Jonker and Schinkel, 2004; Koepsell et al., 2007; Shu et al., 2007; Koepsell, 2015, 2020). In this regard, genetic polymorphisms and DDIs were shown to result in drastically changed serum levels of the respective substrates. Consequently, the latest update of the International Transporter Consortium emphasized OCT1 as a transporter of emerging clinical importance (Zamek-Gliszczynski et al., 2018).

As OCT1 was also shown to be expressed in the human intestine, it was assumed to be involved in the intestinal absorption of drugs. Despite its unequivocal intestinal abundance, the distinct localization in the enterocytes still remains uncertain as two independent studies identified OCT1 either in the apical or the basolateral membrane (Müller et al., 2005; Han et al., 2013). However, only if OCT1 is present in the apical membrane facing the intestinal lumen it can contribute directly to oral drug absorption. There was recently a similar discussion on the localization of OATP2B1 in the human intestine. Targeted proteomics analysis of the intestinal membranes along with functional studies in Caco-2 cells and intestinal tissue from animals and human clarified OATP2B1 as a basolateral carrier (Keiser et al., 2017) and ruled it out to be a transporter involved in intestinal drug absorption. Very recent studies from knockout mice indicate that Oatp2b1 might be involved in intestinal drug absorption (Medwid et al., 2019; Chen et al., 2020). However, considering that human OATP2B1and mice Oatp2b1share only 74.6% amino acid homology, additional transporters are involved in the pharmacokinetics of the investigated drugs (fexofenadine, rosuvastatin and fluvastatin) and that general limitations on the direct comparison of human and rodent pharmacokinetics exist, these findings must be interpreted with caution. Further studies with human intestinal tissue are required, which is also true for OCT1.

Accordingly, most bidirectional transport studies of OCT1 substrates across Caco-2 cells demonstrated a markedly higher secretory transport compared to the opposite direction (B-A > A-B), which suggest a basolateral localization of OCT1 (Lee et al., 2002; Watanabe et al., 2002; Kuwayama et al., 2008). As recently shown, OCT1 also contributes to thiamine uptake (Chen et al., 2014). Here, Oct1 knockout in mice was associated with dramatically reduced uptake of intravenously administered thiamine into intestinal tissues confirming a basolateral localization of OCT1. This assumption is also supported by several other former animal experiments, in which direct excretion of intravenously administered OCT1 substrates into the intestinal lumen was shown to be markedly lower in Oct1-knockout mice (Jonker et al., 2001; Wang et al., 2002; Takano et al., 2017). Moreover, oral administration of OCT1 substrates resulted in unchanged or even substantially increased serum levels in Oct1-knockout mice (Morse et al., 2020). These data are in line with the basolateral localization of Oct1 in the murine intestine as observed by immunohistochemistry (Chen et al., 2001). Considering also the basolateral (sinusoidal) localization of OCT1 in hepatocytes and the fact that most transporters show the same localization in liver, kidney and intestine (e.g., P-gp, MRP2, MRP3, BCRP, MATE1) it appears reasonable to assume OCT1 as a basolateral transporter in human gut. Interestingly,

OCT1 was also speculated to be involved in the efflux of acylcarnitines from the liver to the systemic circulation (Kim et al., 2017). Assuming OCT1 as a bidirectional transporter, it seems possible that it may also be involved in drug absorption on the basolateral membrane of the enterocytes. However, this hypothesis needs to be proven by additional studies.

Finally, also the available pharmacogenetic and DDI studies do not provide evidence for apically localized intestinal OCT1. However, the interpretation of clinical studies is complicated considering the complex contribution of intestinal, hepatic, and renal cation transporters. Moreover, confirmative induction studies as regularly performed for P-gp or cytochrome P450 enzymes are not possible for OCT1.

In conclusion, available evidence from expression studies, *in vitro* and animal experiments as well as data from clinical studies suggest that OCT1 is localized in the basolateral membrane of the enterocytes and cannot be considered as an uptake transporter in the human intestine.

Basolateral OCT1 in the enterocytes would imply its involvement in the intestinal excretion of drug from the systemic circulation. For this secretory net transport across the enterocytes, P-gp can be expected a relevant efflux transporter in the apical membrane because it accepts many OCT1 substrates. Indeed, this intestinal elimination pathway has been observed in several animal studies after intravenous administration of OCT1 substrates (Suttle and Brouwer, 1995; Jonker et al., 2001; Wang et al., 2002; Takano et al., 2017) but also in clinical pilot studies demonstrating direct intestinal secretion of supiride and ranitidine, both substrates of OCT1 and P-gp (Gramatté et al., 1994; Takano et al., 2017). Thus, OCT1 should be considered as a basolateral uptake carrier contributing to the intestinal elimination of cationic compounds from the systemic circulation. However, considering its rather low protein abundance and its mode of action, the allover pharmacokinetic relevance of this elimination pathway appears to be low.

On the contrary, assuming OCT1 in the apical membrane of the enterocytes (Han et al., 2013) would raise the question on the feasibility of an absorptive net transport across the intestinal epithelia because the enterocytes lack cation transporters in the basolateral membrane allowing a flux out of the intercellular space (Proctor et al., 2016).

Given the assumption that OCT1 is not present in the apical membrane of the human enterocytes, which mechanisms may be involved in the intestinal uptake of cationic compounds? Beside mechanisms of paracellular transport as discussed elsewhere (Proctor et al., 2016), the intestinal brush border membrane also expresses several other transporters that have been shown to be involved in the uptake of cationic compounds such as the plasma membrane monoamine transporter (PMAT), the thiamine transporter 2 (THTR2), the choline transporter 1 (CHT1), the norepinephrine transporter 1 (NET1), the serotonin transporter (SERT), and the dopamine transporter 1 (DAT1).

For an unequivocal proof for the localization of intestinal OCT1, targeted proteomic analysis of apical and basolateral membrane fractions of the human intestinal mucosa and bidirectional transport studies of established non-metabolized OCT1 substrates across human intestinal tissue from a sufficient number of volunteers (e.g., carriers of SLC22A1 loss of function alleles vs. carriers of the wild-type or tissue from wild-type carriers in the absence and presence of OCT1 inhibitors) would be required.

## AUTHOR CONTRIBUTIONS

Conceptualization, investigation, writing—original draft preparation, and writing—review and editing, CW, MD, and SO All authors have read and agreed to the published version of the manuscript.

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REVIEW
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# Transport of Drugs and Endogenous Compounds Mediated by Human OCT1: Studies in Single- and Double-Transfected Cell Models

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Organic Cation Transporter 1 (OCT1, gene symbol: *SLC22A1*) is predominately expressed in human liver, localized in the basolateral membrane of hepatocytes and facilitates the uptake of endogenous compounds (e.g. serotonin, acetylcholine, thiamine), and widely prescribed drugs (e.g. metformin, fenoterol, morphine). Furthermore, exogenous compounds such as $MPP^+$, $ASP^+$ and Tetraethylammonium can be used as prototypic substrates to study the OCT1-mediated transport *in vitro*. Single-transfected cell lines recombinantly overexpressing OCT1 (e.g., HEK-OCT1) were established to study OCT1-mediated uptake and to evaluate transporter-mediated drug-drug interactions *in vitro*. Furthermore, double-transfected cell models simultaneously overexpressing basolaterally localized OCT1 together with an apically localized export protein have been established. Most of these cell models are based on polarized grown MDCK cells and can be used to analyze transcellular transport, mimicking the transport processes e.g. during the hepatobiliary elimination of drugs. Multidrug and toxin extrusion protein 1 (MATE1, gene symbol: *SLC47A1*) and the ATP-driven efflux pump P-glycoprotein (P-gp, gene symbol: *ABCB1*) are both expressed in the canalicular membrane of human hepatocytes and are described as transporters of organic cations. OCT1 and MATE1 have an overlapping substrate spectrum, indicating an important interplay of both transport proteins during the hepatobiliary elimination of drugs. Due to the important role of OCT1 for the transport of endogenous compounds and drugs, *in vitro* cell systems are important for the determination of the substrate spectrum of OCT1, the understanding of the molecular mechanisms of polarized transport, and the investigation of potential drug-drug interactions. Therefore, the aim of this review article is to summarize the current knowledge on cell systems recombinantly overexpressing human OCT1.

**Keywords: HEK 293, double-transfected cell line, single-transfected cell line, P-glycoprotein, MATE1, OCT1, SLC22A1 (OCT1), MDCK cell line**

**TABLE 1 |** Substrates of OCT1 (drugs, drug metabolites, endogenous molecules, chemicals) studied in single-transfected cell lines.

| Drug/Compound | Cell model | $K_m$ [μM] | Concentration* [μM] | Reference |
|---|---|---|---|---|
| 1-(2-phenoxyethyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(3-phenylpropyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(4-Phenyl-butyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(*m*-phenoxyphenyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(*p*-chlorophenethyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(*p*-chlorophenyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(*p*-methoxybenzyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-(p-methyl)-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 1-[p-(*p*-phenoxy)phenyl]-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| $^{131}$I-labeled *m*-iodobenzylguanidine | HEK293 | | 37 kBq | Kobayashi et al. (2020) |
| 1-methyl-4-phenylpyridinium ($MPP^+$) | *Xenopus* oocytes | 14.6 ± 4.39 | | Zhang et al. (1997) |
| 1-methyl-4-phenylpyridinium ($MPP^+$) | HEK293 | 32 | | Gründemann et al. (2003) |
| 1-methyl-4-phenylpyridinium ($MPP^+$) | HEK293 | 25.0 | | Umehara et al. (2007) |
| 1-n-pentylbiguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 2-(2,4-dichlorophenyl)ethyl-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 2-(4-biphenyl)ethyl-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 2,2-diphenylethyl-biguanide | HEK293 | 14 ± 2.8 | | Obianom et al. (2017) |
| 2,3-dihydro-1H-inden-2-yl-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| 2-ehylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) | HEK293 | | 1 | Campbell et al. (2015) |
| 3-methoxymorphinan | HEK293 | | 0.05–0.5 | Meyer et al. (2019) |
| 4-4-dimethylaminostyryl-N-methylpyridinium ($ASP^+$) | HEK293 | 2.32 ± 0.29 | | Ahlin et al. (2008) |
| 4-4-dimethylaminostyryl-N-methylpyridinium ($ASP^+$) | HEK293 | 21.2 | | Chen et al. (2017a) |
| 4H-1-benzopyran-4-one-biguanide | HEK293 | | 100 | Obianom et al. (2017) |
| Acebutol-(R) | HEK293 | 19.9 ± 5.7 | | Jensen et al. (2020b) |
| Acebutol-(S) | HEK293 | 21.0 ± 2.5 | | Jensen et al. (2020b) |
| Acetylcholine | *Xenopus* oocytes | | 5 | Lips et al. (2005) |
| Aciclovir | S2 | 151.2 ± 22.1 | | Takeda et al. (2002) |
| Aflatoxin B1 | S2 | | 0.1 | Tachampa et al. (2008) |
| Albuterol | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Amifampridine | HEK293 | 508.1 ± 247.3 | | Jensen et al. (2021) |
| Amiloride | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Amisulpride | HEK293 | 31.3 ± 5.4 | | Dos Santos Pereira et al. (2014) |
| Anisodine | HEK293 | | 1–5 | Chen et al. (2019) |
| AR-H067637 | HEK293 | 26 | | Matsson et al. (2013) |
| AR-H069927 | HEK293 | 116 | | Matsson et al. (2013) |
| Atenolol | MDCK | 3080 | | Mimura et al. (2015) |
| Atenolol racemate | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Atenolol-(R) | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Atenolol-(R) | HEK293 | 201.9 ± 33.1 | | Jensen et al. (2020b) |
| Atenolol-(S) | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Atenolol-(S) | HEK293 | 196.4 ± 23.1 | | Jensen et al. (2020b) |
| Atropine | HEK293 | 5.9 ± 1.4 | | Chen et al. (2017b) |
| Azidoprocainamide | *Xenopus* oocytes | 100.9 ± 43.0 | | van Montfoort et al. (2001) |
| Benzyltriethylammonium | HEK293 | 38.6 ± 9.9 | | Jensen et al. (2021) |
| Berberine | MDCK | 14.8 ± 3.3 | | Nies et al. (2008) |
| Berberrubine | MDCK | 1.27 ± 0.23 | | Li et al. (2016) |
| Bromosulfophthalein | HEK293 | 13.6 ± 2.6 | | Boxberger et al. (2018) |
| Butylscopolamine | HEK293 | 23.4 ± 2.3 | | Chen et al. (2017b) |
| Cimetidine | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| *cis*-Diammine (pyr-idine)chloroplatinum(II) (cDPCP) | MDCK | | 10 | Lovejoy et al. (2008) |
| Cisplatin | HEK293 | | 1000 | Yonezawa et al. (2006) |
| Clidinium | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Coptisine | MDCK | 5.80 ± 1.0 | | Li et al. (2016) |
| Cyclo(His-pro) | HEK293 | 655 ± 191 | | Taubert et al. (2007) |
| Cycloguanil | HEK293 | | 100 | van der Velden et al. (2017) |
| Cycloguanil | HEK293 | 18.3 | | Matthaei et al. (2019) |
| DAPI | MDCK | 8.94 ± 1.26 | | Yasujima et al. (2011) |
| Debrisoquine | HEK293 | | 1 | Seitz et al. (2015) |
| Debrisoquine | HEK293 | 5.9 ± 1.5 | | Saadatmand et al. (2012) |
| Debrisoquine | HEK293 | 24.2 ± 1.3 | | Neul et al. (2021) |
| Dehydrocordaline | MDCK | 11.29 ± 3.3 | | Chen et al. (2020) |
| Denatonium | HEK293 | 12.6 ± 1.0 | | Jensen et al. (2021) |
| Dextrorphan | HEK293 | | 0.05 | Meyer et al. (2019) |
| Dimethylphenylpiperazinium | HEK293 | 62.0 ± 23.3 | | Jensen et al. (2021) |

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**TABLE 1 |** (*Continued*) Substrates of OCT1 (drugs, drug metabolites, endogenous molecules, chemicals) studied in single-transfected cell lines.

| Drug/Compound | Cell model | $K_m$ [μM] | Concentration* [μM] | Reference |
|---|---|---|---|---|
| Dobutamine | HEK293 | 28.4 ± 16.8 | | Jensen et al. (2021) |
| Dopamine | HEK293 | | 100 | Boxberger et al. (2014) |
| Edrophonium | HEK293 | 26.4 ± 9.1 | | Jensen et al. (2021) |
| Epiberberine | MDCK | 4.37 ± 0.42 | | Li et al. (2016) |
| Ethambutol | HEK293 | 526 ± 15.6 | | Parvez et al. (2018) |
| Ethambutol | HEK293 | 686 | | te Brake et al. (2016) |
| Ethidium | CHO and HEK293 | 0.8 ± 0.2 | | Lee et al. (2009) |
| Etilefrine-(R) | HEK293 | 232.9 ± 29.8 | | Jensen et al. (2020b) |
| Etilefrine-(S) | HEK293 | 214.0 ± 24.9 | | Jensen et al. (2020b) |
| Famotidine | HEK293 | 35.7 ± 7.3 | | Jensen et al. (2021) |
| Fenoterol | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Fenoterol | HEK293 | 1.78 ± 0.16 | | Tzvetkov et al. (2018) |
| Fenoterol | HEK293 | 2.9 | | Morse et al. (2020) |
| Fenoterol-(R,R) | HEK293 | 1.7 ± 0.3 | | Jensen et al. (2020b) |
| Fenoterol-(S,S) | HEK293 | 0.8 ± 0.2 | | Jensen et al. (2020b) |
| Fenpiverinium | HEK293 | 8.6 ± 3.2 | | Jensen et al. (2021) |
| Formoterol | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Formoterol-(R,R) | HEK293 | 28.3 ± 6.2 | | Jensen et al. (2020b) |
| Formoterol-(S,S) | HEK293 | 19.1 ± 2.0 | | Jensen et al. (2020b) |
| Frovatriptan | HEK293 | 61.9 ± 10.3 | | Jensen et al. (2021) |
| Furaminidine | CHO | 6.1 ± 1.1 | | Ming et al. (2009) |
| Ganciclovir | S2 | 516.2 ± 70.3 | | Takeda et al. (2002) |
| Glycopyrrolate | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Guanfacine | HEK293 | 8.6 ± 6.1 | | Jensen et al. (2021) |
| Hydromorphone | HEK293 | 56.1 ± 19.1 | | Meyer et al. (2019) |
| Imeglimin | HEK293 | 1130 | | Chevalier et al. (2020) |
| Ipratropium | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Ipratropium | HEK293 | 13.6 ± 1.3 | | Chen et al. (2017b) |
| Jatrorrhizine | MDCK | 4.46 ± 0.4 | | Li et al. (2016) |
| Jatrorrhizine | HEK293 | 4.94 ± 0.55 | | Liang et al. (2020) |
| Ketamine | MDCK | 73.9 ± 15.2 | | Keiser et al. (2018) |
| Lamivudine | CHO | 1250 ± 100 | | Minuesa et al. (2009) |
| Lamivudine | HEK293 | 249 ± 51 | | Jung et al. (2008) |
| Lamivudine | HEK293 | 786 ± 84 | | Arimany-Nardi et al. (2016) |
| Lamotrigin | KCL22 | | 5 | Dickens et al. (2012) |
| Mepenzolate | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Meptazinol | HEK293 | | 0.1–0.5 | Meyer et al. (2019) |
| *meta*-iodobenzylguanidine (*m*IBG) | HEK293 | 15.9 ± 5.3 | | Jensen et al. (2021) |
| *meta*-iodobenzylguanidine (*m*IBG) | HEK293 | 19.5 ± 6.9 | | López Quiñones et al. (2020) |
| Metformin | HEK293 | 1470 ± 190 | | Kimura et al. (2005) |
| Metformin | CHO | 2160 ± 360 | | Nies et al. (2009) |
| Methylnaltrexone | HEK293 | 20.3 ± 5.6 | | Meyer et al. (2019) |
| Methylscopolamine | HEK293 | 23.4 ± 4.0 | | Jensen et al. (2021) |
| Milnacipran | HEK293 | 2.26 ± 1.43 | | Jensen et al. (2021) |
| Monocrotaline | HEK293 | | 1 | Seitz et al. (2015) |
| Monocrotaline | HEK293 | 109.1 ± 17.8 | | Chen et al. (2019) |
| Monocrotaline | MDCK | 25.0 ± 6.7 | | Tu et al. (2013) |
| Morphine | HEK293 | | 0.05–0.5 | Meyer et al. (2019) |
| Morphine | HEK293 | | 0.2 | Zhu et al. (2018) |
| Morphine | HEK293 | | 1 | Seitz et al. (2015) |
| Morphine | HEK293 | 3.4 ± 0.3 | | Tzvetkov et al. (2013) |
| $N^1$-methylnicotinamide | *Xenopus* oocytes | | 300 | Gorboulev et al. (1997) |
| Nadolol | HEK293 | | 1–1000 | Misaka et al. (2016) |
| Naratriptan | HEK293 | | 1000 | Matthaei et al. (2016) |
| N-ethyllidocaine | HEK293 | 51.4 ± 15.4 | | Jensen et al. (2021) |
| Nitidine | MDCK | 0.797 ± 0.17 | | Li et al. (2014) |
| Nizatidine | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| N-methyladenosine | HEK293 | | 100 | Miyake et al. (2019) |
| N-methylquinidine | *Xenopus* oocytes | 11.5 ± 2.1 | | van Montfoort et al. (2001) |
| N-methylquinine | *Xenopus* oocytes | 19.5 ± 7.3 | | van Montfoort et al. (2001) |
| Norfentanyl | HEK293 | 7.7 ± 0.8 | | Meyer et al. (2019) |
| Norlevorphanol | HEK293 | | 0.05–0.5 | Meyer et al. (2019) |
| Noroxycodone | HEK293 | 20.05 ± 6.5 | | Meyer et al. (2019) |
| Norphenylephrine | HEK293 | 994.1 ± 316.5 | | Jensen et al. (2021) |

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**TABLE 1 |** (*Continued*) Substrates of OCT1 (drugs, drug metabolites, endogenous molecules, chemicals) studied in single-transfected cell lines.

| Drug/Compound | Cell model | $K_m$ [μM] | Concentration* [μM] | Reference |
|---|---|---|---|---|
| Octopamine | HEK293 | 388.6 ± 246.4 | | Jensen et al. (2021) |
| O-desmethyl tramadol | HEK293 | | 1 | Tzvetkov et al. (2011) |
| Orciprenaline-(R) | HEK293 | 780.5 ± 285.9 | | Jensen et al. (2020b) |
| Orciprenaline-(S) | HEK293 | 808.8 ± 292.6 | | Jensen et al. (2020b) |
| Oxaliplatin | MDCK | | 10 | Lovejoy et al. (2008) |
| Oxaliplatin | HEK293 | | 1000 | Yonezawa et al. (2006) |
| Oxibutynin | HEK293 | 8.82 ± 0.44 | | Wenge et al. (2011) |
| Oxophenomium | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Oxymorphone | HEK293 | | 0.05 | Meyer et al. (2019) |
| p-(3-Aminoguanidino)-benzoic acid | HEK293 | | 100 | Obianom et al. (2017) |
| *para*-Aminosalicylic acid | HEK293 | 20.3 ± 4.6 | | Parvez et al. (2017) |
| *para*-Hydroxymethamphetamine | HEK293 | 14.5 ± 8.7 | | Wagner et al. (2017) |
| Pazopanib | HEK293 | 3.47 | | Ellawatty et al. (2018) |
| Pentamidine | CHO | 36.4 ± 8.3 | | Ming et al. (2009) |
| Phenformin | HEK293 | | 100 | Obianom et al. (2017) |
| Phenylephrine | HEK293 | 221.2 ± 60.3 | | Jensen et al. (2021) |
| Picoplatin | HEK293 | | 10 | More et al. (2010) |
| Pirbuterol-(R) | HEK293 | 75.3 ± 11.4 | | Jensen et al. (2020b) |
| Pirbuterol-(S) | HEK293 | 72.9 ± 12.3 | | Jensen et al. (2020b) |
| Prenalterol | HEK293 | 13.3 ± 3.4 | | Jensen et al. (2021) |
| Procainamide | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Procaterol | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Proguanil | HEK293 | 17.7 | | Matthaei et al. (2019) |
| Proguanil | HEK293 | 8.1 ± 1.6 | | van der Velden et al. (2017) |
| Prostaglandin $E_2$ | S2 | 0.66 | | Kimura et al. (2002) |
| Prostaglandin $F_{2\alpha}$ | S2 | 0.48 | | Kimura et al. (2002) |
| Prothionamide | HEK293 | 805.8 ± 23.4 | | Parvez et al. (2018) |
| Quercetin | HEK293 | 2.2 ± 0.2 | | Glaeser et al. (2014) |
| Ractopamine | HEK293 | 2.1 ± 0.76 | | Jensen et al. (2021) |
| Ranitidine | HEK293 | | 1 | Bi et al. (2019) |
| Ranitidine | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Ranitidine | HEK293 | 62.9 ± 4.32 | | Meyer et al. (2017) |
| Ranitidine | *Xenopus* oocytes | 70 ± 9 | | Bourdet et al. (2005) |
| Retrorsine | MDCK | | 1 | Tu et al. (2014) |
| Rhodamine 123 | HEK293 | 0.54 ± 0.21 | | Jouan et al. (2014) |
| Ritodrine | HEK293 | 1.67 ± 0.21 | | Jensen et al. (2021) |
| Rizatriptan | HEK293 | | 1000 | Matthaei et al. (2016) |
| Salbutamol | HEK293 | | 0.03–10 | Salomon et al. (2015) |
| Salbutamol-(R) | HEK293 | 224.2 ± 18.4 | | Jensen et al. (2020b) |
| Salbutamol-(S) | HEK293 | 222.5 ± 20.5 | | Jensen et al. (2020b) |
| Salsolinol | HEK293 | 440 ± 209 | | Taubert et al. (2007) |
| Saracatinib | HEK293 | | 10 | Harrach et al. (2017) |
| Sematilide | HEK293 | 102 ± 24.6 | | Jensen et al. (2021) |
| Serotonin | HEK293 | 197 ± 42 | | Boxberger et al. (2014) |
| Sorafenib | CHO | 3,8 | | Swift et al. (2013) |
| Sotalol | HEK293 | 195.9 ± 72.1 | | Jensen et al. (2021) |
| Sparteine | HEK293 | 27.2 ± 2.8 | | Neul et al. (2021) |
| Sulpiride | HEK293 | 259.7 ± 5.4 | | Dos Santos Pereira et al. (2014) |
| Sulpiride | HEK293 | 2.57 ± 0.64 | | Takano et al. (2017) |
| Sumatriptan | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Sumatriptan | HEK293 | 46 | | Morse et al. (2020) |
| Sumatriptan | HEK293 | 55.4 ± 7.8 | | Matthaei et al. (2016) |
| Terbutaline | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Tetraethylammonium (TEA) | *Xenopus* oocytes | | 100 | Zhang et al. (1997) |
| Tetraethylammonium (TEA) | HEK293 | 140 | | Hendrickx et al. (2013) |
| Tetraethylammonium (TEA) | HeLa | 164 ± 17.9 | | Bednarczyk et al. (2003) |
| Tetraethylammonium (TEA) | MDCK | 1750 ± 70 | | Yasujima et al. (2011) |
| Tetraethylammonium (TEA) | HeLa | 229 ± 78.4 | | Zhang et al. (1998) |
| Tetraethylammonium (TEA) | HEK293 | 69.2 | | Umehara et al. (2007) |
| Thiamine | HEK293 | | 1 | Bi et al. (2019) |
| Thiamine | HEK293 | | 0.025 | Liang et al. (2018) |
| Thiamine | HEK293 | 780 ± 64 | | Chen et al. (2014) |
| Thiamine | HEK293 | 1997 ± 174 | | Jensen et al. (2020a) |
| Tiotropium | HEK293 | | 2.5 | Hendrickx et al. (2013) |

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**TABLE 1 |** (*Continued*) Substrates of OCT1 (drugs, drug metabolites, endogenous molecules, chemicals) studied in single-transfected cell lines.

| Drug/Compound | Cell model | $K_m$ [μM] | Concentration* [μM] | Reference |
|---|---|---|---|---|
| Tributylmethylammonium | *Xenopus* oocytes | 53.0 ± 13.9 | | van Montfoort et al. (2001) |
| Trimethylamine N-oxide | HEK293 | 33900 ± 2700 | | Miyake et al. (2017) |
| Tropisetron | HEK293 | | 1 | Tzvetkov et al. (2012) |
| Tropisetron | HEK293 | | 1 | Seitz et al. (2015) |
| Trospium | HEK293 | 106 ± 16 | | Bexten et al. (2015) |
| Trospium | HEK293 | 15.1 ± 3.1 | | Chen et al. (2017b) |
| Trospium | MDCK | 22.0 ± 3.0 | | Deutsch et al. (2019) |
| Trospium | HEK293 | 17.0 ± 4.64 | | Wenge et al. (2011) |
| Tyramine | HEK293 | 94.7 ± 28.2 | | Seitz et al. (2015) |
| Xamoterol (R) | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| Xamoterol (S) | HEK293 | | 2.5 | Hendrickx et al. (2013) |
| YM155 | HEK293 | 22.1 ± 2.5 | | Minematsu et al. (2010) |
| YM155 | S2 | 38.7 | | Iwai et al. (2009) |
| Zalcitabine | HEK293 | 242 ± 56 | | Jung et al. (2008) |
| Zolmitriptan | HEK293 | | 1000 | Matthaei et al. (2016) |

*Concentration* = substance was tested using the stated concentration with an uptake rate ≥2-fold compared to the uptake into control cells.*

## INTRODUCTION

Transport proteins located in different membrane domains are important for the uptake, distribution and excretion of endogenous substances and drugs (International Transporter Consortium et al., 2010; König et al., 2013; Müller et al., 2018a; Koepsell, 2020). Whereas members of the SLC (Solute Carrier) transporter superfamily generally mediate the uptake of substances from the extracellular space into cells, members of the ABC (ATP-binding cassette) transporter superfamily are export proteins responsible for the energy-dependent export of substrates out of cells. SLC and ABC family members are important for the transport of a variety of approved drugs. Therefore, it is important to characterize drugs or drug metabolites as substrates or transport inhibitors. *In vitro* cell models are useful tools for this characterization. The importance of *in vitro* cell models is also highlighted by the fact that they are recommended as tools to study transporter-mediated drug interactions in the guideline/guidance of FDA Food and Drug Administration (2020) and EMA European Medicines Agency (2012).

This article focuses on transport data of the SLC22 family member OCT1 (gene symbol *SLC22A1*) generated by different *in vitro* cell models. OCT1 is predominantly expressed in liver and localized in the basolateral membrane of human hepatocytes (Gorboulev et al., 1997; Nies et al., 2008). It mediates the uptake of several endogenous and exogenous compounds and drugs (**Table 1**). Single-transfected cell models (e.g., HEK-OCT1 cells) recombinantly overexpressing OCT1 were established to study OCT1-mediated transport, to calculate transport parameters (e.g., $K_m$ values), to investigate the impact of genetic variations and to evaluate OCT1-mediated drug-drug interactions *in vitro* (**Figure 1A**; **Table 1**). Since OCT1 has an overlapping substrate spectrum with the apically localized export proteins MATE1 [gene symbol *SLC47A1* (Nies et al., 2011)] and P-glycoprotein [P-gp, MDR1; gene symbol *ABCB1* (Nies et al., 2008; Misaka et al., 2016)], double-transfected cell models have been established (MDCK-OCT1-MATE1 or MDCK-OCT1-P-gp) for investigating the vectorial transport mediated by both proteins (**Table 2**). MATE1 and P-glycoprotein are both localized in the apical (canalicular) membrane of human hepatocytes and responsible for the export of substances out of the cells into bile (Thiebaut et al., 1987; Otsuka et al., 2005). When expressed together with OCT1 in MDCK cells grown as a monolayer, OCT1 localizes in the basolateral and MATE1 or P-gp in the apical membrane (**Figure 1B**). In this experimental setup, substrates of OCT1 and MATE1/P-gp applied to the basolateral compartment will be first taken up into the cells mediated by OCT1 and subsequently exported via MATE1 or P-gp into the apical compartment (**Figure 1B**). Therefore, these cell models can be used to study not only OCT1-mediated uptake into the cells, but also the vectorial transport of substances from the basolateral into the apical compartment mimicking the transport processes during the hepatobiliary elimination e.g. of drugs (Taghikhani et al., 2017). Moreover, the importance of uptake and efflux transporters for perpetrator disposition can be assessed (Müller et al., 2018b). In this review, we summarize transport data related to the hepatocellular uptake transporter OCT1 obtained by studies in different cell models. Furthermore, the advantages and disadvantages of these cell models will be addressed.

## ORGANIC CATION TRANSPORTER 1 AND RELATED EXPORT PROTEINS

### Organic Cation Transporter 1

The rodent orthologue of human OCT1 (rOct1) was first isolated from a rat kidney library and expressed in *Xenopus* oocytes. This rOct1 transporter showed inhibitable and potential-dependent Tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium ($MPP^+$) uptake (Gründemann et al., 1994). Additionally, *in situ*

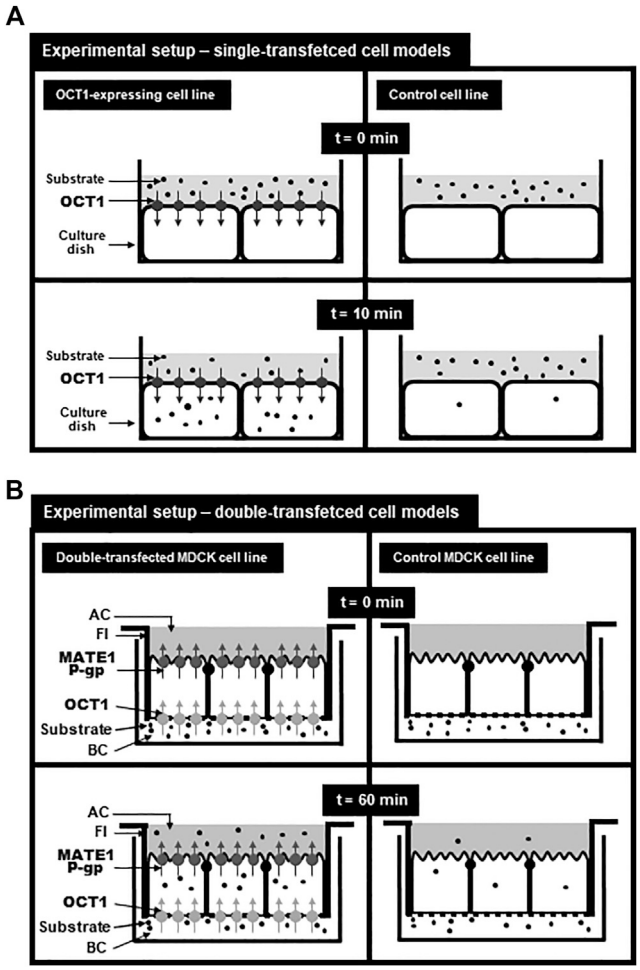


**FIGURE 1 |** Experimental setup for using single-transfected **(A)** and double-transfected **(B)** cell models modified from Taghikhani et al. (Taghikhani et al., 2017). **(A)**: Setup for analyzing the transport function of OCT1 in single-transfected cell lines. At time point 0 min, the donor solution containing the substrate is applied onto the cell layer and after 10 min, the uptake of the substrate into OCT1-expressing cells and into control cells can be determined. By subtracting the uptake into the control cell line from the uptake into the OCT1-expressing cell line, the so called net uptake can be calculated referring to the uptake mediated by recombinantly expressed OCT1. **(B)**: Setup for vectorial transport assays using double-transfected MDCK cell lines expressing OCT1 in the basolateral membrane and MATE1 or P-glycoprotein in the apical membrane. MDCK cells were cultured on filter inserts (FI) separating a basolateral (BC) from an apical (AC) compartment. The substrate was added to the basolateral compartment and after 60 min the substrate concentration in the cells (uptake) or in the apical compartment (vectorial transport) can be calculated and compared to the uptake or the vectorial transport of the control MDCK cell line. Net intracellular substrate concentrations reflects OCT1-mediated substrate uptake and the net substrate concentration in the apical compartment reflects the vectorial transport mediated by OCT1-mediated uptake and MATE1-or P-gp-mediated export.

hybridization and northern blotting analysis demonstrated Oct1 expression in rat hepatocytes and enterocytes. In 1997, human OCT1 (gene symbol: *SLC22A1*) was cloned and characterized by two independent working groups (Gorboulev et al., 1997; Zhang et al., 1997). Although Gorbulev et al. amplified hOCT1 using kidney cDNA, northern blot analysis demonstrated OCT1 expression mainly in the liver (Gorboulev et al., 1997), which was in line with the findings of Zhang et al. using liver cDNA (Zhang et al., 1997). Later, OCT1 was localized at the basolateral membrane of human hepatocytes (Nies et al., 2008). OCT1 facilitates the uptake of organic cations or weak bases (**Table 1**), which comprises approximately 40–67.5% of all drugs (Comer and Tam, 2001; Neuhoff et al., 2003; Manallack, 2007), into human hepatocytes. In the 2018 recommendations of the International Transporter Consortium (ITC), the investigation of OCT1-mediated transport during drug development was added, based on clinically important OCT1-mediated drug-drug interactions (Zamek-Gliszczynski et al., 2018a; Zamek-Gliszczynski et al., 2018b).

## Multidrug and Toxin Extrusion Protein 1

The existence of an organic cation-$H^+$ antiporter was already postulated back in 1985 by studying the transport of $N^1$-methylnicotinamide by the use of membrane vesicles, derived from the brush border membrane of rabbit kidney (Wright, 1985; Inui et al., 2000). The multidrug and toxic compound extrusion family (MATE) was first characterized in bacteria (Pallen, 1999) and Otsuka et al. (Otsuka et al., 2005) identified human and mouse orthologues of the bacterial MATE protein by genomic databank screening. The human MATE family consists of two members, the more widely expressed MATE1 protein and the kidney-specific member MATE2-K. The MATE1 protein is localized in the apical membrane of kidney proximal tubule epithelial cells and in the canalicular membrane of human hepatocytes (Otsuka et al., 2005; Masuda et al., 2006). MATE1 substrates are cations or have a positively charge at physiological pH (Nies et al., 2016). MATE proteins have a strong substrate overlap with the SLC22 family members OCT1, OCT2 and OCT3, indicating an interplay between these transporters in the hepatobiliary and renal elimination of drugs and endogenous compounds. The ITC recommends *in vitro* uptake studies using MATE-transfected cells, if the new molecular entity (NME) shows renal secretion as route of elimination or if the NME is an inhibitor of MATE1/2-K or OCT2 (Hillgren et al., 2013). So far, no criteria are defined for the evaluation of hepatic elimination of drugs mediated by MATE1. Detailed lists of substrates and inhibitors are available in several reviews (Terada and Inui, 2008; Damme et al., 2011; Nies et al., 2011; Motohashi and Inui, 2013; Nies et al., 2016; Koepsell, 2020).

## P-glycoprotein

P-glycoprotein (P-gp) is an ABC transporter and acts as an efflux pump for a variety of drugs such as digoxin, dabigatran etexilate and indinavir. P-gp is due to its ability of extruding drugs an limiting factor for drug bioavailability (Fromm, 2004). The substrate spectrum shows a strong overlap with the substrates of the Cytochrome P450 enzyme CYP3A4 and both proteins together protect the organism from xenobiotics (Kivistö et al., 2004; von Richter et al., 2004). P-gp is expressed in the apical membrane of several tissues such as small intestine, liver and kidney (Thiebaut et al., 1987). Additionally, P-gp plays an

**TABLE 2 |** OCT1 expressing, double-transfectant cell lines and investigated substrates and inhibitors.

| Cell system | Expressed proteins | Working group establishing these cells | References | Tested substrates | Inhibitors |
|---|---|---|---|---|---|
| MDCK | OCT1 and P-gp | Nies et al. | Nies et al. (2008) | Berberine, TEA, $MPP^+$ | LY335979 |
| | | König et al. | Misaka et al. (2016) | Berberine, nadolol | Zosuquidar |
| | OCT1 and MATE1 | Sato et al. | Sato et al. (2008) | TEA, $MPP^+$, metformin, cimetidine, creatinine, guanidine, procainamide, quinidine | $MPP^+$, levofloxacine |
| | | König et al. | König et al. (2011) | $MPP^+$, metformin | |
| | | | Reznicek et al. (2017) | Emtricitabine | Cimetidin, ritonavir |
| | | | Chen et al. (2017b) | Ipratropium | |
| | | | Deutsch et al. (2019) | Trospium | |
| | | | Ceckova et al. (2016) | Lamivudine | Mitoxantrone |
| | | | Ceckova et al. (2018) | $MPP^+$, lamivudine | Efavirenz |
| | | Li et al. | Li et al. (2016); Li et al. (2018) | Metformin | Nuciferine |
| | OCT1 and CYP3A4 | Tu et al. | Tu et al. (2014) | $ASP^+$, Retrorsine | TEA |
| HEK293 | OCT1 and MATE1 | van der Velden et al. | van der Velden et al. (2017) | Proguanil | |
| LLC-PK1 | OCT1 and P-gp | Iwai et al. | Iwai et al. (2011) | YM155, digoxin | YM155, digoxin, cyclosporin A |

important role at blood-tissue barriers such as the blood-brain barrier and placenta, protecting the central nervous system or the unborn child from drugs or other xenobiotics (Fromm, 2004). Furthermore, P-gp is overexpressed in several cancer tissues, leading to multidrug resistance (Gottesman et al., 2002; Leopoldo et al., 2019). Wang et al. (Wang et al., 2003) analyzed by structure activity relationship analysis (SAR) several substrates and inhibitors of P-gp. They postulated that a tertiary nitrogen atom could be beneficial for the binding to P-gp due to the stronger interaction of the formed cation with the binding sites of P-gp. These cationic properties of some P-gp substrates already indicate that there might be an interplay between the OCT1-mediated uptake and the P-gp-mediated efflux during hepatobiliary elimination. Based on the recommendations of the ITC and FDA (International Transporter Consortium et al., 2010; Food and Drug Administration, 2020), a NME should be tested as P-gp substrate using inside-out oriented membrane vesicles or by vectorial transport assays using polarized grown cell lines such as Caco-2 cells or cell lines (MDCK, LLC-$PK_1$) recombinantly overexpressing P-gp.

# CELL MODELS TO STUDY ORGANIC CATION TRANSPORTER 1 TRANSPORT FUNCTION

## Single-Transfected Cell Models for Investigating Organic Cation Transporter 1

Use of single-transfected cell models expressing the transporter of interest is often the first step to gain insights into the substrate spectrum. The transporter is either transiently or stably transfected into a suitable cell line. The most commonly used cell lines for uptake studies are Human Embryonic Kidney 293 cells (HEK293). HEK293 cells are easy to culture and have, due to their human origin, comparable posttranslational protein modification to human tissues (Hu et al., 2018). Additionally, after transfection HEK293 cells are capable of expressing a variety of different proteins (Thomas and Smart, 2005). To study transport proteins, uptake assays can be used to determine transport parameters ($K_m$ or $C_{max}$ values) of the selected substrate (**Figure 1A**) or to perform drug-interaction studies. One limitation of using HEK293 cells is the lack of polarized growth, which excludes them for the analysis of transcellular transport studies. Other frequently used cell lines for establishing single-transfected cell models with the expression of one transport protein are Madin-Darby Canine Kidney cells (MDCK), Chinese Hamster Ovary cells (CHO), *Drosophila* Schneider 2 cells (S2), HeLa cells and *Xenopus* oocytes. *Xenopus* oocytes are a robust cell model, which is derived from *Xenopus laevis* (Zeng et al., 2020). The exogenous mRNA encoding the transport protein of interest is injected into oocytes leading to a functional expression of the protein. However, because of their limited longevity *Xenopus* oocytes cannot be used to generate stable transfectants.

Pioneering work on the characterization of OCT1 was done by Zhang et al. (1997). They were the first to clone OCT1 from human liver and they used *Xenopus* oocytes to analyze OCT1-mediated transport. They calculated the first transport $K_m$ and $V_{max}$ parameters for the uptake of the organic cation $MPP^+$ and measured the $IC_{50}$ values for the inhibition of OCT1-mediated transport of $MPP^+$ by the cations decynium-22, vecuronium and TEA (Zhang et al., 1997). Furthermore, they extended their research by using transiently transfected HeLa cells and characterized the transport of TEA and obtained $IC_{50}$ values for 15 different compounds (Zhang et al., 1998). The first inhibitor analysis using a wide range of compounds was done by Bednarczyk et al. (Bednarczyk et al., 2003). They used OCT1-

transfected HeLa cells and calculated $IC_{50}$ values of 30 structurally diverse organic cations and established a model of inhibitor/OCT1 interaction (Bednarczyk et al., 2003). These findings of structural requirements for OCT1 inhibition were extended by Ahlin and coworkers and their analysis of the inhibitory effect of 191 compounds on the OCT1-mediated uptake of $ASP^+$ (Ahlin et al., 2008). $ASP^+$ [4-(4-(dimethylamino)styryl)-N-methylpyridinium] is a fluorescent cationic model substrate for OCT1, which enables the fast screening of drugs as inhibitors of OCT1-mediated transport by analyzing fluorescence uptake. They identified 62 of the investigated compounds as inhibitors (cutoff value ≥50% inhibition) of which 66% were cations, 32% were neutral and repaglinide was the only anionic compound. Therefore, they estimated that high lipophilicity and a cationic character are the two main physicochemical properties of potent OCT1 inhibitors (Ahlin et al., 2008). A detailed analysis of the 'structure-transport relationship' was missing until Hendrickx et al. analyzed the uptake of 354 (with 83 marketed drugs) compounds into stably transfected HEK293 cells expressing OCT1 using a LC-MS/MS approach (Hendrickx et al., 2013). TEA and ipratropium served as reference compounds. In this study, the molecular volume of a compound was identified as the best descriptor for OCT1 substrates and lipophilicity was identified to be not important (Hendrickx et al., 2013). Recent publications emphasized the use of *in silico* predictions and machine learning approaches for the identification of new OCT1 substrates and their molecular characteristics (Baidya et al., 2020; Jensen et al., 2021). The OCT1 substrate and/or inhibitor spectrum has intensively been studied by various groups [e.g., (Gorboulev et al., 1997; Ciarimboli et al., 2005; Wenge et al., 2011; Tzvetkov et al., 2013; Knop et al., 2015; Otter et al., 2017; Meyer et al., 2019; Jensen et al., 2020b; Koepsell, 2020)].

Single-transfected cell models have also been extensively used to study the influence of genetic polymorphisms in the *SLC22A1* gene on kinetic parameters of the OCT1-mediated transport (Kerb et al., 2002; Shu et al., 2003; Tzvetkov et al., 2011; Tzvetkov et al., 2013; Dos Santos Pereira et al., 2014; Matthaei et al., 2016; Meyer et al., 2017; Jensen et al., 2020b). A detailed list about the *in vitro* analyzed effects of genetic polymorphisms in the *SLC22A1* gene has been published by Koepsell (2020). Furthermore, comparisons of human OCT1 with the orthologues of rat or mouse Oct1 has been performed using single-transfected cell models to gain insights into our understanding of potential substrate binding sites or protein regions involved in substrate recognition (Egenberger et al., 2012; Floerl et al., 2020; Koepsell, 2020; Meyer et al., 2020).

**Table 1** summarizes currently known OCT1 substrates. We included all data where a $K_m$-value was determined or where the uptake was ≥2-fold higher in the OCT1-expressing cells compared to the uptake into the respective control cell line. Potential substrates with uptake ratios between 1.5 and 2 are shown in **Supplementary Table S1**, together with publications that were not able to reproduce uptake experiments with controversial substrates (e.g., imatinib). OCT1 inhibitors are shown in **Supplementary Table S2**. We also included inhibition experiments, where no $IC_{50}$ values were calculated, if the inhibitor was able to reduce the uptake of the substrate to ≤50%. Nevertheless, these lists are not exhaustive.

## Double-Transfected Cell Lines

In contrast to HEK293 cells, MDCK cells form confluent monolayers when seeded on permeable membranes, such as microplate thinserts, separating a basolateral from an apical compartment (**Figure 1B**). These cells can be transfected with two cDNAs, for example one cDNA encoding for a basolaterally localized uptake transporter and one cDNA for an apically localized export protein. This allows a more versatile experimental setup, because these culture conditions enable transcellular transport measurements in combination with the measurement of the intracellular accumulation of the substrates. Furthermore, substrates can be applied either to the basolateral or apical compartment mimicking both routes of substrate transport, the route of excretion with the uptake of substrates from blood across the basolateral membrane and the export across the apical membrane into bile or urine (basal to apical transport) or the route of reuptake of substances across the apical membrane and the export into the blood (apical to basolateral transport e.g., during renal reabsorption). Limitations of this cell line are the expression of endogenous canine transporters such as canine Mdr1, Mrp2 and Oct2, which may affect the transport studies. Additionally, it is absolutely necessary to investigate the tightness of the cell monolayer to avoid paracellular transport of substances (Volpe, 2011).

The first double-transfected MDCK cell line expressing human OCT1 as uptake transporter together with P-gp in the apical membrane was established by Nies et al. [MDCK-OCT1-P-gp, **Table 2** (Nies et al., 2008)]. The protein expression was investigated by immunoblot and immunofluorescence analysis and for the functional testing, TEA and $MPP^+$ served as prototypic substrates for OCT1. Subsequent to the identification of berberine, a quaternary isoquinoline alkaloid, as an OCT1 and OCT2 substrate, the authors used the MDCK-OCT1-P-gp cell line to analyze the transcellular transport of this substance. The transport of berberine from the basal to the apical compartment was 3-fold, 5-fold and 1-fold higher in MDCK-OCT1-P-gp cells compared to the vectorial transport measured with MDCK-OCT1 and MDCK-P-gp single-transfected cells and MCDK control cells, respectively. Furthermore, the addition of the P-gp inhibitor LY335979 resulted in a decrease of the transcellular transport to the level measured in MCDK control cells. Even though the transcellular transport could be inhibited, an increase of the intracellular berberine amount was observed in MDCK-OCT-P-gp cells, indicating that LY335979 specifically inhibits the P-gp mediated export. Misaka et al. also established a MDCK-OCT1-P-gp double-transfectant and this cell line also showed a significant basal to apical transcellular transport of berberine, which could not be measured in the respective single-transfectants (Misaka et al., 2016). They also investigated the transcellular transport of nadolol (10 μM) with and without the addition of 1 μM zosuquidar, a known P-gp inhibitor, demonstrating that zosuquidar was able to significantly inhibit the basal to apical transport of nadolol (Misaka et al., 2016).

Sato et al. (Sato et al., 2008) established an OCT1-MATE1 double-transfected MDCK cell line and investigated the expression and localization by immunofluorescence microscopy. They used TEA as prototypic substrate and measured the transcellular transport from the basolateral to apical (b→a) and from the apical to basolateral (a→b) compartment demonstrating that the cellular accumulation was 66-fold higher, when TEA was applied to the basolateral compartment. Additionally, they were able to reproduce the pH-dependency of MATE1-mediated transport by varying the apical pH and demonstrated that the transcellular transport showed maximal transport rates at extracellular pH 6.5. The addition of 10 mM $MPP^+$ or 1 mM levofloxacin significantly decreased the basolateral to apical transport of TEA. To further analyze the transport of organic cations, Sato and coworkers measured the transcellular transport and cellular accumulation of $MPP^+$, metfomin, cimetidine, creatinine, guanidine, procainamide and quinidine and found significant vectorial transport rates for all substances, applied to the basolateral compartment. Unfortunately, they did not show a comparison between transcellular transport rates and the cellular uptake of substances into the MDCK-OCT1-MATE1 double-transfectant and into the corresponding single-transfectants (MDCK-OCT1 or MDCK-MATE1). The importance of the interplay of OCT1 and MATE1, studied in double-transfected cell lines could also be demonstrated by Sato et al. (Sato et al., 2008). Experiments using HEK293 cells transfected with OCT1 only showed slightly higher uptake rates of quinidine and procainamide (<2 fold) and the HEK-MATE1 cell line showed small uptake rates for quinidine (<2 fold) compared to the uptake into the vector control cell lines. This is contradictory to *in vivo* data that had already shown that quinidine (Notterman et al., 1986) and procainamide (Somogyi et al., 1983) are secreted renally. This underestimation of the role of OCT1 and MATE1 for the transport of both substrates was abolished by the use of double-transfected cell lines where significant transcellular transport rates could be measured for procainamide as well as for quinidine (Sato et al., 2008).

Our working group extended the investigations of Sato et al. by also establishing a MDCK-OCT1-MATE1 double-transfectant (König et al., 2011). The corresponding single-transfected cell lines (MDCK-OCT1 and MDCK-MATE1) were also used for transport assays. The cellular accumulation of $MPP^+$ (10 and 50 μM) and metformin (10 and 50 μM) was highest in MDCK-OCT1 single-transfected cells. Interestingly, the lowest intracellular accumulation was measured in the MDCK-MATE1 single-transfected cells and not in the MDCK control cells. This can be explained by MATE1-mediated efflux of $MPP^+$ or metformin taken up by an endogenous transporter or diffused passively into the cells when applied to the basolateral compartment. Intracellular accumulation in the MDCK-OCT1-MATE1 double-tranfected cell line was also significantly higher compared to the accumulation in the MDCK control cell line demonstrating OCT1-mediated uptake. As expected, there was no significant difference in the transcellular transport of the single-transfected cell lines and the MDCK control cells. In contrast, the MDCK-OCT1-MATE1 double-transfectant showed significantly higher transcellular transport rates for both substrates (10-fold basal to apical over apical to basal transcellular transport of metformin after 60 min). In the following years, several publications used double-transfected OCT1-MATE1 cell models to gain more insights into vectorial transport of organic cations. Reznicek et al. (Reznicek et al., 2017) used emtricitabine as substrate for vectorial transport studies and demonstrated that the transcellular transport is independent of OCT1-mediated uptake. This transport was saturable at very high concentrations (1 mM), temperature- and pH-dependent (decreasing the apical pH significantly increased the b→a transcellular transport). Furthermore, the addition of cimetidine and ritonavir, both known MATE1 inhibitors, resulted in an inhibition of the transcellular transport of emtricitabine by 43 and 35% in the double-transfectant, whereas the intracellular accumulation increased to 143 and 135%, respectively.

Chen et al. (Chen et al., 2017b) demonstrated that the basal to apical transcellular transport of ipratropium (0.5 μM) was 9.9-fold higher in MDCK-OCT1-MATE1 double-transfected cells compared to control cells and Deutsch and colleagues (Deutsch et al., 2019) identified trospium as substrate for both transporters using the same transporter combination. The vectorial basal to apical transport of trospium (1 μM) was 24.5-fold higher compared to the vectorial transport in the control cell line. As expected, the transcellular transport was highest at extracellular pH 6.5, whereas intracellular accumulation was lowest at this pH, demonstrating that OCT1 and MATE1 play an important role in the transcellular transport of trospium.

Ceckova et al. (Ceckova et al., 2016) analyzed the transcellular transport and intracellular accumulation of lamivudine in MDCK-OCT1-MATE1 double-transfected cells and their respective control and single-transfected cell lines. The transcellular transport (b→a) measured in the MDCK-MATE1 and MDCK-OCT1-MATE1 cells was significantly higher in comparison to the MDCK control cells and to the MDCK-OCT1 single-transfectant, whereas the intracellular accumulation of lamivudine was the highest in the MDCK-OCT1 cell line. This transcellular transport could be inhibited by the simultaneous application of lamivudine and mitoxantrone (2 μM) to the basolateral compartment and was reduced to a level which was not significantly different to the MDCK control cells. The fact, that mitoxantrone inhibition led to an increase of the intracellular accumulation of lamivudine, underlines the importance of MATE1 on the transport of lamivudine. Later, Ceckova et al. (Ceckova et al., 2018) used the MDCK-OCT1-MATE1 double-transfectant to study the inhibition of the transcellular transport of 2 nM $MPP^+$ and 10 nM lamivudine by adding efavirenz. In both cases, the presence of 10 μM efavirenz in the basolateral compartment reduces the basolateral to apical transport in all single- and double-transfected cell lines, except in the MDCK control cells. The intracellular accumulation of both substrates was decreased in the MDCK-OCT1 cells but increased in the MDCK-MATE1 cells, confirming the potential of efavirenz as an *in vitro* inhibitor of both transport proteins (Ceckova et al., 2018). Li et al. (Li et al., 2018) addressed a potential drug-drug interaction between

metformin and nuciferine, the active ingredient of lotus leafs (*Folium Nelumbinis*). This herbal drug is used as tea or food supplement for the elderly population suffering from hyperlipidemia and therefore a concomitant use of these herbs with antidiabetic drugs seems quite likely. After the evaluation of nuciferine inhibition (0.01–100 μM) on the OCT1-and MATE1-mediated uptake of metformin (10 μM) in single-transfected cells, they verified these findings by measuring the intracellular accumulation and transcellular transport of 10 μM metformin alone and in the presence of nuciferine (5–80 μM) in the double-transfected cell line. At all investigated time points the basolateral to apical transport of metformin was significantly higher in the MDCK-OCT1-MATE1 double-transfectant, compared to the transport in the MDCK-OCT1 single-transfectant. This transport could be inhibited by adding nuciferine in a concentration-dependent manner. Furthermore, nuciferine also reduced the intracellular accumulation of metformin. In contrast, transcellular transport from the apical to the basolateral compartment was unaltered by the addition of nuciferine. This demonstrates that nuciferine is an inhibitor of both OCT1 and MATE1. Remarkably, when applying the same experimental setup to the MDCK-OCT2-MATE1 double-tranfectant, the transcellular transport of metformin was also decreased but the intracellular accumulation of metformin significantly increased in a concentration-dependent manner after addition of nuciferine. This indicates, that the inhibition of MATE1 is responsible for this effect and nuciferine inhibits OCT1, but not OCT2 (Li et al., 2018).

In an interesting experimental setup van der Velden et al. (van der Velden et al., 2017) were not using MDCK cells to establish double-tranfectants. Instead, they used single-transfected HEK293 cells expressing OCT1 and cotransfected them with MATE1 or with MATE2-K and analyzed proguanil uptake. Because of the lack of polarized growth, vectorial transport studies cannot be performed with the double-transfected HEK293 cells. There was no significant difference in the uptake rate of HEK-OCT1 cells compared to HEK-OCT1-MATE1 cells, but the HEK-OCT1-MATE2-K cells showed a significant lower intracellular accumulation of proguanil, indicating an interplay between OCT1-mediated uptake and MATE2-K-mediated export (van der Velden et al., 2017).

Double-transfected cell models cannot only be used to study the interplay of uptake and efflux transporters, but also to investigate the interplay between transport proteins and metabolizing enzymes. To investigate this, Tu et al. established a double-transfected MDCK cell line, expressing OCT1 together with the phase I drug metabolizing enzyme CYP3A4 (Tu et al., 2014). This CYP enzyme is responsible for the metabolism of approx. 50% of all marketed drugs (Zhou, 2008). They validated the mRNA expression by RT-qPCR and confirmed the OCT1-mediated uptake by using the prototypical substrate $ASP^+$ with or without the presence of TEA as transport inhibitor. The MDCK-OCT1 single-transfectant as well as the MDCK-OCT1-CYP3A4 double-transfectant showed significantly higher $ASP^+$ uptake rates compared to the control cell line, which was strongly reduced by the addition of TEA. The CYP3A4 function in the MDCK-OCT1-CYP3A4 cells was confirmed by a CYP3A4 metabolism activity assay and was comparable to the values determined in MDCK-CYP3A4 single-transfected cells. Subsequently, they tested the cytotoxic activity of retrorsine, a hepatotoxic pyrrolizidine alkaloid, using all established MDCK cell lines. Prior experiments showed that the uptake of retrorsine is significantly higher in MDCK-OCT1 cells compared to the uptake into the MDCK control cells. Furthermore, Fu et al. demonstrated that pyrrolizidine alkaloids exhibit cytotoxicity only after bioactivation, which is mainly mediated by CYP3A4 (Fu et al., 2004). In line with these findings, the cytotoxicity of retrorsine was highest in the MDCK-OCT1-CYP3A4 cell line because of both uptake and bioactivation. There was no difference in the cytotoxicity between control cells and MDCK-OCT1 cells, due to the missing CYP-mediated activation. The MDCK-CYP3A4 single-transfectant also exhibit significantly higher retrorsine sensitivity, but still significantly lower compared to the double-transfectant (Tu et al., 2014).

Instead of MDCK cells, Iwai et al. used Lilly Laboratory Cancer Porcine Kidney 1 cells (LLC-PK1) to establish an OCT1-P-gp double-transfected cell line (Iwai et al., 2011). LLC-PK1 cells form tight monolayers and LLC-P-gp cells are recommended by the FDA as bidirectional transcellular transport system for identifying P-gp substrates and inhibitors Food and Drug Administration (2020). OCT1 function in these double-transfected cells was confirmed by using $MPP^+$ as prototypical substrate and the transport function of P-gp was verified by using digoxin as substrate. The basal to apical transcellular transport of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1*H*-naphtho [2,3-d]imidazolium bromide (YM155, 1 μM), a survivin suppressant and known substrate of OCT1 (Iwai et al., 2009), was much higher in the LLC-OCT1-P-gp double-transfectant compared to LLC-control, LLC-OCT1 and LLC-P-gp single-tranfected cell lines, demonstrated by the high basal to apical flux ratio of 16.6. This transcellular transport decreased by adding cyclosporine A or 1 mM $MPP^+$, respectively, indicating that YM155 is a substrate of both OCT1 and P-gp. The relatively high basal to apical transcellular transport of 1 μM digoxin was unaffected by the addition of 100 μM YM155 but was reduced to the level of the apical to basal transport by adding 10 μM cyclosporine A, demonstrating that YM155 has a low inhibitory effect on P-gp-mediated transport even at higher concentrations. **Table 2** gives an overview about the studies using OCT1 expressing double-transfected cell lines.

## DISCUSSION

*In vitro* cell models expressing transport proteins are useful tools for studies of transporter function and for the identification of transporter substrates and/or inhibitors. Therefore, the FDA and EMA recommend the usage of such cell lines during preclinical drug development. The FDA considers an investigational drug as an *in vitro* substrate for hepatic or renal transporters, 'if uptake is ≥ 2-fold of the drug uptake in empty vector-transfected cells and if a known inhibitor can decrease the drug uptake to ≤50% at a concentration at least 10 times that of the $K_i$ or $IC_{50}$'. To test whether a drug is an inhibitor it is recommended to 'determine

the inhibition potency ($K_i$ or $IC_{50}$) of the drug on the uptake of a known substrate' Food and Drug Administration (2020). In this review we describe cell models for the investigation of the SLC22 family member OCT1. Using single-transfected cell lines expressing OCT1, several drugs could be identified as substrates and inhibitors of this transporter (**Table 1**; **Supplementary Table S2**). Interestingly, it has been demonstrated that OCT1 transport inhibition is substrate-dependent. For example, Boxberger et al. detected substrate-dependent inhibition for several drug (e.g., ranitidine and fluoxetine) by using $MPP^+$, serotonin and TEA as probe substrates in competitive counterflow experiments (Boxberger et al., 2018). Therefore, the use of multiple probe substrates for *in vitro* testings of OCT1 seems reasonable and the use of substrates for the inhibition analysis *in vitro* that can also be used in the subsequent clinical studies as recommended by the FDA Food and Drug Administration (2020).

Despite the frequent use of single- and double-transfected cell lines, *in vitro-in vivo* extrapolations (IVIVE) have still limitations. Many drugs listed in **Supplementary Table S2** only inhibit the transport of substrates at concentrations above their therapeutic plasma concentration or environmentally exposed concentration so that the inhibitory potential is more theoretically relevant (Chedik et al., 2019). *In vitro* studies that analyzed opioids as inhibitors of OCT1, Meyer et al. showed that the calculated maximal unbound plasma concentrations for most of the tested opioids are lower than the obtained $IC_{50}$ values for OCT1 mediated transport (Meyer et al., 2019). Only the maximal portal vein concentration of tapentadol was comparable to the obtained $IC_{50}$ value, indicating a potential drug-drug interaction *in vivo* (Meyer et al., 2019). Furthermore, the influence of endogenous expression of transport proteins in the different cell lines, the use of different cell models (e.g., **Table 1**: $K_m$ TEA determined in MDCK cells, HEK293 cells and HeLa cells) and the independent establishment of several stable transfectants by different working groups lead to interlaboratory variability in the gained $K_m$ and $IC_{50}$ values and to a limited IVIVE. The use of primary human hepatocytes after the *in vitro* validation of drugs as substrates or inhibitors of OCT1, as recommended by Bi et al., could be helpful to gain better predictions of the hepatic clearance or to identify potential DDIs and could help to evaluate the contribution of the OCT1-mediated transport of potential substrates by using selective inhibitors (Bi et al., 2019; Jensen et al., 2020a). Interestingly, strong variations in the uptake of OCT1 substrates ($MPP^+$ and $ASP^+$) were detected comparing human hepatocytes from different donors (De Bruyn et al., 2011; Fattah et al., 2017) and the genetic characterization revealed strong genetic variabilities between the tested batches, where 13 of 27 tested hepatocyte batches showed at least 1 nonfunctional allele of the *SLC22A1* gene (Fattah et al., 2017).

The identification of OCT1 as rate-limiting transporter in the hepatic uptake of clinical important drugs together with *in vivo* data on reported genetic effects led to the update of the ITC recommendations, where OCT1 is now mentioned as transporter of emerging clinical importance (Zamek-Gliszczynski et al., 2018b).

Double-transfected cell lines could lead to an even better understanding of vectorial transport processes during hepatobiliary and renal elimination. They allow the simultaneous measurement of more parameters and are helpful to identify the individual transport protein underlying clinically observed drug-drug interactions and to study the impact of the respective transporters on perpetrator disposition (Müller et al., 2018b). Important double-transfected cell models for investigating the role of OCT1 in the hepatobiliary elimination of drugs are MDCK-OCT1-MATE1 cells expressing OCT1 together with the apically localized export protein MATE1. Both proteins share an overlapping substrate spectrum (Nies et al., 2011) and the vectorial transport of drugs mediated by both transporters has been described (**Table 2**). Interestingly, only by using double-transfected cell models the direction of the MATE1-mediated transport in the double-transfected cell lines resembles the physiological direction (efflux of substrates into the apical compartment), whereas the use of MATE1-transfected HEK293 cells only allows uptake measurements into the cell. In the recent years, several working groups established double-transfected cell lines to analyze the molecular mechanisms underlying polarized transport of endogenous compounds and drugs. Moreover, they are very useful tools for the understanding of the molecular mechanisms underlying clinically relevant drug-drug interactions (**Table 2**).

## AUTHOR CONTRIBUTIONS

BH reviewed the literature, BH and JK drafted the manuscript, MFF revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.662535/full#supplementary-material

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# Effects of a Common Eight Base Pairs Duplication at the Exon 7-Intron 7 Junction on Splicing, Expression, and Function of OCT1

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Organic cation transporter 1 (OCT1, SLC22A1) is localized in the sinusoidal membrane of human hepatocytes and mediates hepatic uptake of weakly basic or cationic drugs and endogenous compounds. Common amino acid substitutions in OCT1 were associated with altered pharmacokinetics and efficacy of drugs like sumatriptan and fenoterol. Recently, the common splice variant rs35854239 has also been suggested to affect OCT1 function. rs35854239 represents an 8 bp duplication of the donor splice site at the exon 7-intron 7 junction. Here we quantified the extent to which this duplication affects OCT1 splicing and, as a consequence, the expression and the function of OCT1. We used pyrosequencing and deep RNA-sequencing to quantify the effect of rs35854239 on splicing after minigene expression of this variant in HepG2 and Huh7 cells and directly in human liver samples. Further, we analyzed the effects of rs35854239 on OCT1 mRNA expression in total, localization and activity of the resulting OCT1 protein, and on the pharmacokinetics of sumatriptan and fenoterol. The 8 bp duplication caused alternative splicing in 38% (deep RNA-sequencing) to 52% (pyrosequencing) of the minigene transcripts when analyzed in HepG2 and Huh7 cells. The alternatively spliced transcript encodes for a truncated protein that after transient transfection in HEK293 cells was not localized in the plasma membrane and was not able to transport the OCT1 model substrate $ASP^+$. In human liver, however, the alternatively spliced OCT1 transcript was detectable only at very low levels (0.3% in heterozygous and 0.6% in homozygous carriers of the 8 bp duplication, deep RNA-sequencing). The 8 bp duplication was associated with a significant reduction of OCT1 expression in the human liver, but explained only 9% of the general variability in OCT1 expression and was not associated with significant changes in the pharmacokinetics of sumatriptan and

fenoterol. Therefore, the rs35854239 variant only partially changes splicing, causing moderate changes in OCT1 expression and may be of only limited therapeutic relevance.

**Keywords: ins/del variant, organic cation transporter 1, SLC22A1, minigene, allelic expression imbalance (AEI), fenoterol, sumatriptan, pharmacokinetics**

## INTRODUCTION

OCT1 (SLC22A1) is by far the most strongly expressed transporter of organic cations in the sinusoidal membrane of the human liver (Nies et al., 2009; Wang et al., 2015; Drozdzik et al., 2019). OCT1 mediates the first step of hepatic clearance of weakly basic or positively charged drugs. Metformin, morphine, sumatriptan or fenoterol and endogenous compounds like thiamine belong to substrates transported by OCT1 (Wang et al., 2002; Tzvetkov et al., 2013; Chen et al., 2014; Matthaei et al., 2016; Tzvetkov et al., 2018). A loss of OCT1 function was shown to increase plasma concentrations of several drugs including sumatriptan and fenoterol (Kerb et al., 2002; Shu et al., 2007; Tzvetkov et al., 2013; Arimany-Nardi et al., 2016; Matthaei et al., 2016; Tzvetkov et al., 2018). Depending on administered drugs, an increase may bear the risk of toxic side effects and may affect drug efficacy.

OCT1 is encoded by the SLC22A1 gene, which is located on the long arm of human chromosome 6 (6q26) and contains 11 exons and 10 introns (Koehler et al., 1997; Zhang et al., 1997). The resulting OCT1 protein has 554 amino acids and is composed of 12 transmembrane helices (TMHs) with intracellularly localized N- and C-termini.

The SLC22A1 gene shows the highest genetic variability within the pharmacologically relevant members of the SLC22 family (Tzvetkov et al., 2016; Schaller and Lauschke, 2019). Fourteen single nucleotide polymorphisms (SNPs) result in amino acid substitutions. Thereof, four common amino acid substitutions (Arg61Cys, Cys88Arg, Gly401Ser, and Gly465Arg) and a deletion of Met420 are known to confer strongly reduced or completely abolished OCT1 activity (Kerb et al., 2002; Shu et al., 2003; Shu et al., 2008; Seitz et al., 2015). Nine percent of Europeans and White Americans are homozygous or compound heterozygous carriers of these reduce function variants (Seitz et al., 2015). These individuals (also referred to as poor OCT1 transporters) have significantly altered pharmacokinetics resulting in altered efficacy and toxicity of clinically relevant drugs like sumatriptan, fenoterol, tramadol and morphine (Shu et al., 2008; Becker et al., 2011; Tzvetkov et al., 2011; Tzvetkov et al., 2012; Fukuda et al., 2013; Tzvetkov et al., 2013; Stamer et al., 2016).

Non-coding variants may also affect OCT1 activity, e.g. by altering OCT1 expression. Indeed, SLC22A1 expression varies strongly between individuals (Nies et al., 2009; O'Brien et al., 2013). The OCT1 mRNA levels differ up to 113-fold and protein levels up to 83-fold between individuals (Nies et al., 2009). However, common promoter variants did not significantly affect the SLC22A1 promoter activity or mRNA expression (Bokelmann et al., 2018).

Another explanation of the high variability in OCT1 expression may be related to genetic variants that cause alternative splicing. Indeed, an 8 base pairs insertion/deletion variant rs35854239 (formerly also designated as rs113569197 or rs36056065) was suggested to affect OCT1 expression and activity by altering splicing (Tarasova et al., 2012; Grinfeld et al., 2013; Kim et al., 2017). This variant is located at the junction of exon 7 and intron 7 of the SLC22A1 gene and represents an 8 bp duplication of the 5' part of intron 7 including the splice donor site (**Figure 1A**). The newly generated donor site results in an 8 bp longer transcript with shift in the open reading frame and a premature stop-codon.

The rs35854239 variant is genetically highly linked to the coding variant Met408Val ($r^2$ of 0.95). In several studies, Met408Val was associated with drug efficacy. This association has been explained by decreasing cellular uptake and thus altering the systemic concentrations or concentrations at the site of action of the drug. However, multiple independent *in vitro* studies demonstrated that the Met408Val substitution does not directly affect OCT1 uptake (Kerb et al., 2002; Shu et al., 2003; Shu et al., 2007; Nies et al., 2014; Tzvetkov et al., 2014; Seitz et al., 2015). Thus, the rs35854239 variant may be the true cause for the observed associations of Met408Val with clinically relevant phenotypes.

The rs35854239 variant is very common. If functional, with its minor allele frequency of 40.6% in Europeans and White Americans, the rs35854239 variant could be the most frequent variant affecting OCT1 expression and activity. However, it is not clear whether the duplicated splicing donor site always leads to alternative splicing, and to what extend the alternatively spliced transcript is functionally active.

In this study, we analyzed to what extend the SLC22A1 8 bp duplication (rs35854239) affects splicing and what are the consequences of the variable splicing on the transporter function *in vitro* and *in vivo*. To this end, first, we quantified the alternatively spliced transcripts both using the minigene assay and direct analyses of human liver samples. Second, we analyzed whether the protein resulting from the alternatively spliced transcripts is active. Finally, we analyzed whether the rs35854239 variant is associated with changes in OCT1 mRNA and protein expression in human livers and pharmacokinetics of sumatriptan and fenoterol in humans.

## MATERIALS AND METHODS

### Materials

Dulbecco's Modified Eagle Medium (DMEM), Hank's Buffered Salt Solution (HBSS), and 4-(4-(dimethylamino)styryl)-N-methylpyridinium ($ASP^+$) were obtained from Life Technologies (Darmstadt, Germany). Poly-D-lysine (1–5 kDa), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), bicinchoninic acid, and copper sulfate pentahydrate were obtained from Sigma-Aldrich (Taufkirchen, Germany). Dulbecco's phosphate-buffered saline (DPBS) and additives for

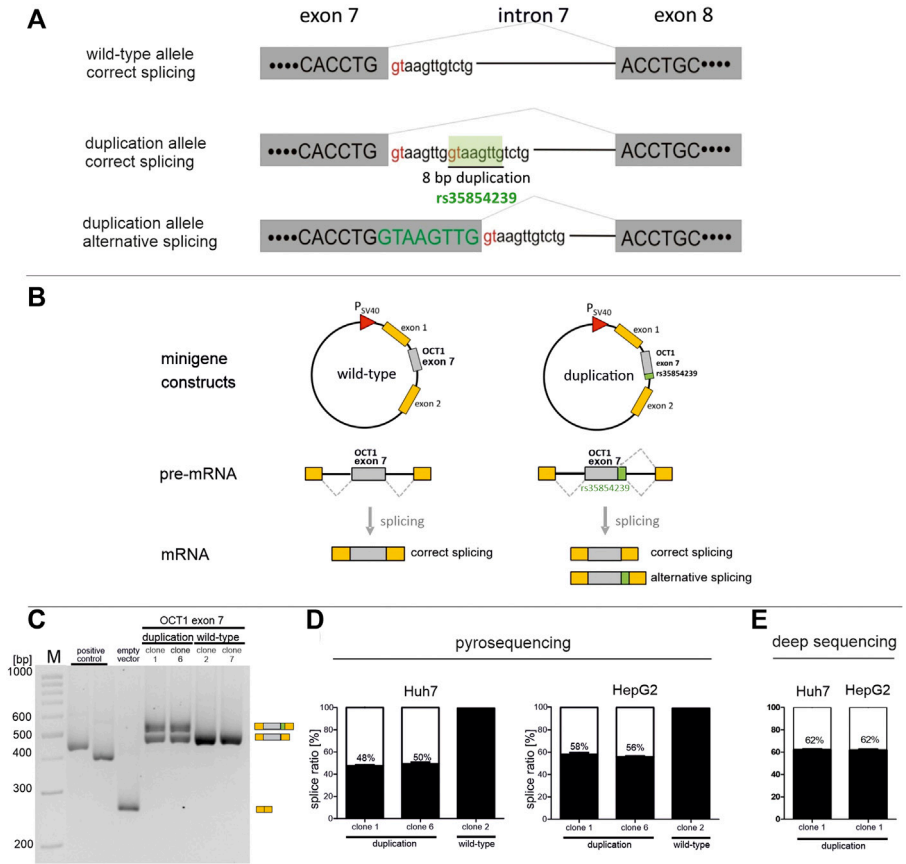



**FIGURE 1 |** Effects of rs35854239 on SLC22A1 exon 7 minigene splicing in Huh7 and HepG2 **(A)** Splicing at the exon 7-intron 7 junction. Splice donor sites within the intronic sequence are shown in red. The 8 bp insertion/deletion variant rs35854239 carries a second splice donor site that is proposed to be spliced alternatively. **(B)** Representation of the pSPL3b splicing vector consisting of two exons of the rabbit β-globulin gene under control of the SV40 promoter (the "minigene"). SLC22A1 exon 7 and its flanking intronic regions with or without rs35854239 (referred to as duplication or wild-type, respectively) were cloned between both exons of the rabbit β-globulin gene. Minigene constructs were transiently transfected into Huh7 and HepG2 cells, and mRNA was isolated 48 h after transfection. As positive control we used the CYP2C19*2 variant, for which alternative splicing is known, (Morais et al., 1994). **(C,D)** Correctly and alternatively spliced OCT1 transcripts were shown **(C)** and quantified using pyrosequencing **(D)**, or deep RNA-sequencing **(E)**. Percentages within boxes represent relative values of correctly spliced minigene transcripts. Data are shown as mean and standard errors of the mean of at least three independent experiments.

cell culturing were obtained from PAN-Biotech (Aidenbach, Germany). Twelve-well plates were obtained from CytoOne (Langenselbold, Germany), 6-well plates from Corning GmbH (Kaiserslautern, Germany), and tissue culture flasks from Sarstedt (Nümbrecht, Germany). All chemicals used in this study were purchased from commercial sources and had purities ≥95%.

## Cell Lines and Cell Culturing

HEK293 (Thermo Fisher Scientific, Darmstadt, Germany), Huh7, and HepG2 (ATCC, Manassas, United States) cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/ml streptomycin at 37°C and 5% $CO_2$.

## Generation of an Alternatively Spliced OCT1 Plasmid

The 8 bp insertion in exon 7 that results from alternative splicing of rs35854239 was introduced into an OCT1 encoding pcDNA5/FRT vector by site-directed mutagenesis as described previously (Seitz et al., 2015). The used primer pair 1 is listed in **Supplementary table S1**. The sequence was validated by capillary sequencing prior to transient transfection into HEK293 cells.

## Cellular Uptake Experiments After Transient Transfection

HEK293 cells were seeded at a density of $5 \times 10^5$ cells per well in a 12-well plate precoated with poly-D-lysine. Twenty-four hours after seeding, cells were transfected with 2 µg of the alternatively spliced OCT1 vector DNA using Lipofectamine™ 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. Transfection efficiency was evaluated by co-transfection with 0.5 µg of the green fluorescent protein coding vector pGFP-tpz (Thermo Fisher Scientific). The next day, uptake experiments were performed at 37°C and pH 7.4 using HBSS+ (HBSS supplemented with 10 mM HEPES buffer). Cells were washed once with pre-warmed

(37°C) HBSS+. Uptake was initiated by adding 20 μM $ASP^+$ diluted in HBSS+ and stopped after two minutes by adding ice-cold HBSS+. Cells were washed twice with ice-cold HBSS+ and lyzed with RIPA buffer. Fluorescence of $ASP^+$ in lysates was measured with an excitation of 485 nm and emission of 612 nm using the Tecan infinite M200 Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). $ASP^+$ fluorescence intensities were normalized to the total protein amount in the samples as measured using the bicinchoninic acid assay (Smith et al., 1985).

## Generation of Minigene Constructs

Splicing of exon 7-intron 7 was analyzed in the splicing vector pSPL3b, further referred to as minigene. Exon 7-intron 7 of OCT1 for both rs35854239 genotypes was amplified with primer pair 2, listed in **Supplementary table S1**. The PCR product was cloned into the pSPL3b vector after restriction of the PCR product and the vector with *Pst*I and *Eco*RV. The Met408 and Val408 were introduced by site-directed mutagenesis using primer pairs 3 and 4, respectively, listed in **Supplementary table S1**. The minigene constructs were validated by capillary sequencing and then used for transient transfection into Huh7 and HepG2 cells.

## Transient Transfection of the Minigene Constructs

Huh7 and HepG2 cells were seeded in six-well plates at a density of $4 \times 10^5$ and $1.7 \times 10^6$ cells per well, respectively. After 24 hours, cells were transfected with 2 μg minigene vector DNA using Lipofectamine™ 2000 as described above. Transfection efficiency was evaluated by co-transfection with pGFP-tpz as described above. Forty-eight hours after transfection, cells were lysed and RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

## PCR Amplification of Spliced Exon 7 Variants

After RNA isolation from transfected cells, complementary DNA (cDNA) was synthesized using the MultiScribe™ Reverse Transcriptase Kit (Applied Biosystems). Spliced exon 7 was amplified with primer pair 5 listed in **Supplementary table S1**. PCR products were separated by gel electrophoresis, bands were visualized under UV light and band intensities were quantified using the Fiji software (ImageJ version 1.52p, National Institutes of Health, Bethesda, United States).

## Analysis of rs35854239 in Human Liver Samples

Human liver samples were obtained from normal liver tissue that had to be removed for technical reasons during liver surgery or from organ donors. Patients gave their informed consent for research use of the removed liver tissue, and the procedures were approved by the ethics committee of the University Medicine Göttingen, Georg-August-Universität Göttingen (application number 26/01/17) and the ethics committee of the Pomeranian Medical University (application number KB-0012/64/12). Deep-frozen human liver samples were homogenized using the Mikro dismembrator S (B. Braun, Melsungen, Germany) at 2500 rpm for 1 min. DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's instructions. For the genotyping of functionally relevant polymorphisms in the SLC22A1 gene, the single base primer extension method was used as described previously (Seitz et al., 2015) using primer 6 listed in **Supplementary table S1**. RNA from human liver samples was isolated from homogenates using the RNeasy Plus Mini Kit and cDNA was synthesized as described above.

## Pyrosequencing

The ratio of correctly vs. alternatively spliced exon 7 in the transfected cell lines with hepatic origin and human liver samples was analyzed using pyrosequencing. Spliced exon 7 from minigene experiments and from cDNA from human liver samples was amplified using primer pair 7 (minigene) and primer pair 8 (liver samples) listed in **Supplementary table S1**. Samples were prepared using PyroMark™ Binding and Annealing Buffer (QIAGEN) and the PyroMark™ Vacuum Prep Station (Biotage, Uppsala, Sweden). Pyrosequencing was carried out on the PyroMark™ Q96 ID (Pyrosequencing AB, Uppsala, Sweden) with PyroMark™ Gold Q96 reagents (QIAGEN) using the primer 9 (minigene) and primer 10 (liver samples).

## Deep RNA-Sequencing and Sequence Mapping

Next-generation DNA and RNA sequencing was performed with cDNA from minigene experiments and DNA and cDNA from human liver samples. Exon 7 and its 3′ flanking region were first amplified using the primer pairs 11 to 14 listed in **Supplementary table S1**. The PCR products were purified by magnetic separation using Agencourt® AMPure® XP reagent (Beckman Coulter GmbH, Krefeld, Germany). Unique indices were attached to the purified amplicons by PCR using the Nextera® XT Index Kit v2 (Illumina Inc., San Diego, United States). The samples were again cleaned up with Agencourt® AMPure® XP reagent. All samples were pooled in appropriate ratios. The pooled library was quantified using the Qubit® 2.0 fluorometer and the Qubit® dsDNA BR assay kit (Thermo Fisher Scientific) and diluted to DNA concentration of 2 nM. DNA was denatured and diluted according to the manufacturer's instructions. As internal control and to increase variability within the sequencing run, 30% PhiX control (Illumina) was spiked in prior to denaturation. The sequencing run was performed using the MiSeq® Reagent Kit v3 (600 cycles) and paired-end 221 reads on the Illumina MiSeq™ (Illumina). The sequencing run was analyzed using the IGV v.2.6.3 software (Broad Institute, Cambridge, United States).

The paired-end sequence reads were merged using PEAR (release 0.9.11; Zhang et al., 2014). The mapping to a reference sequence was performed with Bowtie2 (Langmead et al., 2012)

version 2.3.4.1. DNA was mapped against the human genome assembly (hg19) downloaded from the UCSC Genome Browser. The cDNA from human liver samples was mapped against the "Homo sapiens solute carrier family 22 member 1 (SLC22A1), transcript variant 1, mRNA" (NM_003057.3) with artificially introduced duplication in it in order to better visualize the possible insertion using local mapping mode. The cDNA from minigene was mapped against the minigene itself. For both experiments with human liver samples we calculated subsequently the sequencing depth for each allelic combination of rs35854239 variant and rs628031 (Met408Val) variant with our own script.

## Immunocytochemistry

Five x $10^5$ HEK293 cells were seeded on poly-D-lysine coated cover slips and transfected as described above. One day after transfection, cells were washed twice with PBS and were fixed with 100% ethanol for 20 min at −20°C. After washing three times with PBS, cell membranes were permeabilized with PBS containing 0.4% Tween 20. Cells were washed three times with PBS and subsequently blocked for 3 hours with blocking buffer (5% FCS in PBS). OCT1 was stained using the NBP1-51684 (2C5) antibody (Novus Biologicals, Abingdon, United Kingdom). Cells were co-stained with the EP 1845Y antibody (Abcam, Cambridge, United Kingdom) against the membrane marker $Na^+/K^+$-ATPase. The primary antibodies against OCT1 and $Na^+/K^+$-ATPase were diluted in blocking buffer in a dilution of 1:400 and 1:200, respectively. Per cover slip, 50 µL antibody solution was added, cells were covered with parafilm and incubated in a humid chamber overnight. The next day, after washing three times with PBS, fluorescently-labeled secondary antibodies (Alexa Fluor® 546 goat anti-rabbit IgG (H + L), polyclonal and Alexa Fluor® 488 goat anti-mouse IgG (H + L), polyclonal; Thermo Fisher Scientific) were diluted 1:400 in PBS, added and incubated for 2 hours in the dark. Cells were washed three times with PBS and cover slips were mounted with Roti® Mount Fluor Care DAPI (Carl Roth, Karlsruhe, Germany). The staining was analyzed using the laser scanning microscope LSM780 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Images were processed using the Fiji software.

## Expression Data From Human Liver Samples

OCT1 mRNA and OCT1 protein expression data were extracted from a previous study describing expression of OCT1 and OCT3 in human liver samples (Nies et al., 2009). Analysis was performed on the subset of samples ($n$ = 90) that were from individuals who were non-cholestatic and had no hepatocellular, cholangiocellular or gallbladder carcinoma (Schaeffeler et al., 2011; Nies et al., 2013). The study was approved by the ethics committees of the Charité, Humboldt University (Berlin, Germany) and the University of Tübingen (Tübingen, Germany) in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient.

## Clinical Trial

The clinical trials on the effects of SLC22A1 genetic variants on fenoterol and sumatriptan pharmacokinetics have been described in details before (Matthaei et al., 2016; Tzvetkov et al., 2018). The rs35854239 variant was genotyped using the available DNA from those studies and the single base primer extension method as described previously (Seitz et al., 2015) with the SNaPshot primers listed in **Supplementary table S1**.

## Statistical Analysis

Differences in OCT1 mRNA and protein expression, or drug plasma concentration between homozygous wild-types and homozygous duplication allele carriers were performed using the Mann-Whitney-$U$ test. Differences between DNA and RNA allele frequencies in allelic expression imbalance analyses were performed using the paired sample $t$-test. All analyses were performed using SPSS Statistics version 25 (SPSS INC., IBM, Chicago, IL) Statistical significance was defined as $p < 0.05$. Post-hoc power calculations of the clinical studies were performed with the G*Power software version 3.1.9.4 (Faul et al., 2007).

# RESULTS

## Minigene Analyses of the Effects of SLC22A1 rs35854239 on Splicing

We used minigene assays to quantify the percentage of alternatively spliced transcripts in the 8 bp duplication allele of the rs35854239 variant. For this purpose, exon 7, including 306 bp upstream and 310 bp downstream of the flanking intronic regions, was cloned in the minigene vector pSPL3b between the exons 1 and 2 of the rabbit β-globulin gene. Next to the construct carrying the 8 bp duplication allele, the wild-type allele was also cloned and used as a control in the analyses (**Figure 1B**). Two independent minigene clones containing the duplication allele were analyzed to account for potential artifacts from the quality of the clones and the DNA preparation. The minigene constructs were transiently transfected into HepG2 and Huh7 cells, and the resulting correctly and alternatively spliced transcripts were quantified 48 hours later using three independent quantification techniques: semi-quantitative PCR, pyrosequencing and deep RNA-sequencing. In all cases, first, total RNA was reverse transcribed into cDNA. For the semi-quantitative PCR, the spliced SLC22A1 exon 7 was amplified by PCR using primers within the flanking exons of the rabbit β-globulin gene. The PCR products were separated by gel electrophoresis to enable the selective identification of the correctly and the alternatively spliced transcripts, and the band intensities were quantified (**Figure 1C**). As expected, the wild-type allele was spliced 100% correctly. However, the duplication allele was only spliced 47% correctly (range 42–51%) in transiently transfected Huh7 cells and 52% correctly (range 46–58%) in HepG2 cells (data not shown).

Next, we used pyrosequencing to quantify more precisely the ratio of the alternatively spliced transcripts. The pyrosequencing quantification method was validated by calibration series of vectors encoding the correctly or alternatively spliced minigene (**Supplementary Figure S1**). The pyrosequencing-based quantification showed that in Huh7 cells, the duplication allele

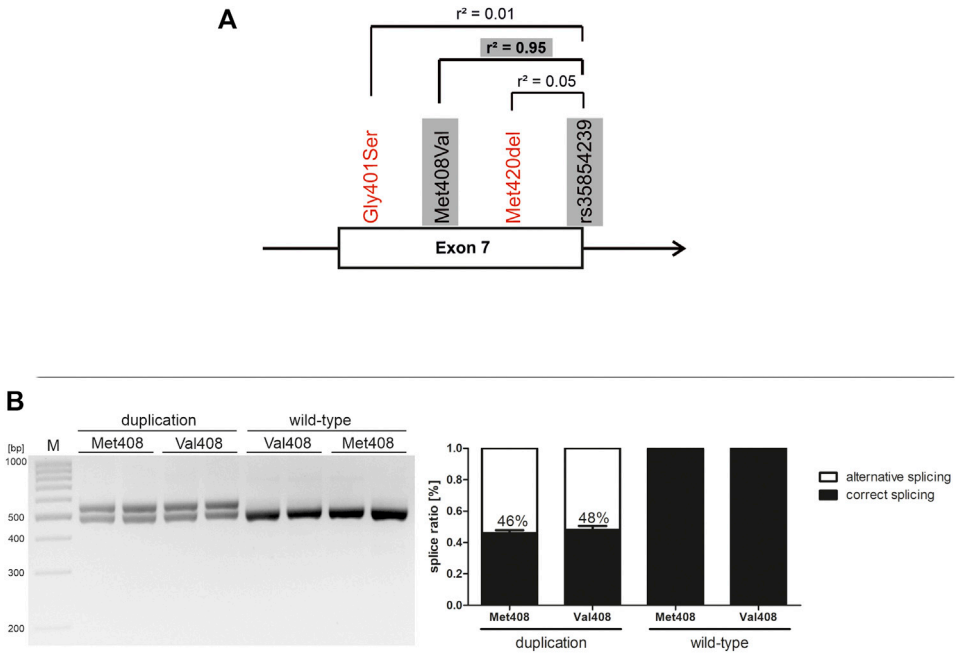



**FIGURE 2 |** Effects of Met408Val on rs35854239 and on exon 7 splicing **(A)** Localization of rs35854239 at the exon 7-intron 7 junction of the SLC22A1 gene. Known variants reducing OCT1 function in exon 7 of the SLC22A1 gene are shown in red. The highly genetically linked variants that are separately analyzed here are highlighted in gray **(B)** The Met408Val polymorphism was introduced by site-directed mutagenesis into both minigenes: harboring the wild-type and the duplication alleles of rs35854239. After transient transfection in Huh7 cells, spliced transcripts were amplified and separated by gel electrophoresis and afterwards, band intensities of correctly and alternatively spliced transcripts were quantified. Percentages within boxes represent relative values of correctly spliced minigene. Shown are means and standard errors of the means of three independent experiments.

was correctly spliced in 49% (range 46–52%) of all transcripts (**Figure 1D**). This was highly comparable with the previously semi-quantitatively determined ratios. In HepG2 cells, the duplication allele was correctly spliced in 57% (range 55–60%) of all transcripts.

Finally, the minigene insertion allele clone 1 spliced in Huh7 or HepG2 cells at 48 h was reanalyzed using deep massive-parallel sequencing (**Figure 1E**). The average depth of targeted RNA sequencing was 59,135 reads (range 32,902–84,691). The quantification of reads carrying the 8 bp insertion as a result of alternative splicing showed a percentage of 62% correctly spliced minigene in both cell lines. These results confirm the alternative splicing of rs35854239. More importantly, these results suggest that the 8 bp duplication causes erroneous splicing in only a part of the transcripts. Estimated by the data of all experiments maximally 52% of the transcripts are erroneously spliced. Thus, our *in vitro* data suggest that even in homozygous carriers of the duplication about the half of OCT1 transcripts will be correctly spliced.

## Effects of SLC22A1 Exon 7 Genetic Variants on rs35854239 Splicing

Within the SLC22A1 gene, exon 7 harbors the highest density of coding functionally relevant polymorphisms. Thereof, the Met408Val substitution is almost completely linked to the rs35854239 duplication (**Figure 2A**). Under native conditions, it could not be excluded that the coding variant substantially contributes to the effects of splicing. Here were took advantage of the minigene technique and addressed separately the effects of the two variants on splicing. The Val408Met substitution alone did not significantly affect splicing, neither on duplication nor on wild-type rs35854239 background (**Figure 2B**). Therefore, it could be concluded that the effects on splicing are completely caused by the rs35854239 duplication and there is no contribution of the highly linked Met408Val.

## Functional Characterization of the Protein Encoded by the Alternatively Spliced Transcript

The alternative splicing leads to an 8 bp longer exon 7, entailing a frame shift. This results in an altered amino acid sequence after codon 425 followed by a premature stop after seven amino acids. The resulting truncated OCT1 protein p. Asp426fs consists of the first nine TMHs only (**Figure 3A**).

To analyze whether the truncated OCT1 protein p. Asp426fs is able to function as an uptake transporter, the 8 bp insertion sequence was introduced between exon 7 and exon 8 of the OCT1 carrying pcDNA5 vector using site-directed mutagenesis. The resulting vector was transiently transfected into HEK293 cells and the uptake of the model OCT1 substrate $ASP^+$ was compared to the uptake of the wild-type OCT1. Three independent clones of p.Asp426fs were analyzed. The alternatively spliced OCT1 protein showed no transport activity (**Figure 3B**). $ASP^+$ uptake of all alternatively spliced OCT1 clones was at same levels as the empty pcDNA5 vector, indicating no OCT1-mediated substrate uptake.

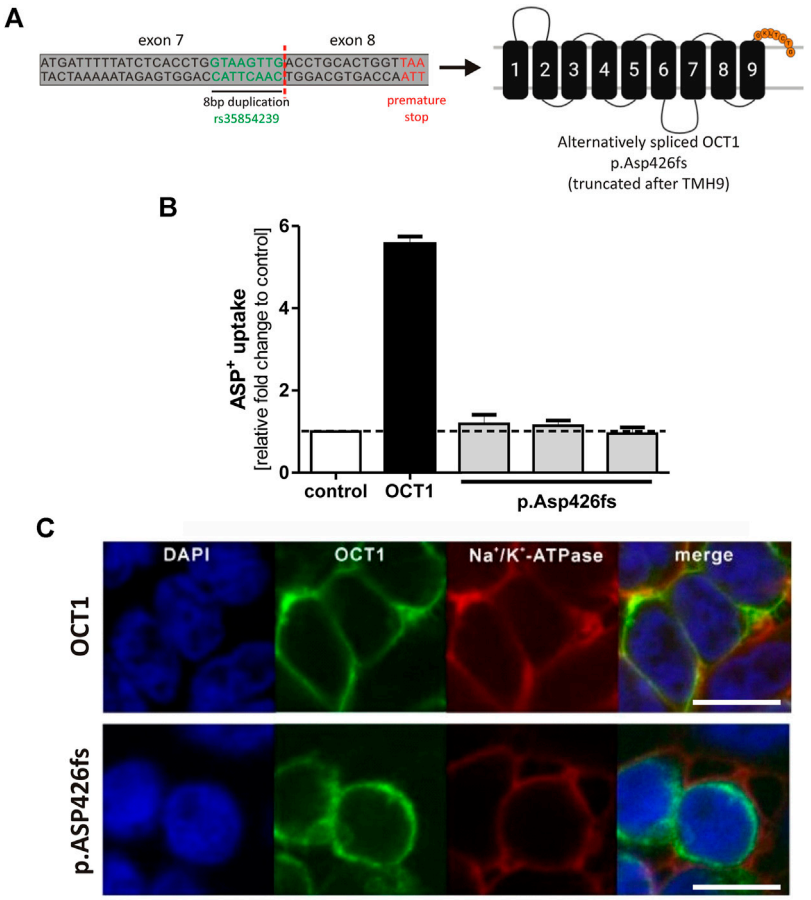



**FIGURE 3 |** Effects of the alternatively spliced OCT1 protein p.Asp426fs on OCT1 function **(A)** Alternative splicing of rs35854239 leads to a premature stop after Asn431, resulting in an OCT1 protein that is truncated after transmembrane helix 9. **(B)** HEK293 cells transiently transfected with a vector coding for the alternatively spliced OCT1 p. Asp426fs were incubated for 2 min with 20 μM $ASP^+$. The OCT1-mediated uptake was calculated as fold change compared to control cells (transfected with the empty vector). Transfection of wild-type OCT1 served as a positive control for functional transporter. Data are shown as mean and standard error of the mean (SEM) of three independent experiments. **(C)** Membrane localization was analyzed using immunofluorescence staining and confocal microscopy (magnification factor 63). The OCT1 antibody used for this purpose recognizes the intracellular loop of the protein between TMH6 and TMH7. OCT1 (green) was co-stained with $Na^+$/$K^+$-ATPase (red) as membrane marker. Scale bar indicates 10 μm. TMH, transmembrane helix.

Immunofluorescence staining revealed aberrant membrane localization of the alternatively spliced OCT1 (**Figure 3C**). This demonstrates that the truncated OCT1 protein, which results from alternative splicing of exon 7, is completely inactive and lacks correct membrane localization.

## Effects of rs35854239 on Splicing in Human Liver Samples

Minigene analyses in cell lines with hepatic origin showed that the 8 bp duplication leads to maximally 52% of alternatively spliced transcripts that encode a non-functional protein. In order to validate these results *in vivo*, we quantified the effects of the 8 bp duplication on OCT1 mRNA splicing in human liver. To this end, DNA from 24 liver samples was genotyped for rs35854239 and the correct splicing of the exon 7-intron 7 junction was quantified using pyrosequencing and deep RNA-sequencing (**Figure 4**).

Using pyrosequencing, we observed 99% correctly spliced SLC22A1 mRNA irrespective of the genotype of the liver donors. Alternative splicing of OCT1 in liver samples from homozygous or heterozygous 8 bp duplication allele carriers appeared with a maximum of 2.1% (**Figure 4A**). This percentage is far below the observed results in minigene experiments (**Figure 1**) but is still substantially higher than the observed 0.4% in the SLC22A1 mRNA from donors with wild-type genotype, which can only be spliced correctly.

Using deep RNA-sequencing, we detected very low levels of alternatively spliced transcripts that were, however, dependent on the rs35854239 genotype (**Figure 4B**). The average depth of sequencing was 74,326 reads per RNA sample (range 41,116–133,715). The liver samples from homozygous duplication allele carriers showed mean values of 0.58% alternatively spliced transcripts (range 0.19–1.14%). In heterozygous genotypes, alternative splicing was detected with a mean of 0.36% (range 0.08–0.83%). The samples of the

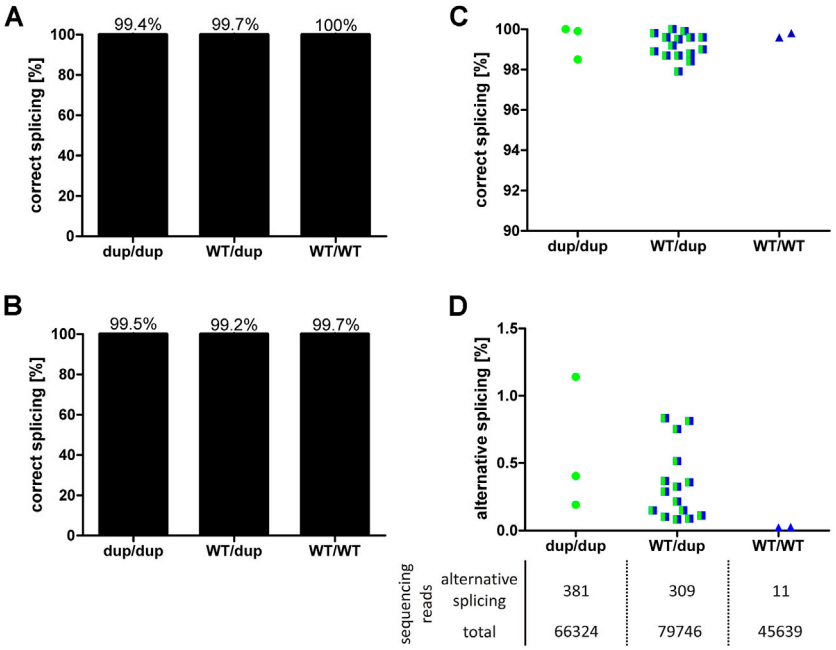


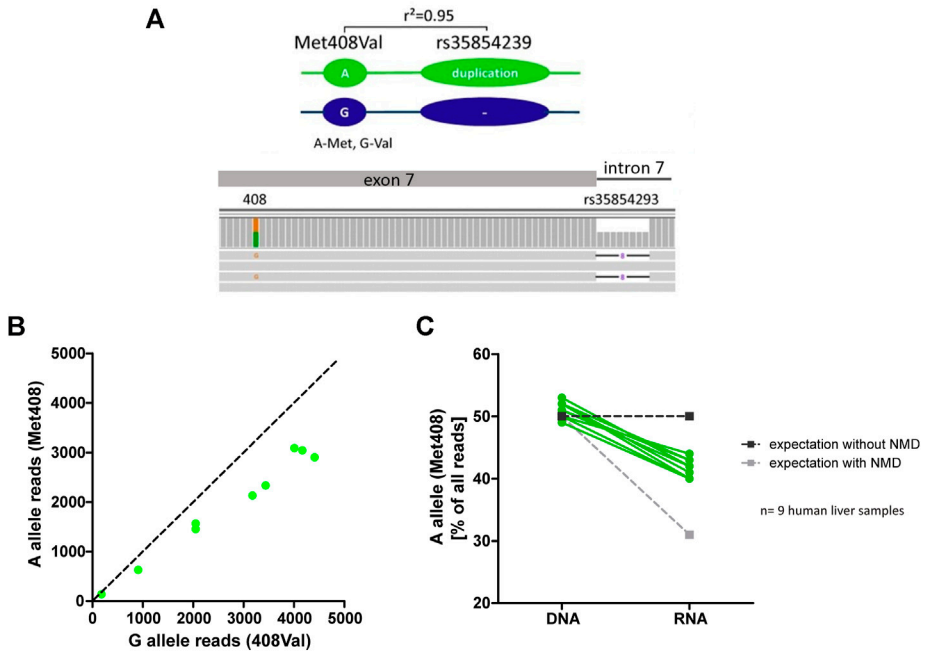

**FIGURE 4 |** Effects of rs35854239 on OCT1 splicing in human liver samples. Splicing of the exon7-intron7 junction of OCT1 mRNA, depending on rs35854239 genotype was quantified using **(A)** pyrosequencing and **(B)** deep RNA-sequencing. On the left, the mean percentage of correctly spliced transcripts depending on the rs35854239 genotype is shown. The percentage of **(C)** correctly and **(D)** alternatively spliced transcripts for each individual sample is shown. **(D)** The mean sequencing depth for alternatively spliced and total transcripts is stated below. Dup, rs35854239 duplication allele; WT, wild-type SLC22A1 allele. Dup/dup: $n = 3$, WT/dup: $n = 15$, WT/WT: $n = 2$.

homozygous wild-type allele carriers showed a mean of 0.02% alternatively spliced transcripts indicating very low levels of possible contamination with this highly sensitive method. In conclusion, both techniques demonstrated that despite the close to 50% probability of alternative splicing of the rs35854239 duplication allele estimated by the minigene assays, alternatively spliced OCT1 could only barely be detected in human liver samples. These results suggest that the alternatively spliced transcripts may be recognized and rapidly degraded under native conditions.

To verify this, we performed an allelic expression imbalance analysis in the human liver samples. We took advantage of the strong genetic linkage between the duplication allele of rs35854239 and the A-allele of the coding variant Met408Val (rs628031, 1222A>G, $r^2 = 0.95$; **Figure 5A**). Based on the strong linkage, in heterozygous carriers of the rs35854239 duplication and Met408Val A-allele haplotype, a degradation of alternatively spliced OCT1 could be detected as a lower abundance of the Met408 A-allele in the RNA transcripts compared to the expected 50% of the DNA reads (**Figure 5B**). We used all nine liver samples from which both DNA and RNA was available and applied deep sequencing for quantification. While the A-allele was detected in 50% of the DNA reads (range 49–53%), the abundance in RNA was significantly decreased to 42% (range 40–44%, $p = 2.77 \times 10^{-7}$, paired *t*-test; **Figure 5C**). This result supports the degradation of the alternatively spliced transcripts and suggests that the presence of the rs35854239 duplication will result in the reduction of OCT1 mRNA levels, and as a consequence OCT1 protein in general.

In addition, more precise analyses of the sequencing reads suggest that the correct "canonical" splicing may be preferred under native conditions. Indeed, reads of alternatively spliced RNA carrying the A-allele of Met408Val were almost not detectable (**Figure 5D**). However, the reads of correctly spliced RNA carrying A-allele Met408Val were 71.3% of the G-allele reads (range from 65.8 to 76.7%) instead of the expected 50% from the minigene analyses. This suggests that in parallel to degradation of the alternatively spliced transcripts also a preference for correct splicing of rs35854239 duplication allele under native conditions may exist.

To address this, we analyzed total OCT1 mRNA and protein expression in 73 human liver samples. The liver samples had been characterized for their OCT1 expression before (Nies et al., 2009). We included only those samples lacking the Arg61Cys substitution, which is known to significantly affect OCT1 protein levels in the liver (**Supplementary table S2** and (Nies et al., 2009) by affecting the correct membrane localization (Seitz et al., 2015). The OCT1 mRNA expression was on median 47% lower in homozygous duplication than in homozygous wild-type rs35854239 allele carriers (median of 0.014 and 0.026 transcripts per beta-actin transcript, respectively; $p = 0.007$; **Figure 6A**). The OCT1 protein levels were on median 35%




**D**

| | DNA | | | | | | | | RNA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplo-type | G-WT | | G-Dup | | A-WT | | A-Dup | | G-WT | | G-Dup | | A-WT | | A-Dup | |
| | reads | [%] | reads | [%] | reads | [%] | reads | [%] | reads | [%] | reads | [%] | reads | [%] | reads | [%] |
| Median | 2655 | 100 | 115 | 4.8 | 121 | 5.5 | 2877 | 105.0 | 3165 | 100 | 14 | 0.4 | 2084 | 71.1 | 13 | 0.6 |
| SD | 588 | 0 | 22 | 0.7 | 19 | 0.8 | 705 | 5.0 | 1490 | 0 | 8 | 0.4 | 1049 | 4.5 | 19 | 0.4 |
| Min | 1754 | 100 | 87 | 3.6 | 101 | 3.9 | 1771 | 97.0 | 180 | 100 | 3 | 0.2 | 138 | 65.8 | 1 | 0.3 |
| Max | 3427 | 100 | 144 | 5.4 | 155 | 6.2 | 3738 | 110.7 | 4389 | 100 | 22 | 1.7 | 3041 | 76.7 | 51 | 1.6 |

**FIGURE 5 |** Allelic expression imbalance in heterozygous carriers of rs35854239 **(A)** The duplication allele of rs35854239 is highly linked to the A-allele of Met408. The abundance of both Met408Val alleles was analyzed on DNA and RNA level in heterozygous Met408Val/rs35854239 human liver samples using deep RNA-sequencing. **(B)** The abundance of both Met408Val alleles on RNA level was analyzed and **(C)** the percentage of the A-allele (Met408) on DNA and RNA levels was compared in nine human liver samples (green). Dashed lines represent expected allele balance without (dark gray) and with (light gray) non sense-mediated mRNA decay (NMD). **(D)** Sequencing depth is given in absolute reads and relative to the Val408 G-allele and rs35854239 wild-type haplotype.

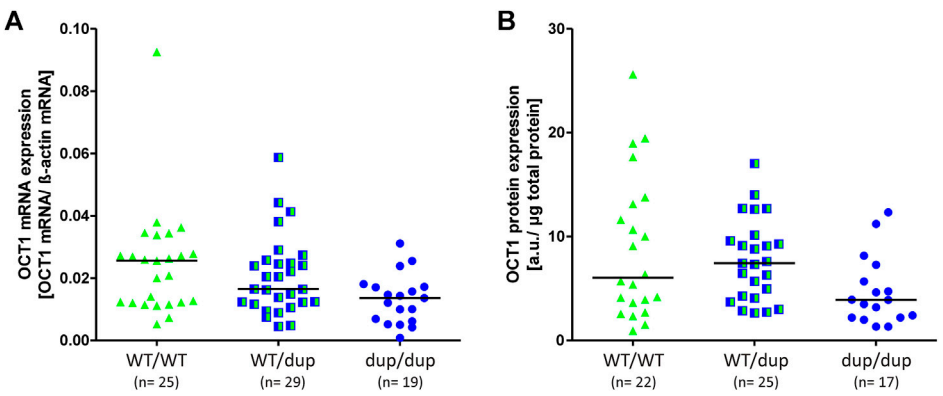



**FIGURE 6 |** Effect of rs35854239 on OCT1 **(A)** mRNA and **(B)** protein expression. Median OCT1 **(A)** mRNA expression and **(B)** protein expression was analyzed in 73 liver samples depending on the rs35854239 genotype. Dup, rs35854239 duplication allele; WT, wild-type OCT1 allele; a. u., arbitrary unit.

lower in homozygous duplication than in homozygous wild-type rs35854239 allele carriers (median of 3.91 and 6.04, respectively; $p = 0.045$; **Figure 6B**). However, although statistically significant, the effects of rs35854239 genotypes could explain only 9% of the variability of mRNA and protein expression in this sample set.

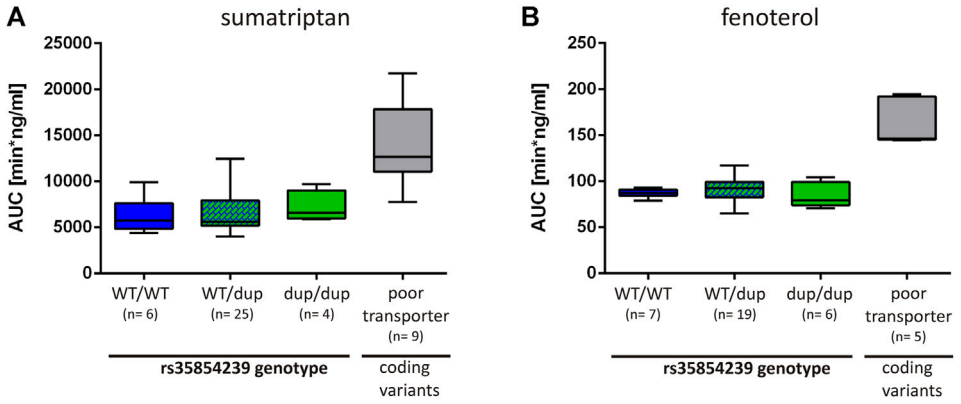



**FIGURE 7 |** Effects of rs35854239 on the pharmacokinetics of **(A)** sumatriptan and **(B)** fenoterol. The AUC of **(A)** sumatriptan and **(B)** fenoterol is depicted depending on the rs35854239 genotype and compared to the OCT1 phenotype (poor transporters). Poor transporters comprise homozygous or compound heterozygous carriers of the OCT1 alleles *3, *4, and *5 harboring the coding variants Arg61Cys, Gly401Ser or Gly465Arg, respectively. Dup, rs35854239 duplication allele; WT, wild-type OCT1 allele; AUC, area under the plasma concentration-time curve. Boxplots show median, lower (25%) and upper (75%) quartiles.

## Effects of rs35854239 Duplication on the Pharmacokinetics of Sumatriptan and Fenoterol

Finally, we analyzed to what extend the decrease of OCT1 expression in carriers of the rs35854239 duplication allele leads to changes in sumatriptan and fenoterol pharmacokinetics in humans. We took advantage of the existing studies on the effects of OCT1 genotypes on the pharmacokinetics of both drugs (Matthaei et al., 2016; Tzvetkov et al., 2018) and analyzed them in the context of the rs35854239 genotype. The AUC of sumatriptan was slightly increased in homozygous rs35854239 duplication allele carriers compared to the wild-type (means of 7187 vs. 6277 min × ng/ ml, respectively, **Figure 7A**). However, this increase was not significant and was on average by 14% compared to the observed 127% increase in poor OCT1 transporters (homozygous or compound heterozygous carriers of the coding variants Arg61Cys, Gly401Ser, Gly465Arg) observed in the same study.

Even more, the AUC of fenoterol was not higher in homozygous carriers of the rs35854239 duplication allele compared to the wild-type (means of 84.25 vs. 86.84 min × ng/ml, respectively; **Figure 7B**). In comparison, poor OCT1 transporters showed 1.89-fold higher AUCs for fenoterol. This data suggests that compared to the well-known loss-of-function coding variants, the 8 bp duplication shows only limited effects on drugs pharmacokinetics.

# DISCUSSION

The eight base pairs duplication at the exon 7-intron 7 junction (rs35854239) has been previously suggested to cause erroneous splicing of OCT1 by introducing an alternative splice site in the intronic sequence of intron 7. In this study, we confirm the alternative splicing and give more precise quantitative information about the effects on OCT1 expression and activity in order to better estimate the contribution of this variant to the highly inter-individual variability in OCT1 activity.

This study built up on the previous findings of Kim et al. (Kim et al., 2017). We confirmed the findings of Kim et al. that the 8 bp duplication causes alternative splicing. We did this both by using minigene assays (**Figure 1**) and by detecting (a low level) of the alternatively spliced transcript in human liver samples (**Figure 4**). We also confirmed the finding of Kim et al. that the alternatively spliced transcript is not leading to a functional protein (**Figure 2**).

The major contribution of this study beyond the previously known is the precise quantification of the effects of the 8 bp duplication rs35854239. We used minigene analyses to quantify the effects on splicing (**Figure 1**) and to confirm that these effects are caused by the 8 bp duplication and not by the highly genetically linked variant Met408Val. We quantified the effects of the 8 bp duplication on total OCT1 expression in human liver both on mRNA and on protein levels (**Figure 6**) and finally we analyzed the association of the splice variants with the pharmacokinetics of drugs that are well known OCT1 substrates (**Figure 7**). This will enable us to better evaluate the contribution of the rs35854239 duplication to the high genetic and thus to the high functional variability of OCT1 in humans.

Our data suggest that the 8 bp duplication allele can cause erroneous splicing of up to 50% of the transcripts, but, probably due to mRNA decay, the number of detectable erroneously spliced transcripts in the human liver is very low. Thus, homozygous carriers of the duplication allele are characterized by decreased expression of the correctly spliced transcripts resulting in a median decrease of OCT1 protein expression by 35% in the human liver. However, the rs35854239 effects explained only 9% of the highly variable SLC22A1 mRNA expression in humans (**Figure 6**), and the 8 bp duplication was not associated with significant changes in the pharmacokinetics of known OCT1 substrates, i.e. sumatriptan and fenoterol (**Figure 7**).

The rs35854239 ins/del variant results in duplication of the originally existing donor splice site of intron 7, giving a possibility of alternative, but also keeping the possibility of correct "canonical" splicing. Here, we demonstrated that both donor splice sites are operative. Depending on the cellular system and the quantification technique used, intensive processing of both splice sites with a slight preference for the usage of the original "correct" donor splice site was suggested (**Figure 1**).

These numbers are in contrast to the almost undetectable alternatively spliced transcripts in the human liver. These discrepancies could be explained by a nonsense-mediated mRNA decay (NMD) as suggested previously by Kim et al. (Kim et al., 2017). Here, we provide multiple confirmations for an mRNA decay of the incorrectly spliced OCT1 transcripts. Firstly, using minigene constructs that use rabbit flanking exons, and thus are not affected by NMD, we observed a substantially higher percentage of the alternatively spliced transcripts. Secondly, we applied allelic expression imbalance analyses, taking advantage of the genetically highly linked coding variant Met408Val, and were able to demonstrate imbalance in the expression of the duplication-linked allele Met408 (**Figure 5**). Finally, we demonstrated significantly reduced expression of the correctly spliced transcripts in homozygous carriers of the duplication allele (**Figure 6**).

Even if not degraded, the alternatively spliced transcript encodes a truncated protein p.Asp426fs. This protein is missing TMHs 10 to 12, which are essential for OCT1 activity (Shu et al., 2003; Gorboulev et al., 2005; Egenberger et al., 2012) and is not localized correctly at in the plasma membrane (**Figure 3**). Therefore, only the correctly spliced transcripts can contribute to OCT1 activity.

A major hypothesis of this study was that the 8 bp duplication that affects splicing may explain a major part of OCT1 expression variability. OCT1 mRNA expression in the human liver varied more than 100-fold between individuals (Nies et al., 2009; O'Brien et al., 2013). In the 73 liver samples analyzed in this study, we observed 23-fold variability in OCT1 expression. Despite a median reduction of OCT1 expression by almost 50% in homozygous duplication carriers, the genetic variant could explain less than 10% of the general variability. Taken together with the lack of strong effects of genetic variants in the SLC22A1 promoter (Bokelmann et al., 2018), only minor effects of *cis*-acting variants could be concluded. Systematic analyses of the genetic component in the variability of OCT1 expression are highly complicated, as multiple sampling of the same individuals or sampling within multiple members of the family are required. However, there are already some data suggesting that *trans*-acting variants or non-genetic factors may play a role. Indeed, genetic variants in transcription factors known to regulate OCT1 expression were associated with OCT1 expression (O'Brien et al., 2013), and disease conditions such as cholestasis may play a role (Nies et al., 2009). There are a number of other transcriptional factors suggested to regulate OCT1 expression, e.g. CCAAT/enhancer binding proteins, pregnane X receptor (PXR), farnesoid X receptor (FXR), and glucocorticoid receptor (GR)(Saborowski et al., 2006; Rulcova et al., 2013; Hyrsova et al., 2016). It will be interesting to study whether genetic variants within them may have *cis*-effects on OCT1 expression.

Regarding potential effects of rs35854239 on pharmacokinetics, our data suggest that the impact on OCT1 expression caused by the duplication does not significantly affect the pharmacokinetics of known OCT1 substrates in humans. We were not able to detect significant differences in the AUCs of sumatriptan or fenoterol depending on the presence or absence of the rs35854239 duplication, in contrast to the clear and highly significant effects of the well-known coding OCT1 variants (**Figure 7**). Indeed, these two studies were not designed to address effects of rs35854239. However, based on the high frequency of this variant, we had a power of 80% to detect the increase in AUC of 47% and higher for sumatriptan, and of 15% or higher for fenoterol. The significant, but less prominent effects of this variant are in line with previous reports (Kim et al., 2017).

One explanation may be that the 50% reduction of OCT1 expression is not sufficient to affect substantially the pharmacokinetics of substrates in humans. Our previous studies on the effects of coding genetic variants on OCT1 activity clearly demonstrated that reduction of the typical OCT1 activity by more than 50% (OCT1 gene dose of less than 1 with a typical gene dose of 2) is necessary to cause measurable changes in drug pharmacokinetics (Tzvetkov et al., 2018; Matthaei et al., 2019). This is in line with the lack of evidence for an independent association of rs35854239 with serum isobutyrylcarnitine levels in the Kim et al. study (Kim et al., 2017). The authors of this study also interpreted this as a consequence of the small effect size of the duplication.

Multiple studies reported an association of the Met408Val variant with pharmacokinetics or efficacy of drugs that are potential OCT1 substrates (White et al., 2006; Shikata et al., 2007; Wang et al., 2008; Kim et al., 2009; Takahashi et al., 2010; Tarasova et al., 2012; Koren-Michowitz et al., 2014; Vaidya et al., 2015). However, those associations were difficult to interpret, as no significant effect of this coding variant on transport activity could be demonstrated (Shu et al., 2003; Shu et al., 2007; White et al., 2010; Nies et al., 2014; Tzvetkov et al., 2014; Seitz et al., 2015). The rs35854239 variant is almost completely genetically linked to Met408Val ($r^2$ of 0.95). Our data strongly suggest the rs35854239 variant as causative variant due to its effects on splicing and thus on OCT1 expression. However, we also demonstrated that these effects are less prominent than the influences of other common coding variants of the gene, which lead to reduced or loss of OCT1 function (Arg61Cys, Cys88Arg, Gly401Ser and Gly465Arg). Therefore, the observed strong association with Met408Val or directly with rs35854239, but absence of association with the highly functional amino acid substitutions listed above are still difficult to explain.

In conclusion, using minigene analyses we were able to quantify that the common naturally occurring 8 bp duplication at the exon 7-intron 7 junction (rs35854239) causes alternative splicing in approximately 50% of the cases. The alternatively spliced transcripts are degraded under native conditions in the liver, but even if stable they are not able to encode the active protein (as demonstrated here using expression in HEK293 cells), and thus result in a significant decrease in OCT1 expression detectable both on mRNA and protein levels. However, although very common (minor allele frequency of 40.6%), the decrease in expression, taken together with the high general variability of OCT1 expression, was not

sufficient to cause strong effects on drug pharmacokinetics (as demonstrated by analyzing the effects of the variant on pharmacokinetics of sumatriptan and fenoterol in healthy individuals).

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI repository, accession number: PRJNA720275 (https://www.ncbi.nlm.nih.gov/sra/PRJNA720275)

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of the University Medicine Göttingen, Georg-August-Universität Göttingen (application number 26/01/17) by the ethics committees of the Charité, Humboldt University (Berlin, Germany) and the University of Tübingen (Tübingen, Germany). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SR, KK, LS performed experiments. SR, MT, KK, AT, AN analyzed data. JM, AT, JL-R, JG, MD, AN contributed new reagents or analytic tools. SR, MT, MM, KK, AN wrote the manuscript. All authors read and edited the manuscript. MM, MT and JB participated in research design.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.661480/full#supplementary-material

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# Isobutyrylcarnitine as a Biomarker of OCT1 Activity and Interspecies Differences in its Membrane Transport

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Genome-wide association studies have identified an association between isobutyrylcarnitine (IBC) and organic cation transporter 1 (OCT1) genotypes. Higher IBC blood concentrations in humans with active OCT1 genotypes and experimental studies with mouse OCT1 suggested an OCT1-mediated efflux of IBC. In this study, we wanted to confirm the suggested use of IBC as an endogenous biomarker of OCT1 activity and contribute to a better understanding of the mechanisms behind the association between blood concentrations of carnitine derivatives and OCT1 genotype. Blood and urine IBC concentrations were quantified in healthy volunteers regarding intra- and interindividual variation and correlation with OCT1 genotype and with pharmacokinetics of known OCT1 substrates. Furthermore, IBC formation and transport were studied in cell lines overexpressing OCT1 and its naturally occurring variants. Carriers of high-activity OCT1 genotypes had about 3-fold higher IBC blood concentrations and 2-fold higher amounts of IBC excreted in urine compared to deficient OCT1. This was likely due to OCT1 function, as indicated by the fact that IBC correlated with the pharmacokinetics of known OCT1 substrates, like fenoterol, and blood IBC concentrations declined with a 1 h time delay following peak concentrations of the OCT1 substrate sumatriptan. Thus, IBC is a suitable endogenous biomarker reflecting both, human OCT1 (hOCT1) genotype and activity. While murine OCT1 (mOCT1) was an efflux transporter of IBC, hOCT1 exhibited no IBC efflux activity. Inhibition experiments confirmed this data showing that IBC and other acylcarnitines, like butyrylcarnitine, 2-methylbutyrylcarnitine, and hexanoylcarnitine, showed reduced efflux upon inhibition of mOCT1 but not of hOCT1. IBC and other carnitine derivatives are endogenous biomarkers of hOCT1 genotype and phenotype. However, in contrast to mice, the mechanisms underlying the IBC-OCT1 correlation in humans is apparently not directly the OCT1-mediated efflux of IBC. A plausible explanation could be that hOCT1 mediates cellular concentrations of specific regulators or co-substrates in lipid and energy metabolism, which is supported by our *in vitro* finding that at baseline intracellular IBC concentration is about 6-fold lower alone by OCT1 overexpression.

**Keywords: OCT1, SLC22A1 (OCT1), isobutyrylcarnitine, efflux, organic cation transporter, carnitine, Biomarker**

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## INTRODUCTION

The organic cation transporter 1 (OCT1) is strongly expressed in human hepatocytes (Nies et al., 2009) and accelerates membrane transport of numerous endogenous metabolites, drugs and toxins (Nies et al., 2011; Koepsell, 2013). The *SLC22A1* gene, coding for human OCT1 (hOCT1), is genetically highly variable (Kerb et al., 2002; Shu et al., 2003; Seitz et al., 2015). In the European population, five common loss-of-function polymorphisms are known, which have significant consequences for the pharmacokinetics of drugs, such as fenoterol (Tzvetkov et al., 2018), metformin, and sumatriptan (Matthaei et al., 2016). Thiamine (vitamin B1) was identified as natural substrate of murine OCT1 (mOCT1) and hOCT1 (Chen et al., 2014) but thiamine pharmacokinetics are not dependent on hOCT1 genotype (Jensen et al., 2020).

There is significant interest in the discovery of endogenous biomarkers reflecting the *in vivo* activity of drug metabolizing enzymes and the *in vivo* activity of drug membrane transport (Yee et al., 2016; Chu et al., 2017; Chu et al., 2018). For instance, human blood concentrations of N-methyl-nicotinamide, *N*-methyladenosine, glycochenodeoxycholate sulfate, and 6-beta-hydroxycortisol may reflect the *in vivo* activity of MATE, OCT2, OATP1B1, and OAT3, respectively. In genome-wide association studies, isobutyrylcarnitine (IBC) was strongly associated with *OCT1* genetic polymorphisms (Suhre et al., 2011) and may thus be a suitable endogenous biomarker of OCT1 activity (Luo et al., 2020). IBC is a metabolite of valine, when its acyl residue is transferred from isobutyryl-CoA to carnitine (Ramsay et al., 2001) (**Figure 1**). Acylcarnitines, in general, are amino acid or fatty acid breakdown products. Conversion of acyl-CoA to the acylcarnitine ester *via* carnitine acyltransferase is essential to maintain the pool of free coenzyme A (Ramsay et al., 2001). Because of the role of carnitine conjugation in buffering excessive fatty acids, acylcarnitine species are biomarkers of congenital metabolic diseases with disruption in peroxisomal or mitochondrial oxidation processes (Pedersen et al., 2006; Giesbertz et al., 2015).

The mechanisms of membrane transport of carnitine derivatives are controversial. The zwitterionic carnitine itself is transported by the almost ubiquitously expressed organic cation transporters OCTN1 and OCTN2 (Tamai et al., 1998; Ramsay et al., 2001; Koepsell, 2013; Salomon et al., 2019). Recently, an efflux function of OCT1 for IBC was proposed (Kim et al., 2017) based on transport experiments with mOCT1 and the relationship between human plasma carnitine derivatives and human OCT1 genotype was explained by that finding with murine OCT1. However, that explanation did not consider known major species differences in substrate selectivity and transport kinetics between mOCT1 and hOCT1, which are well-known and quite extensive (Gorboulev et al., 1997; Zhang et al., 1997; Green et al., 1999; Schmitt et al., 2003; Meyer et al., 2020). Short-chain acylcarnitine species are very hydrophilic with negative $\log D_{7.4}$ values ranging from −6.80 to −1.64 (**Supplementary Table S1**), indicating the necessity for transporter-mediated cell membrane passage. However, experimental evidence for IBC transport *via* organic cation transporters currently exists only for mOCT1.

With the studies presented here, we wanted to assess the suitability of IBC as endogenous biomarker of OCT1 genotype and phenotype. But most importantly, we wanted to elucidate the mechanisms behind the association between hOCT1 genotype and blood concentrations of IBC and other carnitine derivatives. Thus far, transport of IBC had only been studied with murine OCT1 but not with human OCT1 and efflux transport had been incompletely characterized. While performing these experiments, we soon discovered that IBC is not transported *via* human OCT1, neither into the cell nor out of the cell. Therefore, we performed several additional experiments to elucidate the mechanisms behind the association between OCT1 genotype and IBC blood concentrations. In this context we hypothesized that other OCT1-dependent endogenous substrates might regulate carnitine metabolism. Therefore, we studied the uptake of

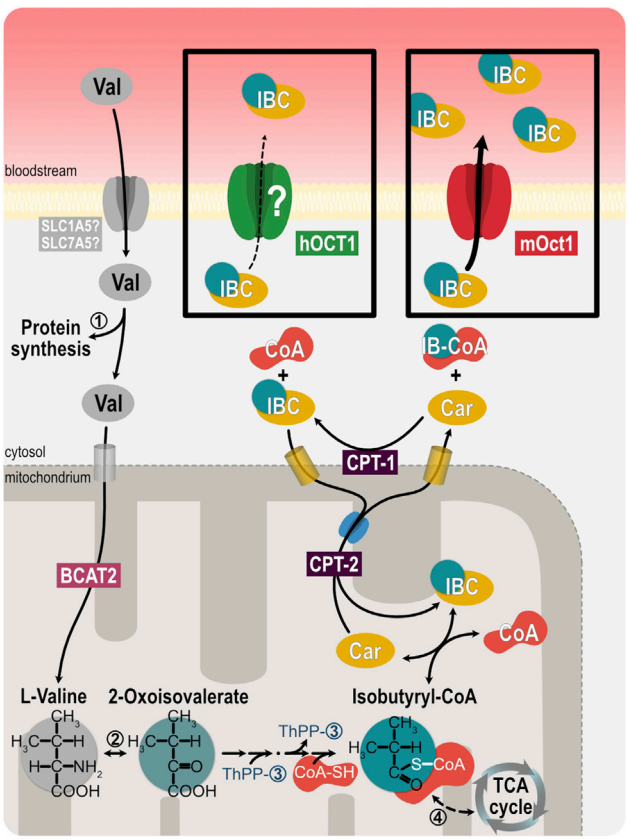


**FIGURE 1 |** Illustration of isobutyrylcarnitine metabolism. Oxidation of branched-chain amino acids, such as valine, after uptake into mitochondria by SLC25A44 and the branched-chain amino acid transaminase 2 (BCAT2), lead to acylcarnitine intermediates, such as isobutyrylcarnitine. Exchange of carnitine and acylcarnitines across mitochondrial membranes exports the acyl residues into the cytosol. ① valyl tRNA ligase, ② branched-chain amino acid transaminase, ③ 3-methyl-oxo-butanoate dehydrogenase, ④ acyl-CoA dehydrogenase, CPT-1 carnitine-palmitoyltransferase 1, CPT-2 carnitine-palmitoyltransferase 2.

substances from human plasma into OCT1 active and OCT1 deficient cells by untargeted and semi-targeted metabolomics.

# MATERIALS AND METHODS

## Clinical Study

In 65 healthy male and female individuals, IBC was measured in plasma after overnight fasting. Blood was taken in mornings on up to eight occasions with intervals of at least 1 week to compare intra- vs. interindividual variation and relation to OCT1 genotypes. Urine samples and corresponding plasma samples at the beginning and at the end of the urine collection period were collected by another 30 unrelated healthy volunteers after overnight fasting (3 h collection period). Renal clearance of IBC was calculated as the ratio of the amount of IBC excreted within the 3 h collection period over the plasma area under the concentration time curve of IBC from the same 3 h interval. Ethylenediaminetetraacetic acid was used as the anticoagulant for blood sampling in both studies. Plasma samples from volunteers that had participated in studies on the effect of OCT1 genotype on pharmacokinetics of fenoterol (Tzvetkov et al., 2018), sumatriptan (Matthaei et al., 2016), and proguanil (Matthaei et al., 2019) were used to correlate IBC blood concentrations with pharmacokinetics of these drugs. The studies were approved by the ethics committee of the University Medicine Göttingen and the relevant regulatory authories (EudraCT 2012-003546-33) and all volunteers had given written informed consent.

## Organic Cation Transporter 1 Genotyping

OCT1 genotyping was performed on DNA extracted from blood samples by solid-phase extraction. The genotyping procedure was described detailed elsewhere (Matthaei et al., 2016). In brief, primer extension assays were performed for the variants Ser14Phe (rs34447885), Arg61Cys (rs12208357), Cys88Arg (rs55918055), Pro117Leu (rs200684404), Ser189Leu (rs34104736), Gly401Ser (rs34130495), Met420del (rs202220802), and Gly465Arg (rs34059508). Almost all study samples were genotyped in duplicate, with 100% concordant results.

## Isobutyrylcarnitine Blood and Urine Concentration Analyses

Quantification of IBC plasma and urine concentrations was performed *via* liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) after precipitation. The detailed protocol is provided in the supplementary methods.

## Uptake and Efflux of Carnitine, Acylcarnitines, Valine, or Known Substrates

Transport experiments were performed with primary human hepatocytes (Thermo Fisher Scientific, Darmstadt, Germany) or HEK293 cells stably transfected to overexpress hOCT1 or mOCT1. As a control, cells transfected with the empty vector pcDNA5 were used. The generation and validation of the cell lines was described previously (Saadatmand et al., 2012; Meyer et al., 2020). Uptake or efflux of carnitine, acylcarnitines, or valine were performed with radiolabeled or deuterated substrates and quantified by scintillation counting or LC-MS/MS. For the latter, specific mass transitions and voltages were used (**Supplementary Table S1**). The cell number of each experiment was normalized by total protein measurement in representative wells by using the bicinchoninic acid assay (Smith et al., 1985). Detailed descriptions of the methods are provided in the supplementary files.

## Metabolomics

To identify endogenous substrates of mouse and human OCT1, we performed untargeted metabolomics. Plated HEK293 cells overexpressing mOCT1, hOCT1 or the empty vector were incubated with pooled fresh frozen plasma. After lysis and protein quantification for normalization purposes, lipids and proteins were removed by a modified Bligh and Dyer method (Bligh and Dyer, 1959). The detailed protocol of sample workup is provided in the Supporting Information section online. Detection of metabolites was performed by mass spectrometry on a *Xevo G2-S QToF*. Analysis was performed using *MassLynx 4.1* (Waters, Milford, United States), *Progenesis QI 2.4* (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) as well as *Metaboanalyst 4.0* (Chong et al., 2019). Identification of metabolites was achieved by an in-house database as well as the HMDB database *via Progenesis* software (Wishart et al., 2007).

## Statistics

Linear regression was used to determine correlation between IBC plasma concentrations and known OCT1 substrates or metabolites. Statistical significance of differences between two groups was analyzed using the Student's *t*-test and presentation of means and standard errors of mean (SEM). Comparisons between more than two groups were analyzed by one-way ANOVA with Tukey post-hoc test. $^{*}p < 0.05$; $^{*}p < 0.01$; $^{***}p < 0.001$. Trends of mean IBC plasma concentrations were analyzed by linear regression analysis. Unless noted otherwise, all *in vitro* analyses were performed at least with 3 independent replicates. The entire study sample available was used to compare IBC concentrations between carriers or two, one or zero fully active OCT1 alleles.

# RESULTS

## Clinical Studies Confirmed Association of Plasma Isobutyrylcarnitine and Organic Cation Transporter 1 Activity

To confirm the association between low plasma IBC and loss-of-function polymorphisms in OCT1, we analyzed the plasma IBC concentrations in 65 healthy volunteers. Plasma IBC concentrations were significantly higher in individuals carrying two wild-type alleles [22.6 ± 2.6 ng/ml (mean ± SEM)], compared to carriers of one (13.8 ± 1.1 ng/ml) or zero fully active OCT1 alleles (7.4 ± 0.7 ng/ml, $p < 0.0001$ in linear regression analysis,

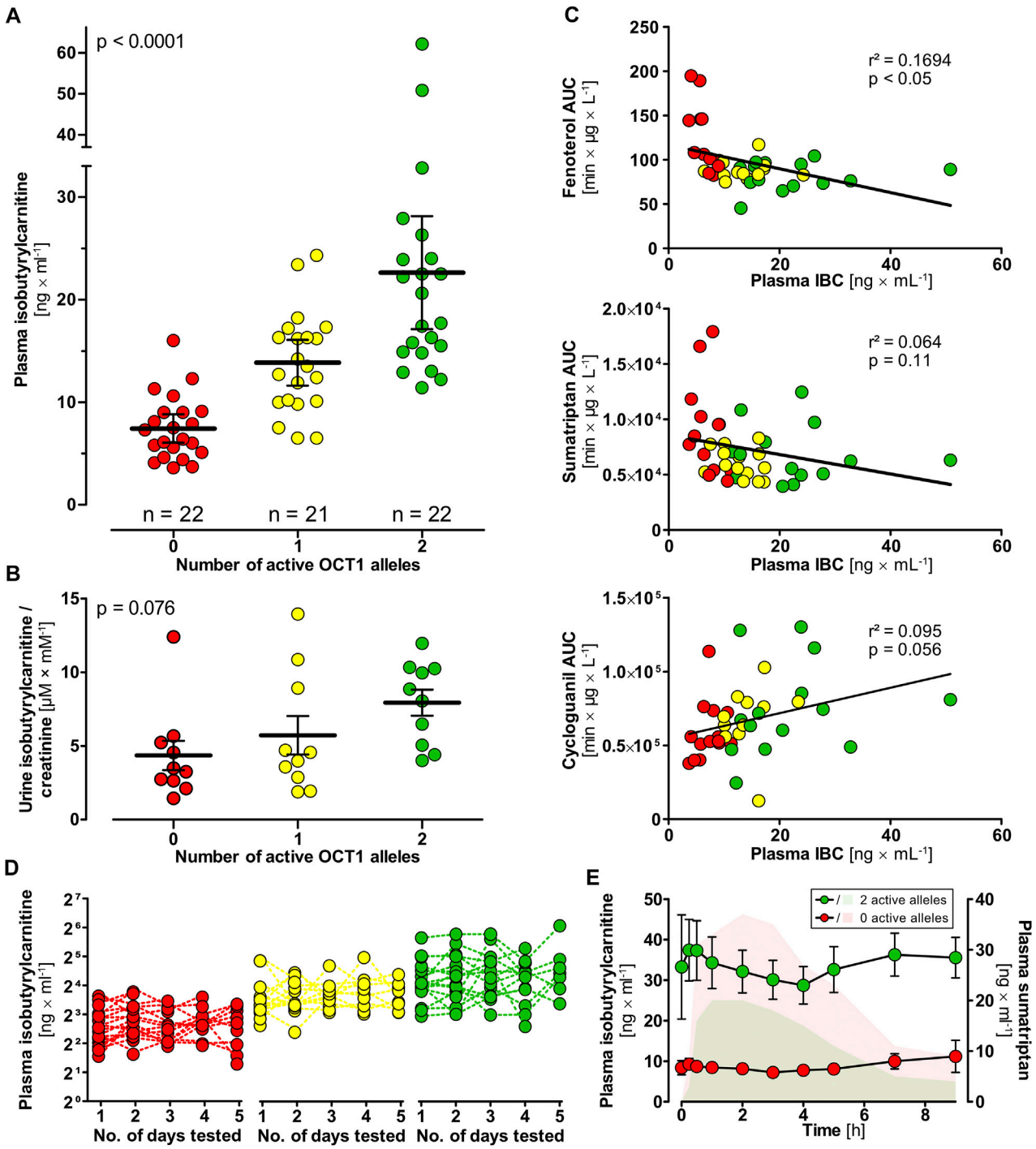


**FIGURE 2 |** Isobutyrylcarnitine plasma concentrations in healthy volunteers (red circles, 0 active OCT1 alleles; yellow circles, 1 active OCT1 allele; green circles, 2 active OCT1 alleles). **(A)** Higher IBC plasma concentrations could be observed in individuals with active OCT1 compared to OCT1-deficient individuals (statistical testing performed using one-way ANOVA). **(B)** IBC urine concentrations were higher in individuals with active OCT1 compared with deficient OCT1 as well, however, not statistically significant ($p$ = 0.076, linear regression analysis). **(C)** Mean IBC plasma concentrations showed genotype-dependent correlation with AUCs of the known OCT1 substrates fenoterol, sumatriptan, and cycloguanil (linear regression statistics). **(D)** High interindividual but low intraindividual variation in of plasma IBC was found supporting a strong genetic background for human plasma IBC. **(E)** IBC plasma concentrations (circles) after a single oral dose of 50 mg sumatriptan. Sumatriptan plasma concentrations of individuals with two reference alleles (green AUC) and individuals with zero active alleles are indicated. This data indicates that the IBC-OCT1 correlation is not only mediated by OCT1 genotype but also be OCT1 activity. Data are represented as the mean ± SEM.

**Figure 2A**). It is to note, that the genotypes of the groups with one or zero active alleles are more diverse and comprise the variants OCT1*2 to OCT1*6 as inactive alleles (**Supplementary Table S2**). The measurements over time showed a stable course in each group classified by genotype (**Figure 2D**). Standard deviation between the means of all subjects was 8.73 ng/ml compared with a much lower standard deviation within all subjects of 4.24 ng/ml. The corresponding genetic component was 0.76, indicating that as much as 76% of the variation in IBC blood concentrations may be due to heritable factors (Kalow et al., 1998; Kalow et al., 1999). Amongst all tested factors possibly affecting the IBC blood concentrations, the OCT1 genotype was the most important one (multiple regression: $\beta = 0.60$, $p = 3.9 \times 10^{-7}$, $r = 0.64$). Other factors, such as age, sex, weight or body height were not significant.

Isobutyrylcarnitine concentrations in urine were highest in individuals carrying two reference alleles, followed by individuals with one and zero active alleles (not significant in one-way ANOVA, **Figure 2B**). Unlike in blood, the other acylcarnitines (acetylcarnitine, propionylcarnitine, 2-methylbutyrylcarnitine, succinylcarnitine) were statistically not significantly associated with OCT1 genotype in urine. Mean renal clearances were 139 ml × $min^{-1}$, 104 ml × $min^{-1}$, and 234 ml × $min^{-1}$ with an SEM of 10, 15, and 45 for volunteers with two, one and zero wild-type active OCT1 alleles ($p < 0.01$, linear regression analysis).

Plasma IBC concentrations correlated with the pharmacokinetics of the known OCT1 substrates fenoterol ($r^2 = 0.169$, $p < 0.05$) and sumatriptan ($r^2 = 0.064$, $p = 0.11$) as well as with cycloguanil, the metabolite of the OCT1 substrate proguanil ($r^2 = 0.095$, $p = 0.056$; **Figure 2C**). In healthy volunteers who had received a single oral dose of 50 mg sumatriptan (Matthaei et al., 2016), we observed a reduction of IBC occurring slightly delayed after the peak drug concentrations, but in volunteers with two active OCT1 alleles only (**Figure 2E**).

## Mechanisms Underlying the Isobutyrylcarnitine Organic Cation Transporter 1-Genotype Association

First, we wanted to characterize transport kinetics of IBC with human OCT1 (hOCT1). In HEK293 cells overexpressing hOCT1, uptake of IBC did not show saturated transport characteristics (**Figure 3A**). Moreover, uptake of IBC in these cells could not be inhibited by addition of the well-established OCT1 inhibitors 1-methyl-4-phenylpyridinium (MPP+) or 4-[4-(dimethylamino) styryl]-N-methylpyridinium (ASP+, **Supplementary Figure S1**). Human OCT1 is therefore unlikely to be a mediator of IBC cell uptake. In contrast, mOCT1 showed low affinity-high capacity influx transport with a $K_M$ value of 1.47 ± 0.17 mM (mean ± SEM) and a $v_{max}$ of 8.50 ± 0.41 nmol × mg $protein^{-1}$ × $min^{-1}$. The hOCT1 and mOCT1 cell lines were validated with numerous substrates (Matthaei et al., 2016; Tzvetkov et al., 2018; Matthaei et al., 2019) with an excellent correlation between *in vitro* intrinsic clearance and *in vivo* pharmacokinetic data, indicating that this model cell line is reflecting membrane transport in humans quite well.

The corresponding transport experiments with the human carnitine transporter hOCTN2 showed a $K_M$ of 72.7 ± 18.6 μM (mean ± SEM) and a $v_{max}$ of 2.06 ± 0.08 nmol × mg $protein^{-1}$ × $min^{-1}$ for IBC, which underlines the well-known capabilities of hOCTN2 to transport not only carnitine but also acylcarnitine species in humans. Altogether, in contrast to mOCT1, hOCT1 was not capable of accelerating cell uptake of IBC, but IBC may be transported by hOCTN2.

## Substantial Interspecies Differences in Carnitine Efflux Transport

The IBC-OCT1 genotype association might also be explained by an effect of OCT1 genotype on hepatocellular availability of the precursors carnitine and valine. However, the efflux of carnitine was strongly increased upon overexpression of mOCT1 but not, or only to a very moderate extent, by hOCT1 (**Figure 3B**) when normalized by total carnitine after the incubation phase. Compared to reference hOCT1, cell lines expressing common loss-of-function hOCT1 allelic variants (hOCT1*2 and *3) showed even reduced carnitine efflux and ranged between reference hOCT1 and the empty-vector control cell line (**Supplementary Figures S2A,B**). However, absolute [$^3$H]-carnitine efflux into the cell culture medium was not significantly increased by overexpression of mOCT1 or hOCT1 (**Figure 3C**), which indicates that seemingly existing differences in carnitine efflux occurred solely by unequal preloading. Preloading was much higher with mOCT1 compared with hOCT1. This shows that simple normalization after unequal preloading is not sufficient and conveys a misinterpreting message compared to efflux with similar intracellular starting concentrations. However, the capacity of hOCT1 to catalyze carnitine influx was shown earlier (Kim et al., 2017) and confirmed by the higher preloading in hOCT1 active cells compared with control cells. Thus, one explanation why IBC was higher with high OCT1 activity might simply be a better supply of carnitine in hepatocytes.

To provide direct evidence for mOCT1-and hOCT1-mediated IBC efflux transport, we investigated the efflux of IBC after preloading with IBC. Because substance efflux is always the sum of (linearly concentration dependent) intracellular concentration of the substance undergoing efflux plus the transporter mediated acceleration of efflux, normalization of the starting condition is very important to characterize transporter-mediated efflux. Normalized efflux was high in mOCT1 overexpressing cells, limited only by intracellular amounts (**Figure 4A**). After 30 min, almost all preloaded IBC was effluxed into the extracellular medium. In contrast, mOCT1 did apparently not accelerate IBC efflux. As illustrated, there was only a minor difference between hOCT1 and the empty-vector control cell line. However, much higher intracellular IBC concentrations could be found after the 30 min preloading period in mOCT1 overexpressing cells, which reflects the differences in IBC uptake between mOct1 and hOCT1 (**Figure 4A**). As a consequence, absolute IBC efflux was dramatically stronger *via* mOCT1, compared to human OCT1 and negative control. Thus, the observed efflux activity published

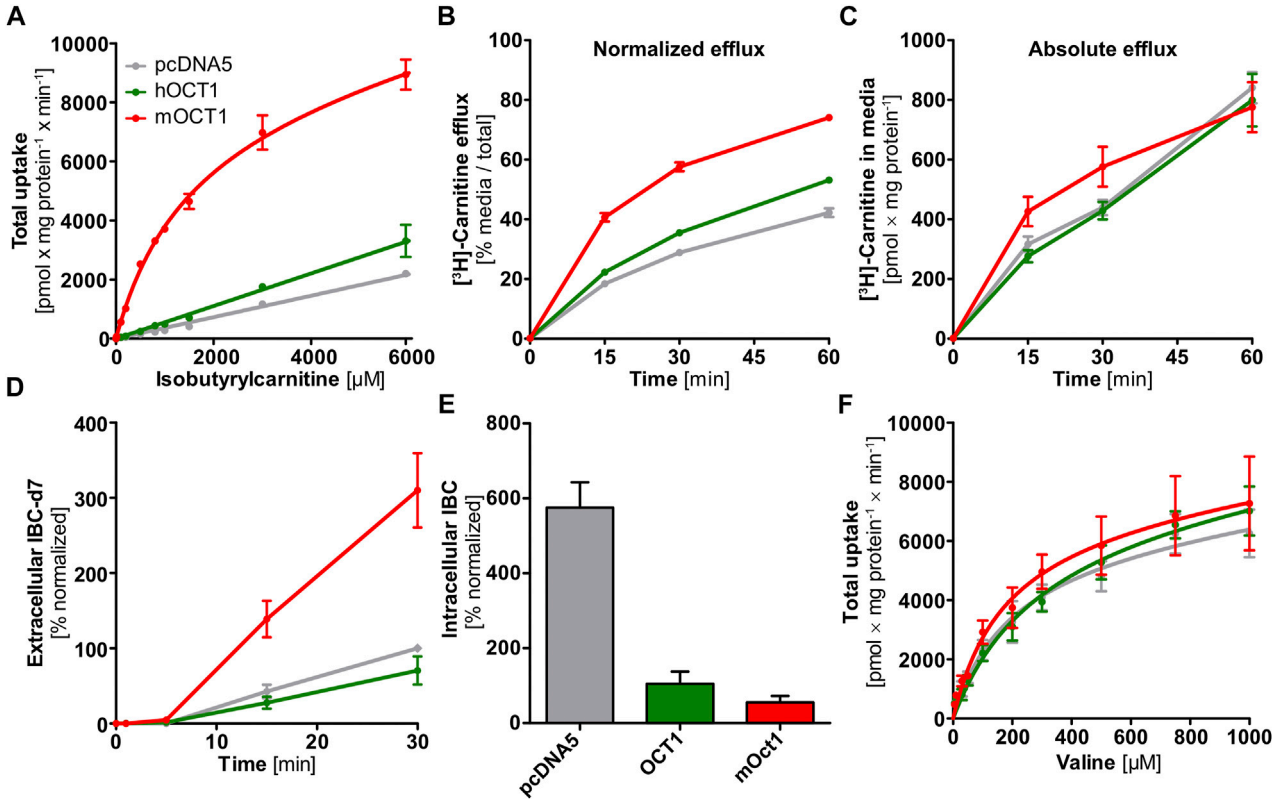


**FIGURE 3 |** *In vitro* uptake and efflux experiments with carnitine, isobutyrylcarnitine, or valine. **(A)** In contrast to HEK293 cells overexpressing mOCT1, hOCT1 did not show uptake of IBC significantly above the uptake seen in empty-vector control cells (pcDNA5). **(B)** HEK293 cells overexpressing mOCT1 showed increased efflux of radiolabeled carnitine, compared to hOCT1 and empty vector control (pcDNA5) when calculated in relation to total carnitine in medium and cells. **(C)** Absolute carnitine efflux did not differ between cells overexpressing hOCT1 and empty vector control and also with mOCT1 there was a moderate effect only. **(D)** Efflux of IBC-d7 was observed after incubation with valine-d8 only for mOCT1. **(E)** Intracellular IBC concentrations under regular culturing conditions depended strongly on the overexpression of OCT1 and are higher without hOCT1 or mOCT1 ($p < 0.0001$, one-way ANOVA with Tukey post-hoc test). **(F)** Valine was studied as the precursor of IBC, Valine uptake by mouse and human OCT1 compared to empty-vector control cell line (pcDNA5). **(A–F)** Data are represented as the mean ± SEM from at least three independent experiments. Total uptake implies the intracellularly accumulated substance normalized by total protein and time, without subtraction of uptake into empty vector-overexpressing cell line "pcDNA5".

earlier (Kim et al., 2017) and found in our experiments normalized by total [$^3$H]-carnitine (**Figure 3B**) might be the result of the increased preloading prior to measurement of efflux.

To differentiate whether the observed efflux by mOCT1 was the result of higher preload or a really higher efflux activity, IBC concentrations for pre-incubation were adjusted to match the concentration in empty-vector control cells after the preloading phase. After adjustment, not any efflux of IBC was found by hOCT1, neither with nor without normalization to total IBC. In contrast, mOCT1 overexpressing cells showed an increased efflux into the medium, especially in absolute terms (**Figure 4B**; **Supplementary Figure S3B**).

## Formation and Efflux of d9-Acylcarnitines

OCT1-dependent differences in plasma concentrations of IBC and other acylcarnitines could depend on their biosynthesis. To track the intracellular formation and subsequent efflux of acylcarnitine species, cells overexpressing mouse or human OCT1 were incubated with deuterated carnitine (carnitine-d9). By this, also the produced acylcarnitines were deuterated and could be specifically quantified (**Supplementary Table S1**) showing that human OCT1 overexpressing cells did not differ from empty-vector control cells with respect to efflux of acylcarnitines without or with inhibition of OCT1. In contrast, cells overexpressing mOCT1 revealed lower intracellular concentrations of IBC-d9, independent of glucose concentrations in the medium (**Figure 5A** and **Supplementary Figure S4**). Additional experiments with high and low glucose in the media were performed because catabolism of lipids and amino acids might significantly depend on the glucose supply. Correspondingly, the extracellular concentrations of these acylcarnitines were elevated compared to cells overexpressing hOCT1 or empty-vector control cells. These effects of mOCT1 could be reduced or even inverted by addition of the OCT1 inhibitor desipramine during the efflux phase of the experiment. Similar observations were also made for other acylcarnitine species, such as butyrylcarnitine-d9, hexanoylcarnitine-d9, and many more (**Supplementary Figure S4**). Interestingly, the concentration of glucose in the medium did significantly influence the amount of formed product. However, the effect

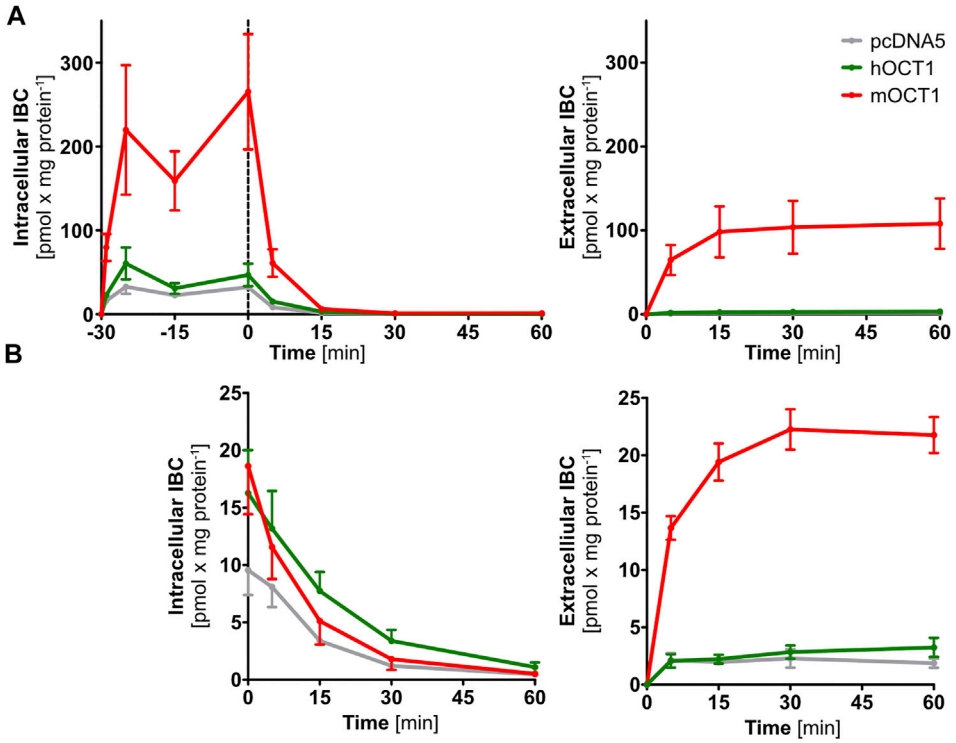


**FIGURE 4 |** Time-dependent isobutyrylcarnitine efflux. Absolute efflux of IBC without **(A)** and with **(B)** adjustment of preloading IBC concentrations revealed IBC efflux capabilities of mOCT1 but not hOCT1. In the upper right part, intracellular concentrations are shown during the course of preloading and then, after stop of preloading by changing the media, the efflux is shown. In the other three figures, only the efflux phase is shown. Data are represented as the mean ± SEM and result from at least three independent experiments.

of OCT1 was similar with high and low glucose (**Figure 5A**). Again, this experiment confirmed that only mOCT1, but not hOCT1 acts as efflux transporter of acylcarnitine derivatives.

## Comparative Efflux of Acylcarnitine Species by Murine and Human Primary Hepatocytes

In addition to cells overexpressing mouse or human OCT1, primary hepatocytes were used to evaluate the capability of both species to facilitate efflux of acylcarnitines. Results showed that—in those cases in which the concentrations differed significantly—remaining intracellular amounts of acylcarnitine species were lower in mouse hepatocytes and higher in human hepatocytes after the 30 min efflux period (**Supplementary Figure S5**). Vice versa, extracellular amounts of acylcarnitine species were higher in the medium of mouse hepatocytes and lower in the medium of human hepatocytes, which resulted in increased extracellular/intracellular ratios (**Figure 5C**). The most striking results were found for butyrylcarnitine, of which extracellular concentrations were more than twice as high in the medium of mouse hepatocytes, while intracellular concentrations were about thrice as high in human hepatocytes. This confirmed the comparatively enhanced acylcarnitine efflux capability of mouse hepatocytes compared to human hepatocytes, as it was shown by stable transfected HEK293 cells overexpressing mouse or human OCT1.

## Tracing Organic Cation Transporter 1 Depending Isobutyrylcarnitine by Use of Deuterated Valine

Uptake of valine, the IBC precursor, was not different between the empty vector control and OCT1 overexpressing cells, which excludes an increased valine uptake as the direct cause for elevated IBC levels (**Figure 3F**). Saturation of uptake was similarly overserved for the empty-vector control cell line as well—probably mediated by amino acid transporters.

Use of valine-d8 offered another chance to trace the mOCT1 vs. hOCT1 dependent cellular fate of isobutyrylcarnitine. Conversion to isobutyryl-d7-CoA and the subsequent formation of IBC-d7 from valine-d8 was tracked based on the deuteration-altered mass. The intracellular accumulation of IBC-d7 was lower in mOCT1 and hOCT1 overexpressing cells compared to empty-vector control cells, while the extracellular culmination of IBC-d7 was the highest in mOCT1 overexpressing cells (**Figure 3D**). These findings indicate the involvement of mouse and human OCT1 in transport of substances influencing cell metabolism. However, in connection with the other experiments (**Figures 3**,**4**) this does neither proof IBC uptake nor efflux by hOCT1. It is to note that a general imbalance was seen in HEK293 cell lines under regular culturing conditions. Non-deuterated IBC was increased intracellularly in the control cell line by about ten-fold compared to the same cell line

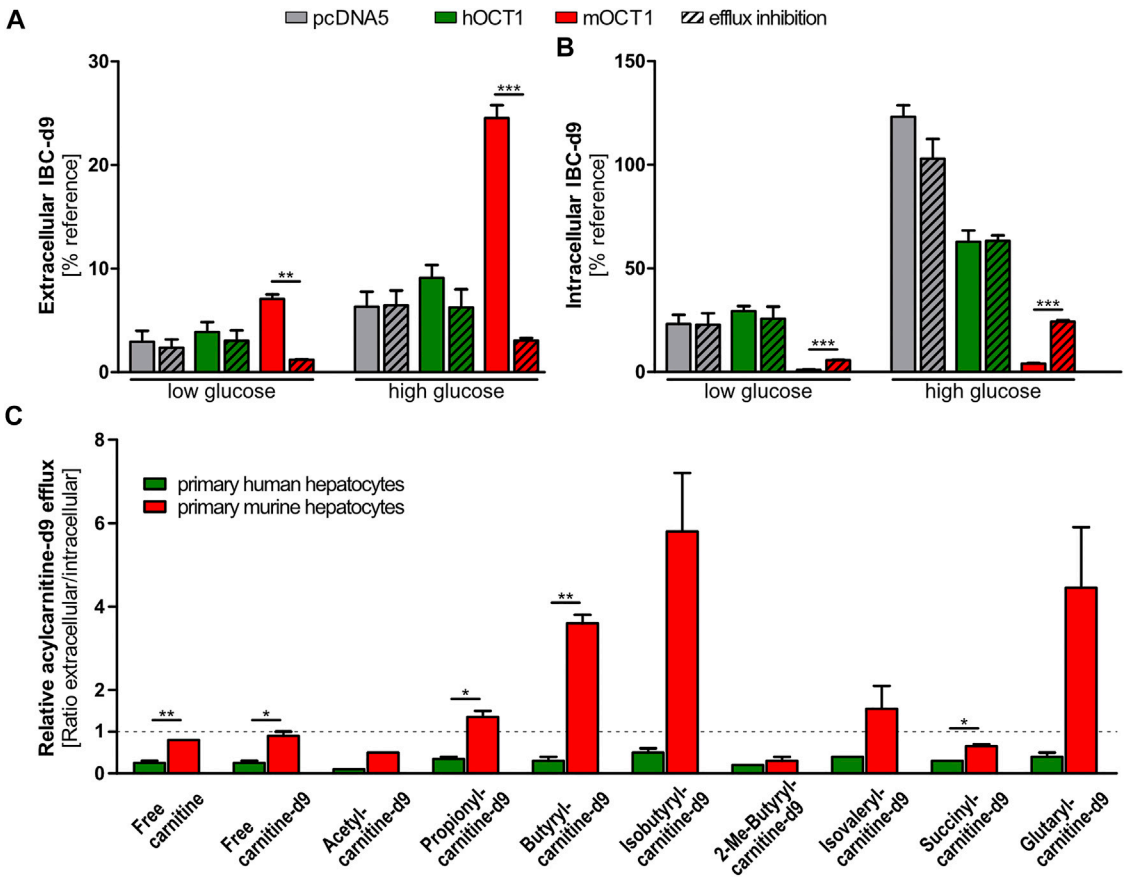


**FIGURE 5 |** Efflux of acylcarnitines. **(A)** Efflux of acylcarnitines after incubation with carnitine-d9 led to increased extracellular and reduced intracellular isobutyrylcarnitine and other acylcarnitine species. Inhibition of OCT1 by desipramine (indicated with '+') reduced or hindered this efflux. **(B)** Murine and human primary hepatocytes revealed distinguishable patterns of intra- and extracellular acylcarnitine species after preloading with deuterated carnitine (carnitine-d9). Again, only experiments with mouse OCT1 showed a consistent efflux activity of mOCT1 (red columns). Glucose concentration in the medium may change metabolic pathways but as seen, the relative effects of hOCT1 and mOCT1 were the same irrespective of glucose supply. **(A)** and **(B)** Data are represented as the mean ± SEM from at least three independent experiments. Normalization was conducted by comparison to intracellular carnitine-d9 post efflux. **(C)** Efflux from primary murine or human hepatocytes appears different for several acylcarnitine species. Measurement in supernatant medium and in cells showed increased efflux by murine hepatocytes for most investigated acylcarnitines. Data are represented as the mean ± SEM. Statistical analysis of two groups were performed using the Student's *t*-test,*$p < 0.05$; *$p < 0.01$; ***$p < 0.001$.

overexpressing mOCT1 and about five-fold compared to the cell line overexpressing hOCT1 (**Figure 3E**). This indicates a constant efflux of IBC or general differences in metabolism, leading to varying formation.

## Comparative Efflux of Other Known Substrates by Mouse and Human Organic Cation Transporter 1

To evaluate a potentially diverging capability of OCT1 to facilitate efflux in general, we investigated the efflux of five known OCT1 substrates. After adjusting intracellular preloading concentrations to those in the empty-vector control cell line, the efflux was characterized by quantifying the substances in the supernatant and the cell lysate. None of the tested substrates were subject to efflux by mOCT1 (**Supplementary Figure S6**) and only metformin efflux transport was enhanced by hOCT1 overexpression compared with the control cell line (**Supplementary Figure S6D**). Lower extracellular concentrations of proguanil in cells overexpressing hOCT1 compared to control cell line point towards the simultaneous uptake (**Supplementary Figure S6B**). Overall, the differences between mOCT1 and hOCT1 concerning IBC efflux could not be observed for other OCT1 substrates. In particular, these experiments with 5 drugs may indicate that there is no generally higher propensity of mOCT1 to catalyze efflux transport compared with hOCT1.

## Metabolomics Mouse and Human Organic Cation Transporter 1 Uptake Profile

The association between OCT1 genotype and blood IBC concentrations may be explained by a more complex metabolic crosstalk with involvement of other substances not

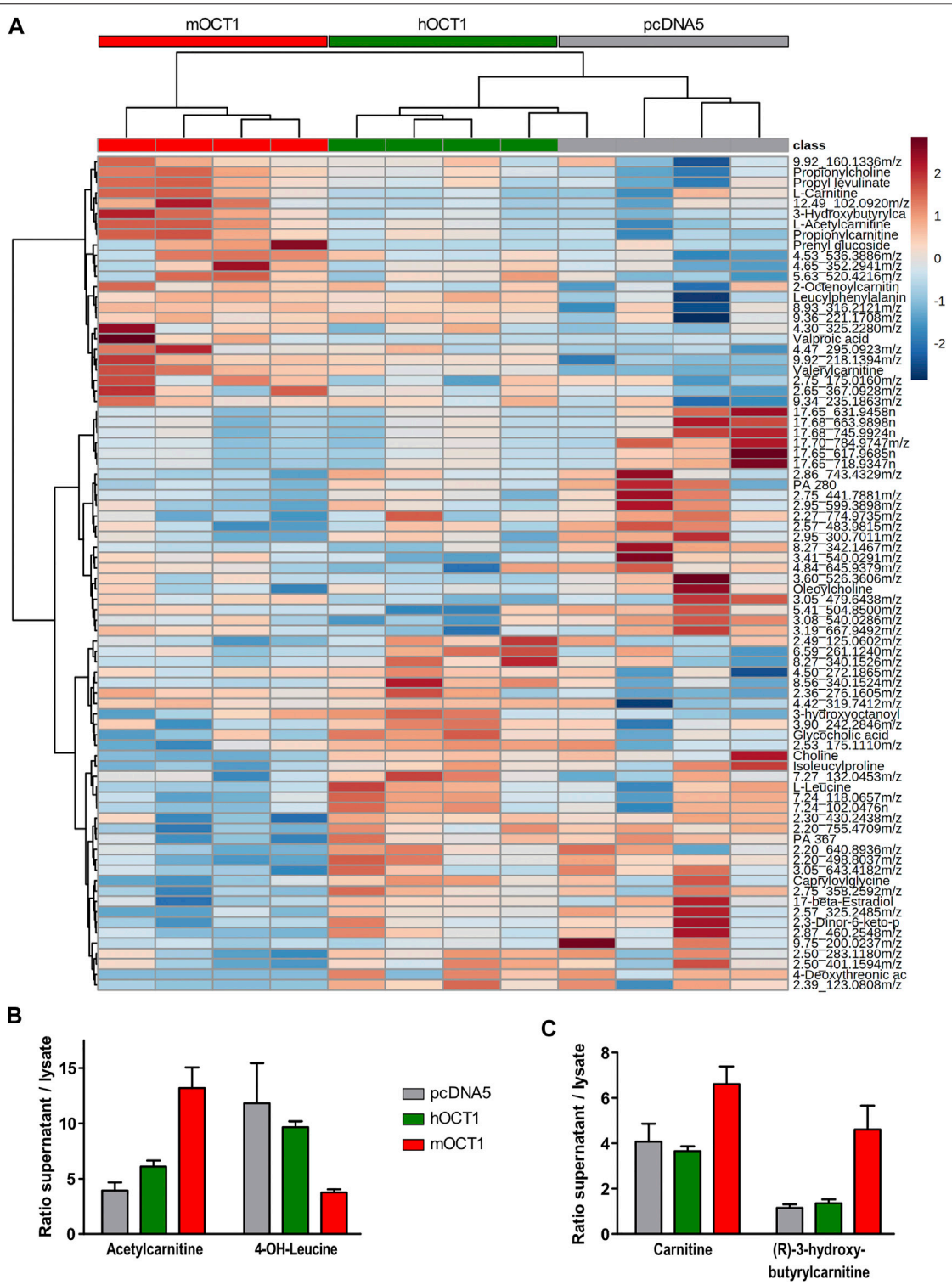


**FIGURE 6 |** Metabolomic analyses from hOCT1 or mOCT1 overexpressing HEK293 cells **(A)** Hydrophilic interaction chromatography coupled with positive ionization mode mass spectrometry led to identification of metabolites differentially taken up from human normal donor blood plasma. If substances could not unambiguously be identified based on their masses, the retention times and the mass-to-charge ratios are provided. Data for heatmap was normalized and clustering of the 80 features with lowest ANOVA *p*-value is shown. Euclidean distance and Ward-clustering were used. As indicated by the red, green and gray bar showing the mOCT1, hOCT1 and empty vector transfected cell lines, with one exception clustering reflected the different effects of mOCT1 and hOCT1 very well **(B)** Identification by retention time and mass revealed varying uptake by hOCT1 and mOCT1. Mean ± SEM, $n = 4$ **(C)** Differences were also observed for substances identified only by mass database. Data are represented as the mean ± SEM from four independent experiments.

yet known to us in the present context. Therefore, untargeted and semi-targeted metabolomics analyses were performed on human plasma and lysates of HEK293 cells overexpressing mouse or human OCT1 after incubation with human plasma. The extracellular/intracellular ratio was used to identify the top 80 metabolites, which were differentially transported into the cells (**Figure 6A**). Amongst the positively ionized metabolites, the most striking differences were observed for acetylcarnitine and 4-OH-L-leucine, for which mOCT1 overexpressing cells showed an increased (acetylcarnitine) or decreased (4-OH-leucine) extracellular-to-intracellular ratio compared to both, hOCT1 overexpressing and empty-vector control cells (**Figure 6B**). Also 3-hydroxybutyrylcarnitine (an intermediate in the catabolism of lysine and tryptophan) and L-carnitine were subject to mOCT1 efflux with a 3.5-fold and a 1.5-fold increase of the ratio, respectively (**Figure 6C**).

Also several anionic substances were affected by hOCT1 or mOCT1 overexpression. The extracellular-to-intracellular ratio for OCT1 was increased for 14-hydroxymyristic acid (14-hydroxytetradecanoic acid) by about 2.5-fold, compared to negative control and mOCT1. Furthermore, the ratio for 5-methyl cytidine, docosahexaenoic acid and 9-oxodecanoic acid was reduced by mOCT1 overexpression (data not shown).

Since our *in vitro* systems could not identify any relevant IBC transport by human OCT1, we speculated that the IBC-OCT1 correlation may be due to a more complex metabolic or signaling crosstalk. It might well be that OCT1 transports endogenous regulators of energy metabolism and the resulting differences might then result in the correlation between OCT1 activity and IBC. A strong regulator of energy metabolism is PPARα. Therefore, we studied the effects of the PPARα agonist fenofibrate and the antagonist MK886 on intracellular IBC concentrations. As seen in **Figure 7**, there was a remarkable effect of these transcriptional modulators on IBC, but the effect did not significantly differ depending on presence or absence of human OCT1.

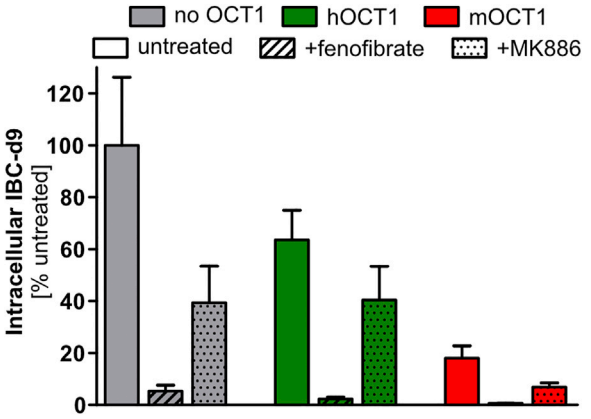


**FIGURE 7 |** Isobutyrylcarnitine depletion inducible by PPARα activation. Pretreatment for 24 h with 33 μM fenofibrate, an activator of PPARα, led to strong reduction of intracellular IBC. But somehow unexpectedly, the PPARα inhibitor MK886 also reduced intracellular IBC. Data are represented as the mean ± SEM from four independent experiments. This data illustrates that the OCT1 effect on IBC concentrations may also be due to more complex signaling interactions and not due to an OCT1 mediated membrane transport of IBC.

## DISCUSSION

Human organic cation transporter OCT1 is characterized by extensive mostly genetic variation with complete lack of activity in several percent of many populations on the one hand and very high expression and activity in the remaining population. Understanding the endogenous physiological implications of this variation is a most exciting issue of pharmacogenomics. This study confirmed that carriers of hOCT1 genotypes conferring reduced or deficient activity have on average more than threefold lower IBC blood concentrations than carriers of OCT1 reference genotype. IBC blood concentrations showed high interindividual but low intraindividual variation, indicating significant heritability of IBC formation and/or transport. IBC blood concentrations correlated with pharmacokinetics of known OCT1 substrates confirming suitability of IBC as an endogenous *in vivo* biomarker of OCT1 activity. With all the clinical and experimental data presented here we aimed to elucidate the mechanisms behind the relationship between human OCT1 activity and concentrations of carnitine derivatives in human blood. Experiments with cell lines overexpressing hOCT1 and mOCT1 and with more "naturalistic" primary human hepatocytes should contribute to the understanding of the mechanisms behind the OCT1 genotype-dependent differences in IBC concentrations (**Figures 2**,**4**,**5**) (Suhre et al., 2011; Kim et al., 2017). These experiments revealed that hOCT1, in contrast to mOCT1, did not exhibit any efflux activity for acylcarnitines. Experiments with primary hepatocytes from both species underlined these interspecies differences between hOCT1 and mOCT1, while other hepatic transporters might contribute in these primary cells as well. None of the results were compatible with the previously suggested explanation for the IBC-OCT1-genotype association, namely that hOCT1 would be an IBC efflux transporter (Kim et al., 2017). Apparently the simple experimental systems of HEK cells overexpressing hOCT1 or primary human hepatocytes expressing OCT1 (**Figure 5B**) cannot mimic or explain the human IBC OCT1-genotype association. Since all clinical data presented here are compatible with the hypothesis that OCT1 is an efflux transporter for IBC, the most obvious explanation is that OCT1 behaved in all our cell culture experiments differently than in the human body. While we cannot finally exclude this, we and other investigators have long lists of OCT1 substrates where the *in vitro* data correlated excellently with the human pharmacokinetic data (Shu et al., 2008; Tzvetkov et al., 2011; Fukuda et al., 2013; Tzvetkov et al., 2013; Matthaei et al., 2016; Tzvetkov et al., 2018). Based on that we still have to seek for other mechanisms behind the association between human blood plasma IBC concentrations and OCT1 genotype and OCT1 activity as well (**Figure 2**). However, the fact that known inhibitors had no effect on the uptake of IBC *in vitro* (**Supplementary Figure S2**) while the intake of sumatriptan

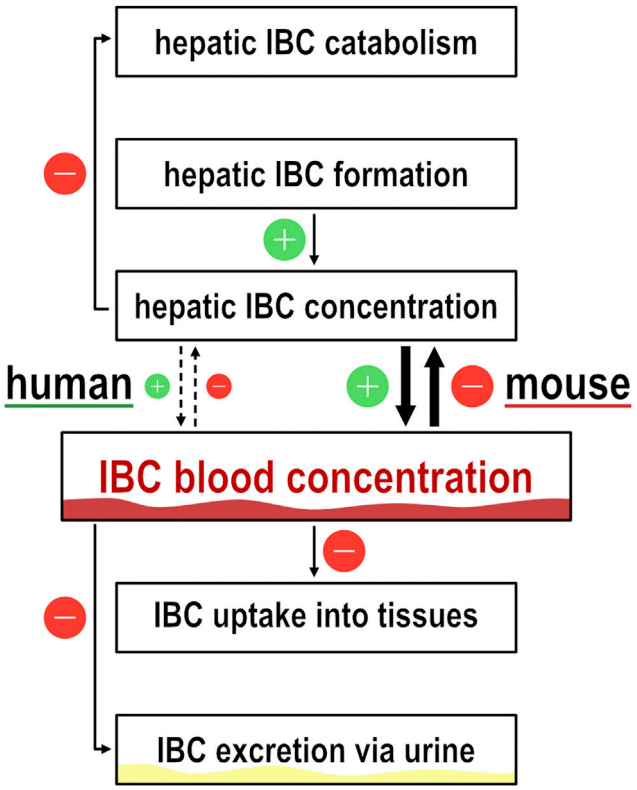


**FIGURE 8 |** Illustration of isobutyrylcarnitine dynamics. Flow chart to illustrate the dynamics of isobutyrylcarnitine blood concentrations. Icons with '+' and '–' illustrate the effect on IBC blood concentration by the respective pathway.

led to a decrease in IBC plasma concentrations *in vivo* (**Figure 2E**) suggests that the connection between OCT1 activity and increased IBC plasma concentrations cannot simply be the result of inhibition of IBC transport by OCT1. Since OCT1 is also a carnitine transporter, inhibition of carnitine hepatocellular uptake might be an explanation why carnitine derivatives are lower when OCT1 activity is low. However, there is at least one other strong carnitine uptake transporter, OCTN2, and it is questionable why hOCT1 should be limiting in this situation.

Generally, high IBC blood concentrations, and high blood concentrations of other carnitine derivatives as well, may be explained by higher formation rate and/or by lower elimination rate (**Figure 8**). The first alternative, formation rate of IBC depending on expression of OCT1 may be higher if OCT1 mediates efflux or simply only intracellular binding of IBC and thus preventing degradation of IBC *via* the citric acid cycle. The second alternative, elimination of IBC, is mostly *via* renal glomerular filtration and tubular secretion processes (Rebouche and Paulson, 1986; Mancinelli et al., 1995). Expression of OCT1 in renal tubular cells is controversial. But if it is true that OCT1 is expressed at the apical side of renal tubular cells (Tzvetkov et al., 2009), high OCT1 activity could result in higher re-absorption and thus lower elimination rate. This could be observed in our measurements of IBC in urine (**Figure 2B**) and resulting renal clearances were indeed higher in carriers of two functionally inactive OCT1 alleles than in those with active OCT1. This would be compatible with tubular reabsorption of IBC. However, the extent of differences in IBC plasma concentration cannot be explained by a moderate renal reabsorption and this concept again would require OCT1 mediated influx transport which was not observed in our hOCT model cell lines.

High human IBC blood concentration was interpreted to be the result of high OCT1 efflux activity, but numerous other explanations may similarly explain these correlations. Generally, high concentrations of carnitine derivatives may reflect the status of cell energy metabolism (Roe et al., 1984; Roe et al., 1998; Sass et al., 2004). A relative thiamine deficiency might result in a shift from oxidative metabolism to fermentation, since several biochemical reactions within the citric acid cycle, but also the conversion of 2-oxoisovalerate to isobutyryl-CoA, depend on the cofactor thiamine pyrophosphate (Perham, 2000). Reduced activity of isobutyryl-CoA dehydrogenase leads to increased plasma levels of isobutyrylcarnitine (Koeberl et al., 2003). Similarly, elevated isovalerylcarnitine could be found as a result of impaired isovaleryl-CoA dehydrogenase activity (Roe et al., 1984). Thus, at a first glance thiamine might be the clue for the IBC-OCT1 relationship in humans. However, under thiamine deficiency, blood concentrations of acylcarnitine derivatives should be lower in OCT1 deficiency-coding than in active OCT1 genotypes. In addition, as shown by several clinical and cell culture experiments, OCT1 is not relevant for thiamine pharmacokinetics in humans and is only one of several thiamine transporters in human cell lines (Jensen et al., 2020).

Another interesting point is, whether or not OCT1 can function as an efflux transporter. Based on current mechanistic understanding of the alternating access model proposed for OCT1 (Koepsell, 2015), both influx and efflux transport may be mediated by this transporter but Hendrickx *et al.* identified only 3 out of 354 substances potentially transported out of the cell by hOCT1 (Hendrickx et al., 2013). Here we tested this in more detail for known OCT1 substrates, such as fenoterol, metformin, proguanil, ranitidine, and sumatriptan, and interestingly only for metformin an efflux transport activity was seen with hOCT1 (**Supplementary Figure S6**).

The experiments on $^{3}$H-carnitine efflux that led to the recently proposed efflux of IBC through hOCT1 as the explanation for elevated blood concentrations (Kim et al., 2017) were in general resembled in this work. Here, for the first time, the efflux of explicitly IBC revealed strong interspecies differences, with hOCT1 being much less capable of carnitine efflux than mOCT1, which stand in contradiction to the previous explanation. In addition, differences in uptake and intracellular preloading were considered and implied into *in vitro* transport experiments. These interspecies differences of OCT1 are well known and have been shown in multiple occasion, so that the transfer of findings across species cannot be performed unseen (Gorboulev et al., 1997; Zhang et al., 1997; Green et al., 1999; Schmitt et al., 2003; Meyer et al., 2020).

Eventually, alternative explanations for the correlation of elevated plasma levels and an active OCT1 are needed.

The relationship between blood concentrations of IBC might be mediated by OCT1-dependent uptake of valine, the precursor of isobutyryl-CoA/IBC, by OCT1—surplus of valine might lead to a surplus of IBC, which gets exported. But this hypothesis was not supported by our experiments (**Figure 3**), there is no valine transport *via* OCT1. IBC formation might be regulated by other metabolites, therefore we searched for other metabolic differences using metabolomics analyses. Indeed, there were numerous differences between OCT1 expressing cells compared with empty-vector control cells (**Figure 6**; **Supplementary Table S3**).

The plasma acylcarnitine composition in general reflects the cellular acyl-CoA pattern (Costa et al., 1999) and fasting increases acylcarnitines at the expense of free carnitine (Frohlich et al., 1978). Therefore, the presence or absence of a functional OCT1 could induce a shift in cellular metabolism, which becomes manifest as discriminative plasma levels of isobutyrylcarnitine. The metabolomics data presented in this study provides a reasonable picture underpinning the differences between human and murine OCT1 in uptake and efflux of their substrates. Intracellular concentrations of probably more than 100 endogenous substances was different depending on hOCT1 or mOCT1 overexpression, but extensive further research is needed to disentangle which of these differences are physiologically relevant.

Upregulation of PPARα is known to lead to an increase of short-chain acylcarnitines in urine (Patterson et al., 2009). There are some striking similarities between these PPARα effects on carnitine derivatives described by Patterson et al., 2009, and the effects of OCT1 genotype in humans found here. Also, our finding from the untargeted metabolomics assay that some longer-chained fatty acid derivatives (substances very unlikely to be transported *via* OCT1) were different depending on OCT1 expression might be compatible with the hypothesis that concentrations of endogenous regulators of PPARα are modulated by OCT1 explaining the IBC-hOCT1 relationship and this might also explain the association between OCT1 and lipid metabolism (Liang et al., 2018). In contrast to previously reported increased short-chain acylcarnitines upon PPARα activation *in vivo* (Patterson et al., 2009), in our *in vitro* experiments PPARα activation led to a strong reduction of IBC, but independent of hOCT1/mOCT1 overexpression. The experiments with a PPARα agonist and an antagonist did, however, not support that the OCT1 effect on IBC is mediated by an endogenous regulator of PPARα. However, effect of PPARα modulators being much bigger than that of OCT1 (**Figure 7**) illustrates that generally a more complex metabolic or signaling crosstalk could be the basis of the OCT1/IBC association.

In conclusion, here we confirmed the previous findings (Suhre et al., 2011; Rodrigues et al., 2018) that plasma IBC is strongly associated with OCT1 activity (**Figure 2**). However, the physiological basis of the association remains unclear. In this study, we showed that the previous suggested explanation that OCT1 acts as a hepatic efflux transporter for IBC (Kim et al., 2017) is of only limited validity in humans. We showed that mouse OCT1 is both, an uptake and efflux transporter of IBC, but human OCT1 is neither an uptake transporter nor an efflux transporter of IBC. Once again, this example shows the difficulties in the translation from mouse models to human conditions, and simple explanations of human physiology based on data from mice may sometimes point the wrong way. The precise mechanisms leading to the association between OCT1 activity and plasma IBC in humans have to be elucidated in the future, but plasma IBC association with OCT1 levels have been confirmed in multiple studies and thus plasma IBC could be regarded a valid biomarker for OCT1 activity.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University Medical Center Göttingen. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

OJ, JM, JB, and MT contributed to conception and design of the study. OJ, JM, HK, and MM conducted the research. OJ, HK, JB, and MT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.674559/full#supplementary-material

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# Effects of Genetic Polymorphism in CYP2D6, CYP2C19, and the Organic Cation Transporter OCT1 on Amitriptyline Pharmacokinetics in Healthy Volunteers and Depressive Disorder Patients

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The tricyclic antidepressant amitriptyline is frequently prescribed but its use is limited by its narrow therapeutic range and large variation in pharmacokinetics. Apart from interindividual differences in the activity of the metabolising enzymes cytochrome P450 (CYP) 2D6 and 2C19, genetic polymorphism of the hepatic influx transporter organic cation transporter 1 (OCT1) could be contributing to interindividual variation in pharmacokinetics. Here, the impact of OCT1 genetic variation on the pharmacokinetics of amitriptyline and its active metabolite nortriptyline was studied *in vitro* as well as in healthy volunteers and in depressive disorder patients. Amitriptyline and nortriptyline were found to inhibit OCT1 in recombinant cells with $IC_{50}$ values of 28.6 and 40.4 µM. Thirty other antidepressant and neuroleptic drugs were also found to be moderate to strong OCT1 inhibitors with $IC_{50}$ values in the micromolar range. However, in 35 healthy volunteers, preselected for their OCT1 genotypes, who received a single dose of 25 mg amitriptyline, no significant effects on amitriptyline and nortriptyline pharmacokinetics could be attributed to OCT1 genetic polymorphism. In contrast, the strong impact of the CYP2D6 genotype on amitriptyline and nortriptyline pharmacokinetics and of the CYP2C19 genotype on nortriptyline was confirmed. In addition, acylcarnitine derivatives were measured as endogenous biomarkers for OCT1 activity. The mean plasma concentrations of isobutyrylcarnitine and 2-methylbutyrylcarnitine were higher in participants with two active OCT1 alleles compared to those with zero OCT1 activity, further supporting their role as endogenous *in vivo* biomarkers for OCT1 activity. A

**Abbreviations:** $ASP^+$, 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide; AT, amitriptyline; AUC, area under the curve; CYP, cytochrome P450; IBC, isobutyrylcarnitine; NT, nortriptyline; OCT, organic cation transporter; SLC, solute carrier.

moderate reduction in plasma isobutyrylcarnitine concentrations occurred at the time points at which amitriptyline plasma concentrations were the highest. In a second, independent study sample of 50 patients who underwent amitriptyline therapy of 75 mg twice daily, a significant trend of increasing amitriptyline plasma concentrations with decreasing OCT1 activity was observed ($p$ = 0.018), while nortriptyline plasma concentrations were unaffected by the OCT1 genotype. Altogether, this comprehensive study showed that OCT1 activity does not appear to be a major factor determining amitriptyline and nortriptyline pharmacokinetics and that hepatic uptake occurs mainly through other mechanisms.

**Keywords: amitriptyline, CYP2C19, CYP2D6, drug transport, nortriptyline, OCT1, organic cation transporter 1, SLC22A1**

## INTRODUCTION

Amitriptyline (AT) is a tricyclic antidepressant that has been in use for the therapy of major depression and other psychiatric disorders since the 1960ies. It is still frequently used today but usually as second-line therapy, due to its risk for severe adverse reactions. In addition, AT and its active metabolite nortriptyline (NT) show large interindividual variation in pharmacokinetics and, accordingly, there is substantial interest in therapy individualisation by drug monitoring and using molecular genetic biomarkers for polymorphic drug membrane transport and biotransformation (Hiemke et al., 2018). The mechanism of action involves reuptake inhibition of serotonin and noradrenaline in the synaptic cleft (Gillman, 2007). Adverse reactions are concentration-dependent and can result from antagonism of H1 histamine, alpha-1-adrenergic, and muscarinic receptors (Richelson, 1979; Kachur et al., 1988; Goldman et al., 1989; Ramakrishna and Subhash, 2012). Apart from being an antidepressant, AT is also used at lower doses for migraine prophylaxis, the management of neuropathic pain, in irritable bowel syndrome, and for the treatment of fibromyalgia (Moore et al., 2015; Rico-Villademoros et al., 2015; Silberstein, 2015; Schneider et al., 2019).

Upon systemic absorption, AT is subject to extensive hepatic metabolism, with less than 5% excreted unchanged in urine (Rudorfer and Potter, 1999). AT is metabolised mainly by cytochrome P450 (CYP) 2D6 into hydroxylated metabolites and by CYP2C19 to NT (**Figure 1**), which itself is also a tricyclic antidepressant (Breyer-Pfaff, 2004; Hicks et al., 2017). In fact, both, the more serotoninergic AT and its more noradrenergic metabolite NT, contribute to the therapeutic effects after AT administration (Hiemke et al., 2018). Both CYP2D6 and CYP2C19 are genetically highly polymorphic (Dalén et al., 1998; Zhou, 2009; Bahar et al., 2017; Sienkiewicz-Oleszkiewicz and Wiela-Hojeńska, 2018), and the substantial impact this has on AT and NT pharmacokinetics has been known for several decades (Mellström et al., 1983; Baumann et al., 1986; Steimer et al., 2004; Steimer et al., 2005; Milosavljevic et al., 2021). It has since been strongly recommended to implement regular CYP2D6 and CYP2C19 genotyping in AT therapy and consider personalised dose adjustments (Kirchheiner et al., 2001; Hicks et al., 2017).

For hepatic metabolism to occur, AT must first enter the hepatocytes. With a $pK_a$ of 9.4, approximately 99% of AT is positively charged at physiological pH and, despite its lipophilicity ($logD_{7.4}$ of 3), it may traverse cell membranes more efficiently by carrier-mediated transport than by non-ionic diffusion. One possible influx transporter with particular relevance for hepatic uptake could be the organic cation transporter 1 (OCT1; *SLC22A1*), a member of the Solute Carrier (SLC) family with a very broad substrate profile (Koepsell, 2020). OCT1 is abundantly expressed at the sinusoidal membrane of hepatocytes (Nishimura and Naito, 2005), where it mediates the hepatic uptake of organic, mostly cationic endogenous and exogenous small molecule compounds. A large number of inherited variants in the gene coding for OCT1 with comparatively high population frequencies have been described, and carriers of some of these variants showed greatly reduced or completely deficient transport activity (Seitz et al., 2015). OCT1 polymorphism may thus partially account for interindividual differences in the pharmacokinetics of numerous drugs (Tzvetkov et al., 2011; Tzvetkov et al., 2013; Tzvetkov et al., 2018; Matthaei et al., 2019; Koepsell, 2020; Jensen et al., 2021). The increased plasma concentrations of these drugs in some patients as a result of OCT1 (partial or complete) deficiency may lead to more severe adverse reactions. This could potentially be the case for AT and NT as well, and the aim of this study was to explore this possibility.

Genome-wide association studies have found a strong association between the *SLC22A1* locus and plasma concentrations of acylcarnitines, which are intermediate metabolites of mitochondrial oxidation reactions (Suhre et al., 2011). This provides further insights into the potential physiological functions of OCT1. It is also of medical relevance, as plasma acylcarnitine concentrations have been associated with metabolic disorders, including obesity and diabetes (Adams et al., 2009; Mihalik et al., 2010; Mai et al., 2013). Isobutyrylcarnitine (IBC) has been proposed to function as an endogenous biomarker for studying OCT1 *in vivo* (Luo et al., 2020). Thus, the effects of AT on plasma IBC concentrations were studied here as well.

The purpose of this study was to investigate whether OCT1 polymorphism may determine the pharmacokinetics of AT and its clinically relevant metabolite NT. This was studied here

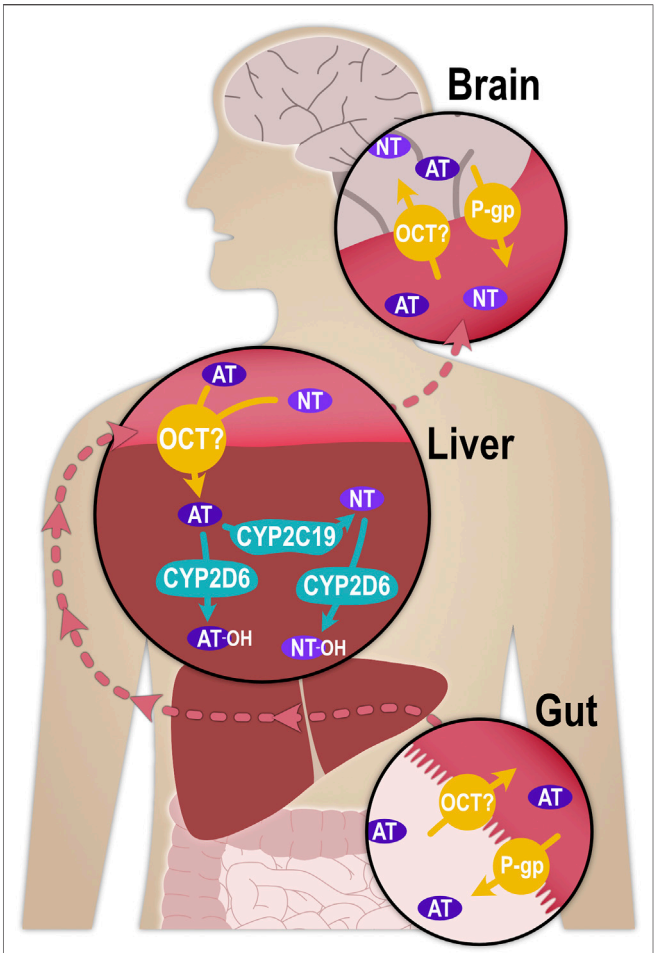


**FIGURE 1 |** Schematic illustration of the processes that determine the pharmacokinetics of AT and NT. P-glycoprotein (P-gp, MDR1, ABCB1), as part of the blood-brain-barrier, was shown in mice to transport AT and NT from the central nervous system into brain capillaries, thereby determining their concentrations at the synapse (Uhr, 2000; Grauer and Uhr, 2004; Uhr et al., 2007).

*in vitro*, in healthy volunteers, and in depressive disorder patients. In addition, the impact of CYP2D6 and CYP2C19 genetic polymorphism on AT and NT pharmacokinetics was characterized further and possible effects of AT on plasma IBC concentrations were explored.

## MATERIALS AND METHODS

### *In vitro* OCT1 Inhibition Experiments

The inhibition of OCT1 by different psychotropic drugs was studied in transport experiments using HEK293 cells stably transfected with wild-type OCT1. The cells were generated by targeted chromosomal integration using the Flp-In system (Thermo Fisher Scientific, Darmstadt, Germany), as has been described in detail before (Saadatmand et al., 2012). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C, 5% $CO_2$, and 95% relative humidity. Cells were kept in culture for no more than 30 passages. All buffers and reagents were purchased from Thermo Fisher Scientific (Darmstadt, Germany).

Approximately 48 h before the transport experiments, recombinant OCT1-expressing cells and empty vector-transfected control cells were seeded at a density of $4 \times 10^5$ cells/well in 12-well plates coated with poly-D-lysine and incubated as described above. On the day of the experiment, the cells were washed twice with prewarmed (37°C) Hank's Balanced Salt Solution (HBSS). They were subsequently incubated for 3 min at 37°C with 1 µM of the fluorescent OCT1 substrate $ASP^+$ (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide) and increasing concentrations of the antidepressants in 500 µl HBSS. The reaction was stopped by adding 2 ml ice-cold HBSS. This was subsequently removed and the cells were lyzed in 500 µl radioimmunoprecipitation assay (RIPA) buffer for 10 min under shaking. The cell uptake of $ASP^+$ was quantified using a Tecan Ultra microplate reader (Tecan Group AG, Männedorf, Switzerland) at excitation wavelength 485 nm and emission wavelength 612 nm. The intracellular $ASP^+$ concentrations were normalised to the total amount of protein in the sample that was determined using the bicinchoninic acid assay (Smith et al., 1985). $IC_{50}$ values were calculated using SigmaPlot 11 (Systat Software GmbH, Erkrath, Germany) and Prism 5 (GraphPad Software, San Diego, CA, United States).

### Subjects and Study Designs

#### Study in Healthy Volunteers

In this open-label study, the pharmacokinetics of 25 mg AT were analysed in relation to OCT1, CYP2D6, and CYP2C19 genotypes. In total, 35 unrelated healthy volunteers participated in this study. When considering the frequent OCT1 alleles *2, *3, *4, *5, and *6 to be functionally deficient, approximately 25% of Europeans are carriers of at least one deficient OCT1 variant and about 7% are homozygously deficient with respect to OCT1 (Seitz et al., 2015). In order to enrich the study sample with the less frequent functionally deficient OCT1 variants, participants were selected based on OCT1 genotype from an internal database at the Institute of Clinical Pharmacology of the University Medical Center Göttingen. All volunteers who are listed in the internal database had agreed to it and the database was approved by the ethics committee of the University of Göttingen. The number of participants for each group (carriers of 2, 1, and 0 active OCT1 alleles; **Tables 1, 2**) was calculated to achieve 80% power to identify a 50% difference in the area under the plasma concentration-time curve (AUC; the primary parameter in this study) in the carriers of 2 compared to the carriers of 0 active OCT1 alleles with a type-I (alpha) error of 5% and assuming a 35% standard deviation of the AUC in both groups. A 50% decrease in clearance is a reasonable effect size in comparison with known effects of CYP2D6 polymorphism on the AUCs of AT and NT (Kirchheiner et al., 2004) and considering that clinical drug dose adjustments are typically by about 50% or more. Additional subjects with heterozygous genotypes were included to provide a better understanding of the effects of specific variants and the mode of inheritance. All volunteers gave their written

**TABLE 1 |** Demographic data of the healthy volunteer study population stratified by OCT1 genotype.

| Parameter | 2 active OCT1 alleles ($n$ = 14) | 1 active OCT1 allele ($n$ = 9) | 0 active OCT1 alleles ($n$ = 12) | Total study population ($n$ = 35) |
|---|---|---|---|---|
| Mean age (years) | 25 | 27 | 29 | 27 |
| Sex | 7 (50%) male | 4 (44%) male | 5 (42%) male | 16 (46%) male |
| Mean body height (cm) | 177 | 178 | 172 | 175 |
| Mean body weight (kg) | 71 | 72 | 70 | 71 |
| Mean body mass index | 23 | 23 | 24 | 23 |
| Ethnicity | All caucasian | All caucasian | All caucasian | All caucasian |
| Smoking habit | 1 smoker | 0 smokers | 1 smoker | 2 smokers |

informed consent before participation in the study. The study was approved by the ethics committee of the University of Göttingen and the German Federal Institute for Drugs and Medical Devices (BfArM). It was registered at the clinical trials databases Clinicaltrials.gov (NCT02054299) and EudraCT (number 2012-003546-33).

Healthy male and female volunteers aged between 18 and 50 y with a body mass of at least 48 kg and a body mass index of 17–32 were eligible for inclusion. Volunteers who underwent regular drug treatments other than oral contraceptives or who suffered from any relevant chronic illness, as well as pregnant or lactating women, were not included. All subjects were healthy according to a detailed medical history, medical examination, electrocardiogram, urine status and clinical chemistry, and haematology parameters (sodium, potassium, total bilirubin, aspartate aminotransferase, alanine-aminotransferase, creatinine, C-reactive protein, thyroid-stimulating hormone, haemoglobin, erythrocyte, thrombocyte, and leucocyte counts).

After overnight fasting, a single dose of 25 mg AT (Amitriptylin-dura®, Mylan dura GmbH, Darmstadt, Germany) was orally administered to each subject. Blood samples were taken before AT administration and at 1, 2, 4, 6, 8, 12, 24, and 48 h after administration. The blood samples were centrifuged at room temperature for 10 min and the plasma was stored at −20°C before the concentration analyses. Blood pressure and heart rate were measured, a resting electrocardiogram was taken, pupillometry measurement was taken, and the participants were asked to report on any adverse events and possible symptoms (specifically, we asked for possible headache, fatigue, sleepiness, visual or hearing impairments, restlessness, nausea, dizziness, dry mouth, tremor, and a sensation of cold) using visual analogue scales 1 h before AT administration and at the following time points after administration: 65 min, 3, 5, 7, 11.5, 23.5, and 47.5 h.

### Study in Depressive Disorder Patients

In addition to the study in healthy volunteers described above, possible effects of the OCT1 genotype on AT pharmacokinetics were also investigated in 50 patients suffering from at least medium-grade depressive disorder. These patients had been recruited within a previous study, in which the effects of different CYP2D6 and CYP2C19 genotypes on AT and NT pharmacokinetics as well as on adverse effects and therapy response were investigated. A detailed description of the study sample, the study design, and the results pertaining to CYP2D6 and CYP2C19 polymorphism are found in the respective publications (Steimer et al., 2004, 2005). Briefly, 75 mg AT was administered twice daily at 12 h dosing intervals. Any drugs or dietary ingredients that might interfere with CYP2D6 or CYP2C19 metabolism were avoided whenever possible. Blood samples (12-hour-trough levels) were taken on days 0, 7, 14, 18, and 21, centrifuged, and stored at 4°C for genotyping (CYP2D6 and CYP2C19) and concentration analyses. The blood samples were subsequently stored at −20°C and later genotyped for OCT1. The study has been approved by the ethics committee of the Technical University of Munich, Germany.

## Bioanalytics

### Study in Healthy Volunteers

The peripheral venous blood samples of the healthy volunteers were treated with ethylenediaminetetraacetic acid (EDTA) for anticoagulation, centrifuged within 30 min after withdrawal (3,100 × $g$, 10 min, room temperature), and the plasma was stored at −20°C. For determining the plasma concentrations of AT, NT, IBC, 2-methylbutyrylcarnitine, and propionylcarnitine, plasma samples were mixed with twice the volume precipitation reagent of 10% (v/v) methanol and 90% (v/v) acetonitrile that included the corresponding deuterated internal standards AT-d6 (Biozol Diagnostica GmbH, Eching, Germany), NT-d3, IBC-d6, 2-methylbutyrylcarnitine-d9, and propionylcarnitine-d3 and d9 (all Santa Cruz Biotechnology, Heidelberg, Germany) and shaken for 15 min. After centrifugation (13,000 rpm, 15 min, room temperature), two-thirds of the supernatant were transferred to a new reaction tube and evaporated at 40°C under nitrogen flow. The residue was reconstituted under shaking in 0.1% methanoic acid and briefly centrifuged before quantification using a Nexera UHPLC system (Shimadzu, Kyoto, Japan) coupled to an API 4000 tandem mass spectrometer (AB Sciex, Darmstadt, Germany). Separation was done using a Brownlee SPP RP-Amide column (4.6 × 100 mm inner dimensions, 2.7 µm particle size; PerkinElmer, Rodgau, Germany) with a Phenomenex C18 pre-column (4 × 2 mm, Phenomenex, Aschaffenburg, Germany). For AT and NT, the mobile phase consisted of 0.1% (v/v) methanoic acid, 5.3% (v/v) methanol, and 31.7% (v/v) acetonitrile in water. For the carnitine derivatives, it consisted of 0.1% (v/v) methanoic acid, 0.43% (v/v) methanol, and 2.57% (v/v) acetonitrile in water. The lower limit of quantification was 0.5 ng/ml for AT and 0.1 ng/ml for NT. Precision and accuracy were controlled by additional control samples spiked with 2 and 20 ng/ml of AT and NT, resulting in

coefficients of variation of 6.0 and 3.8% (means of 2.02 and 19.5 ng/ml) for AT and of 3.8 and 2.7% (means of 1.91 and 18.8 ng/ml) for NT. The mass spectrometry detection parameters are listed in **Supplementary Table S1**.

### Study in Depressive Disorder Patients

The serum concentrations in depressive disorder patients were determined either by the Emit® immunoassay specific for AT and NT or a commercial high-performance liquid chromatography assay (Bio-Rad Laboratories GmbH, Feldkirchen, Germany), as described before (Steimer et al., 2004; Steimer et al., 2005).

## Genotyping

For both studies, genomic DNA was isolated from venous blood samples via automated solid phase extraction (EZ1 DNA Blood kit; Qiagen, Hilden, Germany). The following genetic variants were analysed using single-base primer extension using fluorescence-labelled dideoxynucleotides (described by Seitz et al. (2015) and Kirchheiner et al. (2008)) for OCT1: *1 (wild-type), *2 (M420del, rs72552763), *3 (R61C, rs12208357), *4 (G401S, rs34130495), *5 (G465R, rs34059508 in combination with M420del, rs72552763), *6 (C88R, rs55918055 in combination with M420del, rs72552763), *7 (S14F, rs34447885), *9 (P117L, rs200684404), and *10 (S189L, rs34104736); for CYP2D6: *1, *2, *3, *4, *5, *6, *9, *10, *35, *41, and gene duplication. The CYP2C19 variants *2 (rs4244285) and *17 (rs12248560) and the OCT1 variant *8 (Arg488Met, rs35270274) were genotyped using a TaqMan SNP genotyping assay (Life Technologies). Almost all samples were genotyped in duplicate, with 100% concordant results.

## Statistics

For the study in healthy volunteers, the primary endpoints were the AUCs of plasma AT and NT concentrations. Secondary endpoints were the other pharmacokinetic parameters of AT and NT, as well as heart rate, blood pressure, pupil size effects, and possible adverse events (headache, fatigue, visual or hearing impairments, restlessness, nausea, dizziness, dry mouth, tremor, as well as sensations of hypothermia and heart palpitations determined using a visual analogue scale test). Pharmacokinetic parameters were calculated by non-compartmental analysis using Phoenix 64 WinNonlin version 6.3 (Certara Inc., Princeton, NJ, United States). $AUC_{inf}$ of AT was calculated from the time of dosing using the linear/log trapezoidal rule and extrapolated to infinity based on the last predicted concentration and using the terminal elimination rate constant (lambda z). AUC of NT was calculated from the time of dosing until the last measurement at 48 h using the linear/log trapezoidal rule, as a decline in NT concentrations was not observed in some subjects and extrapolation to infinity thus not possible. Further parameters that were studied included the total plasma clearance after oral administration (CL/F) and the terminal half-life ($t_{1/2}$), which were calculated as CL/F = dose/$AUC_{inf}$ and $t_{1/2}$ = ln (2)/lambda z.

The correlation between AT and NT plasma AUC (study in healthy volunteers) or mean plasma concentration per dose unit (study in depressive disorder patients) and OCT1, CYP2D6, and CYP2C19 genotypes were calculated using the Jonckheere-Terpstra non-parametric analysis, which takes into consideration the *a priori* ordering (or trend) in gene activities from zero to normal to ultra-rapid (for CYP2C19 and CYP2D6). To do so, the genotypes were categorized into 0, 1, or 2 active alleles for OCT1, into 0, 0.5, 1, 1.5, 2, 2.5, or 3 active alleles for CYP2D6, and into 0, 1, 1.5, 2, 2.5, or 3 active alleles for CYP2C19, depending on their known effects on transporter/enzyme activity. OCT1 alleles *2, *3, *4, *5 were classified as being zero active. However, given the substrate-dependent effects of OCT1*2, calculations were repeated with OCT1*2 classified as being fully active. A semi-quantitative gene dosage was calculated for CYP2D6, as has been described earlier (Steimer et al., 2004). For calculating a CYP2C19 activity score, CYP2C19*2 was regarded as zero active, CYP2C19*1 was classified as 1, and CYP2C19*17 as 1.5. Additional multiple linear regression analyses included sex, age, body mass index, and glomerular filtration rate.

## Dose-Adjustment Calculations

The genotype-based dosage adjustment recommendations were calculated by using the equations described in Stingl et al. (2013) (supplementary data), modified to base these calculations on AUC data instead of clearance values. The adjusted dose was thereby calculated for CYP2D6 extensive metabolisers (EM) as $D_{EM} = 100/(0.1 \times AUC_{EM}/AUC_{PM} + 0.4 \times AUC_{EM}/AUC_{IM} + 0.5)$ and for CYP2C19 EM as $D_{EM} = 100/(0.03 \times AUC_{EM}/AUC_{PM} + 0.27 \times AUC_{EM}/AUC_{IM} + 0.7)$. The dose adjustments for the poor (PM), intermediate (IM), and ultra-rapid (UM) metaboliser phenotypes were calculated as follows: $D_{PM\ or\ IM\ or\ UM} = D_{EM} \times AUC_{EM}/AUC_{PM\ or\ IM\ or\ UM}$. The multipliers in the EM calculations account for the typical population frequencies of the respective genotypes in European populations (e.g. 0.1 for 10% of CYP2D6 PM). The rationale behind these calculations is that the average recommended drug dose usually determined without considering the genotypes was chosen as the average optimum for populations with the given genotype frequencies (Kirchheiner et al., 2001).

# RESULTS

## *In vitro* Inhibition of OCT1 by Different Psychotropic Drugs

Thirty-two clinically relevant antidepressants, neuroleptics, and an anticholinergic drug for the treatment of Parkinson's disease were screened for OCT1 inhibition. These have been selected based on their positive charge at physiological pH, as charged compounds are mostly unable to efficiently traverse biological membranes through passive diffusion and their pharmacokinetics might depend on transport proteins, such as OCT1. An inhibitor for a transporter does not necessarily have to be a substrate as well, but for many compounds, this is indeed the case. The psychotropic drugs were assessed for their potential to inhibit cell uptake of the fluorescent OCT1 model substrate $ASP^+$ (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide) in OCT1-overexpressing cells. As shown in **Figure 2**, most of the tested compounds showed inhibitory potencies in the low to mid-micromolar range, indicating that these are moderate to strong inhibitors of OCT1. AT showed a mean $IC_{50}$ value of 28.6 ±

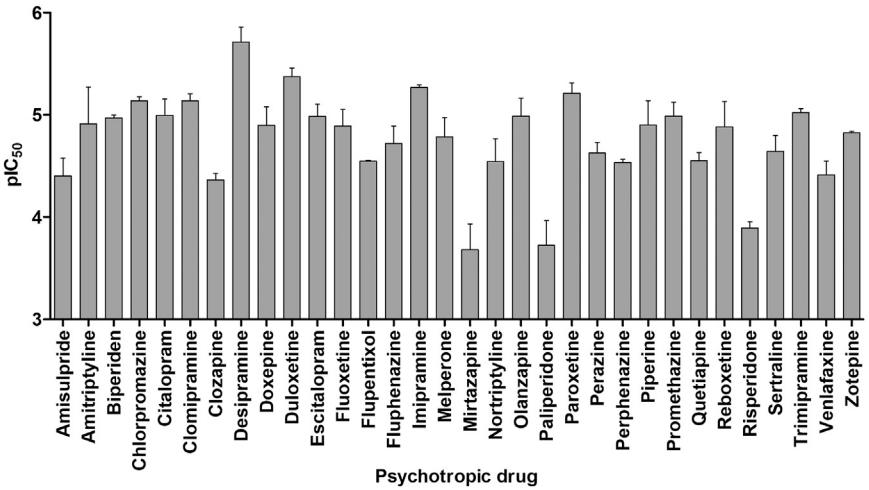


**FIGURE 2 |** *In vitro* assessment of OCT1 inhibition by a range of different antidepressant and neuroleptic drugs. Shown is the mean negative logarithm of the $IC_{50}$ values of 3–4 independent experiments, the error bars indicate the SEM.

18.9 μM and NT of 40.4 ± 16.2 μM, which is in agreement with previously reported data (Haenisch et al., 2012; Zhu et al., 2018). Because of this interaction with OCT1 and its widespread clinical use, AT was further analyzed with respect to the impact of genetic variation in OCT1 in volunteer and patient studies.

## Pharmacokinetics of Amitriptyline and Nortriptyline in Relation to OCT1, CYP2D6, and CYP2C19 Genotypes in Healthy Volunteers

In the clinical study in healthy volunteers, preselected according to their OCT1 genotype, 35 volunteers (19 female and 16 male) received 25 mg of AT as a single dose. The study participants were between 18 and 48 y of age, with a mean age of 27 y. The mean body mass index was 23.0 kg/$m^2$. Stratified by OCT1 genotype, 14 subjects were homozygous carriers of the active OCT1*1 (wild-type) allele, nine subjects carried one active allele (OCT1*1) and one allele with no or reduced activity (*2,*3, *4), and 12 subjects were carriers of two OCT1 alleles with no or reduced activity (*2, *3, *4, *5). There were no significant differences in demographic data between the OCT1 genotypes (**Table 1**).

Large variation was seen in the pharmacokinetics of AT and, even more so, for its therapeutically active metabolite NT. The $AUC_{inf}$ of AT varied about fourfold (range: 109.9–429.9 h*μg/L) and the $AUC_{48h}$ of NT approximately sevenfold (range: 39.3–283.7 h*μg/L). However, these variations were apparently not a result of OCT1 polymorphism, as differences in AUC between carriers of two, one, or zero active OCT1 alleles were not statistically significant (**Figure 3**; **Table 2**, **Supplementary Figure S1**; **Supplementary Table S2**). The only statistically significant difference in relation to OCT1 genotype was observed for the $T_{max}$ of NT ($p$ = 0.016, Jonckheere-Terpstra test), which was almost twofold higher in the group comprised of the carriers of two active OCT1 alleles as compared to the other two groups. However, this difference is likely explained by one subject with particularly high plasma NT concentrations, who had low CYP2D6 activity and very high CYP2C19 activity (**Figure 3**). Any differences in the $AUC_{48h}$ of the 'active moiety' (sum of the $AUC_{48h}$ of AT and NT) between the OCT1 genotypes were not significant ($p$ = 0.059, Jonckheere-Terpstra test).

Interestingly, if OCT1*2 would be considered as being fully active, $T_{max}$, $C_{max}$, and $AUC_{48h}$ for NT differed significantly based on OCT1 genotype ($p$ = 0.050, 0.018, and 0.011, respectively), whereas any differences in AT pharmacokinetic parameters were still statistically not significant.

The CYP2D6 genotype had a strong effect on the pharmacokinetics of AT and NT. The plasma concentrations of AT and NT increased with decreasing CYP2D6 activity (**Figure 4**), and subjects with lower CYP2D6 activity showed a higher $AUC_{inf}$ and $AUC_{48h}$ as well as a longer plasma half-life and a lower AT clearance (**Table 3**). The CYP2C19 genotype had no significant effect on AT pharmacokinetics (**Figure 4**) but subjects with higher CYP2C19 activity showed a higher NT $AUC_{48h}$ and $C_{max}$ compared to subjects with lower CYP2C19 activity (**Figure 4**; **Table 4**).

A multiple linear regression analysis confirmed statistically significant effects of CYP2D6 genotype on AT pharmacokinetics (**Table 5**). CYP2D6 genotype accounts for 43% of the variation. Concerning NT, both CYP2D6 and CYP2C19 genotypes had statistically significant effects on the $AUC_{48h}$ and could explain 58% of the variation. In contrast, OCT1 genotype, gender, age, body mass index, and glomerular filtration rate had no significant effects on the variation in both the $AUC_{inf}$ of AT and the $AUC_{48h}$ of nortiptyline.

## Adverse Effects of Amitriptyline

AT was generally well-tolerated and no serious adverse events occurred during the entire study. Using visual analogue scales, the participants reported symptoms of fatigue, which peaked at 3 h after AT administration at which plasma AT concentrations were generally the highest (**Figure 5**). However, it should be taken into consideration that no placebo control was used in this

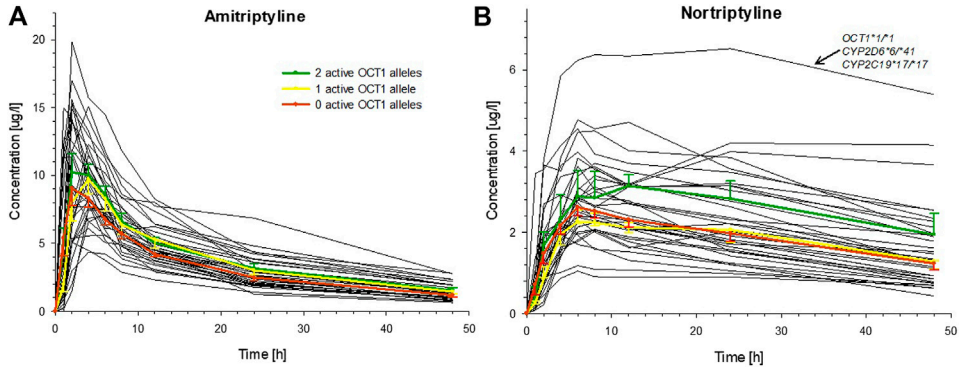


**FIGURE 3 |** Individual plasma concentrations of **(A)** AT and **(B)** NT over time for all healthy volunteers (black curves), indicating the large interindividual variation for these tricyclic antidepressants. The mean (±SEM) concentrations for carriers of two (green), one (yellow), or zero (red) active OCT1 alleles are superimposed (OCT1-dependent differences in AUC were not statistically significant). The single participant with the highest NT concentrations had wild-type OCT1 genotype, reduced activity CYP2D6 genotype, and a very high activity CYP2C19 genotype.

**TABLE 2 |** Pharmacokinetic parameters stratified by OCT1 genotype.

| Parameter | 2 active OCT1 alleles ($n$ = 14) | 1 active OCT1 allele ($n$ = 9) | 0 active OCT1 alleles ($n$ = 12) | $p$-value[a] |
|---|---|---|---|---|
| Amitriptyline | | | | |
| $t_{1/2}$ (h) | 21.0 ± 4.0 | 20.5 ± 3.9 | 20.3 ± 3.8 | 0.715 |
| $T_{max}$ (h) | 2.9 ± 1.4 | 3.1 ± 1.0 | 3.4 ± 1.6 | 0.725 |
| $C_{max}$ (µg/L) | 11.6 ± 4.0 | 10.5 ± 3.3 | 9.9 ± 3.9 | 0.301 |
| $AUC_{inf}$ (h*µg/l) | 242.6 ± 87.5 | 228.5 ± 72.4 | 199.3 ± 60.0 | 0.235 |
| $AUC_{48h}$ (h*µg/l) | 194.2 ± 66.3 | 184.2 ± 56.0 | 161.6 ± 40.5 | 0.260 |
| CL (L/min) | 1.9 ± 0.6 | 2.0 ± 0.8 | 2.3 ± 0.6 | 0.235 |
| $V_z$ (L) | 3,401 ± 1,041 | 3,610 ± 1,633 | 3,828 ± 800 | 0.260 |
| Nortriptyline | | | | |
| $t_{1/2}$ (h) | 40.1 ± 38.2 | 56.4 ± 31.3 | 46.1 ± 24.4 | 0.742 |
| $T_{max}$ (h) | 11.5 ± 8.7 | 6.7 ± 1.3 | 6.2 ± 1.4 | **0.016** |
| $C_{max}$ (µg/L) | 3.4 ± 1.4 | 2.6 ± 0.9 | 2.7 ± 0.7 | 0.223 |
| $AUC_{48h}$ (h*µg/L) | 125.7 ± 63.5 | 88.3 ± 32.2 | 90.2 ± 27.1 | 0.100 |

*Data are shown as the mean.*

[a]*Differences were analysed for statistical significance using the Jonckheere-Terpstra test. Significant values are highlighted in bold.*

pharmacokinetic study, and a fully valid assessment of adverse effects was thus not possible (i.e. reported adverse effects might not exclusively be due to AT administration but could be a result of the 'placebo effect' as well). The intensity of fatigue was not dependent on OCT1, CYP2D6, or CYP2C19 genotypes ($p > 0.05$, Jonckheere-Terpstra test). Statistically significant time- and concentration-related adverse effects like dry mouth, visual or hearing impairment, restlessness, headache, nausea, dizziness, or a sensation of cold reported using visual analogue scales as well as potential anticholinergic effects studied by pupillometry were not observed after the 25 mg AT dose.

## Pharmacokinetics of Amitriptyline and Nortriptyline in Relation to OCT1 Genotype in Depressive Disorder Patients

Possible differences due to OCT1 polymorphism on the pharmacokinetics of AT and its metabolite NT were additionally studied in 50 patients suffering from medium-grade to severe depressive disorder that were recruited as part of a previous study on the impact of CYP2D6 and CYP2C19 polymorphism on AT and NT pharmacokinetics, adverse effects, and therapy response (Steimer et al., 2004; Steimer et al., 2005). These underwent a therapy of 75 mg AT twice daily at 12 h dosing intervals. Out of these 50 patients, 27 were carriers of two active OCT1 alleles (OCT1*1/*1; **Supplementary Table S3**), 17 were carriers of one active OCT1 allele (*1 in combination with *2, *3, or *4), and six patients carried zero active OCT1 alleles (*2, *3, or *4). Different CYP2D6 and CYP2C19 genotypes were found to be similarly distributed across all three groups (**Table 6**). A trend of increasing plasma concentrations with decreasing OCT1 activity was seen for AT (**Figure 6**, **Supplementary Figure S2**; **Supplementary Table S3**). Although the differences in mean AT concentrations between the three groups were rather modest, they showed statistical significance ($p$ = 0.018, Jonckheere-Terpstra test). In contrast, mean plasma NT concentrations per dose unit were relatively similar for all

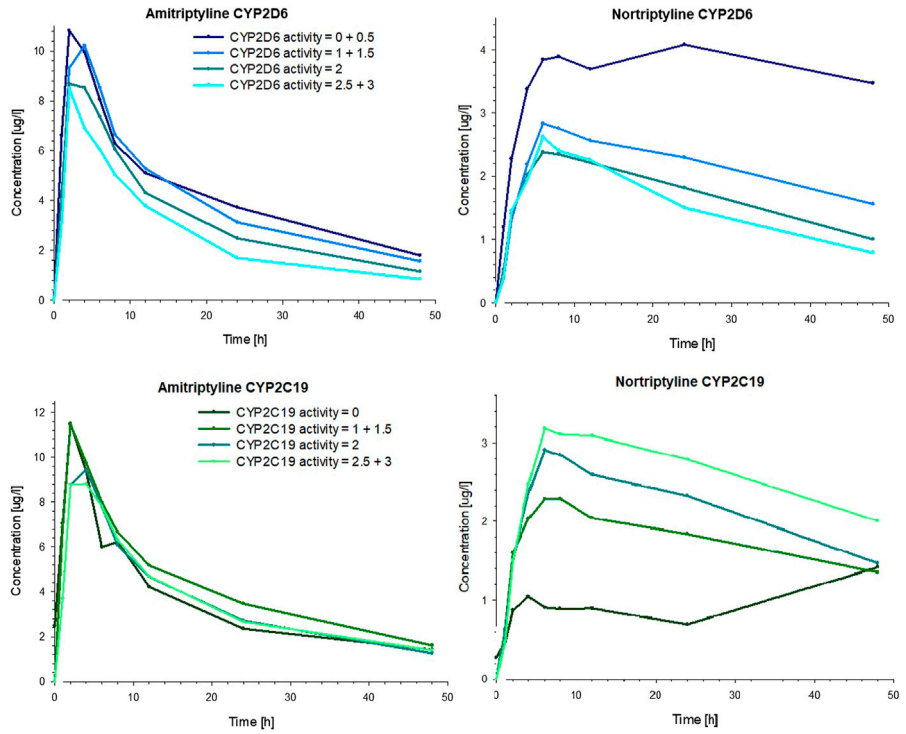


**FIGURE 4 |** Mean plasma concentrations of AT **(left)** and NT **(right)** in healthy volunteers over time, stratified by the number of active alleles for CYP2D6 **(top)** and CYP2C19 **(bottom)**.

**TABLE 3 |** Pharmacokinetic parameters stratified by CYP2D6 genotype.

| | CYP2D6 activity (semi-quantitative gene dosage)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Parameter** | **0** (*n* = 3) | **0.5** (*n* = 2) | **1** (*n* = 10) | **1.5** (*n* = 6) | **2** (*n* = 11) | **2.5** (*n* = 1) | **3** (*n* = 2) | ***p*-value[b]** |
| Amitriptyline | | | | | | | | |
| $t_{1/2}$ (h) | 23.7 | 24.2 | 21.4 | 19.8 | 19.5 | 14.0 | 20.6 | **0.046** |
| $T_{max}$ (h) | 3.4 | 2.0 | 2.7 | 3.7 | 3.4 | 6.0 | 2.0 | 0.677 |
| $C_{max}$ (µg/L) | 10.9 | 12.7 | 11.9 | 10.3 | 10.0 | 7.0 | 10.2 | 0.243 |
| $AUC_{inf}$ (h*µg/L) | 310.6 | 214.4 | 250.8 | 225.6 | 195.5 | 157.7 | 157.0 | **0.011** |
| $AUC_{48h}$ (h*µg/L) | 236.2 | 165.9 | 198.8 | 183.9 | 162.2 | 141.5 | 130.0 | **0.037** |
| CL (L/min) | 1.45 | 2.09 | 1.81 | 2.03 | 2.25 | 2.64 | 2.92 | **0.011** |
| $V_z$ (L) | 2,982 | 4,189 | 3,269 | 3,446 | 3,768 | 3,192 | 5,361 | 0.269 |
| Nortriptyline | | | | | | | | |
| $t_{1/2}$ (h) | 92.0[c] | 105.1 | 64.2 | 51.9 | 29.9[c] | 25.0 | 26.9 | < **0.001** |
| $T_{max}$ (h) | 18.9 | 15.5 | 6.2 | 7.0 | 7.7 | 6.0 | 6.0 | 0.074 |
| $C_{max}$ (µg/L) | 3.48 | 5.54 | 2.94 | 2.79 | 2.57 | 3.11 | 2.49 | **0.019** |
| $AUC_{48h}$ (h*µg/L) | 144.6 | 222.2 | 103.8 | 98.3 | 81.8 | 89.1 | 71.1 | **0.001** |

*Data are shown as the mean. The study population was not selected based on their CYP2D6 genotypes.*

[a]*The genotype-based CYP2D6 activity is based on the semi-quantitative gene dosage, as described earlier (Steimer et al., 2004).*

[b]*Differences were analysed for statistical significance using the Jonckheere-Terpstra test. Significant values are highlighted in bold.*

[c]*In 2 subjects carrying zero active CYP2D6 alleles and in one subjects with a CYP2D6 gene activity of 2, no decrease in NT concentration was observed and, therefore, no terminal elimination rate could be calculated.*

OCT1 genotypes. Differences in the 'active moiety' (sum of AT and NT plasma concentrations) between OCT1 genotypes were significant ($p = 0.036$, Jonckheere-Terpstra test). Multiple linear regression analysis showed significant effects for OCT1 and CYP2C19 on AT and highly significant effects for CYP2D6 on NT mean plasma concentrations per dose unit (**Table 7**).

If OCT1*2 would be considered as being fully active, 44 patients would be carriers of two active OCT1 alleles, five patients would be carriers of one active OCT1 allele, and one patient would be a carrier of zero active OCT1 alleles. With this classification, the mean plasma concentrations per dose unit were not significantly different between OCT1 genotypes for both AT

**TABLE 4 |** Pharmacokinetic parameters stratified by CYP2C19 genotype.

| CYP2C19 activity score[a] | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Parameter** | **0** ($n$ = 1) | **1** ($n$ = 5) | **1.5** ($n$ = 2) | **2** ($n$ = 16) | **2.5** ($n$ = 10) | **3** ($n$ = 1) | ***p*-value[b]** |
| Amitriptyline | | | | | | | |
| $t_{1/2}$ (h) | 26.7 | 21.6 | 20.3 | 20.1 | 19.8 | 28.3 | 0.359 |
| $T_{max}$ (h) | 4.0 | 2.6 | 2.0 | 3.2 | 3.6 | 2.0 | 0.639 |
| $C_{max}$ (µg/L) | 11.5 | 11.1 | 16.2 | 10.5 | 9.5 | 13.9 | 0.303 |
| $AUC_{inf}$ (h*µg/L) | 320.9 | 231.0 | 317.0 | 210.0 | 210.7 | 271.0 | 0.254 |
| $AUC_{48h}$ (h*µg/L) | 229.0 | 183.2 | 255.6 | 171.8 | 171.3 | 198.4 | 0.238 |
| CL (L/min) | 1.30 | 1.94 | 1.5 | 2.1 | 2.2 | 1.54 | 0.254 |
| $V_z$ (L) | 3,004 | 3,525 | 2,559 | 3,696 | 3,740 | 3,762 | 0.340 |
| Nortriptyline[c] | | | | | | | |
| $t_{1/2}$ (h) | 106.3 | 48.2 | 25.1 | 47.7 | 44.1 | 145 | 0.983 |
| $T_{max}$ (h) | 6.0 | 6.0 | 14.1 | 6.6 | 9.9 | 24.9 | 0.452 |
| $C_{max}$ (µg/L) | 1.05 | 2.1 | 3.5 | 3.0 | 3.1 | 6.5 | **0.012** |
| $AUC_{48h}$ (h*µg/L) | 39.3 | 69.4 | 123 | 102.8 | 107.8 | 283.7 | **0.008** |

*Data are shown as the mean. The study population was not selected based on their CYP2C19 genotypes.*

*[a]For calculating the CYP2C19 activity score, CYP2C19*2 was regarded as zero active, CYP2C19*1 was classified as 1, and CYP2C19*17 as 1.5.*

*[b]Differences were analysed for statistical significance using the Jonckheere-Terpstra test. Significant values are highlighted in bold.*

*[c]In two subjects with CYP2C19*1/*17 genotype and in one subject with CYP2C19*1/*1 genotype, no decrease in NT concentration was observed and, therefore, no terminal elimination rate could be calculated.*

**TABLE 5 |** Multiple linear regression analysis to determine the individual factors that influence AT and NT AUC in healthy volunteers.

| | **Amitriptyline $AUC_{inf}$** (all factors: $r$ = 0.66, $r^2$ = 0.43) | | **Nortriptyline $AUC_{48h}$** (all factors: $r$ = 0.76, $r^2$ = 0.58) | |
|---|---|---|---|---|
| **Individual factors** | **Coefficient** | ***p*-value** | **Coefficient** | ***p*-value** |
| Sex | −42.5 | 0.10 | 4.58 | 0.74 |
| Age (years) | −1.13 | 0.59 | −1.39 | 0.23 |
| Body mass index (kg/m$^2$) | 1.65 | 0.78 | −4.11 | 0.21 |
| Glomerular filtration rate (ml/min/1.73 m$^2$) | 1.02 | 0.28 | −0.02 | 0.97 |
| OCT1 activity | 10.40 | 0.45 | 11.56 | 0.14 |
| CYP2C19 activity | −25.29 | 0.18 | 35.94 | **0.001** |
| CYP2D6 activity | −53.07 | **0.002** | −30.94 | **0.001** |

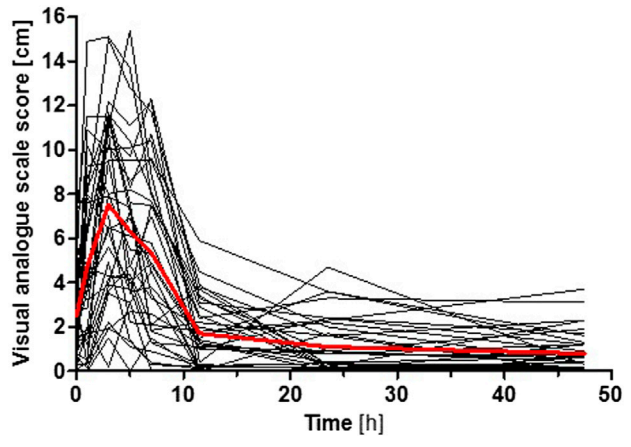


**FIGURE 5 |** Intensity of fatigue after AT administration reported by the participants using a visual analogue scale. Shown is the time course for each participant (black curves) and the mean superimposed (red curve). The intensity of fatigue was not dependent on OCT1, CYP2D6, or CYP2C19 genotypes ($p$ > 0.05, Jonckheere-Terpstra test).

($p$ = 0.216, Jonckheere-Terpstra test), and NT ($p$ = 0.800, Jonckheere-Terpstra test), but a trend of increasing plasma concentrations with decreasing OCT1 activity was still seen for AT.

## Effects of OCT1 Activity on Plasma Concentrations of Acylcarnitine Derivatives

In order to investigate the proposed suitability of IBC as a human *in vivo* biomarker for OCT1 activity (Luo et al., 2020), plasma concentrations of IBC as well as of 2-methylbutyrylcarnitine and propionylcarnitine were determined in a subgroup of 18 volunteers who participated in the study on AT pharmacokinetics. Because of the ambiguous role of OCT1*2 with respect to several OCT1 substrates, carriers of OCT1*2 were not included. Baseline IBC plasma concentrations were 2.9- to 4.9-fold and 2-methylbutyrylcarnitine plasma concentrations were 1.3- to 2.3-fold higher in participants with two active OCT1 alleles compared to the participants with zero active OCT1 alleles ($p$ < 0.0001 for both, unpaired $t$ test; **Figures 7A,C**), whereas plasma propionylcarnitine concentrations were similar for both groups (**Figure 7D**; $p$ = 0.386, unpaired $t$ test). At time points 2 and 4 h after AT administration, at which plasma AT concentrations

**TABLE 6 |** Distribution of CYP2D6 and CYP2C19 activity across the study sample of 50 depressive disorder patients, stratified by OCT1 genotype.

| | 2 active OCT1 alleles ($n$ = 27) | 1 active OCT1 allele ($n$ = 17) | 0 active OCT1 alleles ($n$ = 6) |
|---|---|---|---|
| **CYP2D6 activity** | | | |
| **3.0** | 0 (0%) | 1 (6%) | 0 (0%) |
| **2.0** | 10 (37%) | 8 (47%) | 3 (50%) |
| **1.5** | 8 (30%) | 2 (12%) | 1 (17%) |
| **1.0** | 7 (26%) | 5 (29%) | 2 (33%) |
| **0.5** | 2 (7%) | 1 (6%) | 0 (0%) |
| **CYP2C19 activity** | | | |
| **2.0** | 16 (59%) | 11 (65%) | 4 (67%) |
| **1.0** | 10 (37%) | 6 (35%) | 2 (33%) |
| **0.0** | 1 (4%) | 0 (0%) | 0 (0%) |

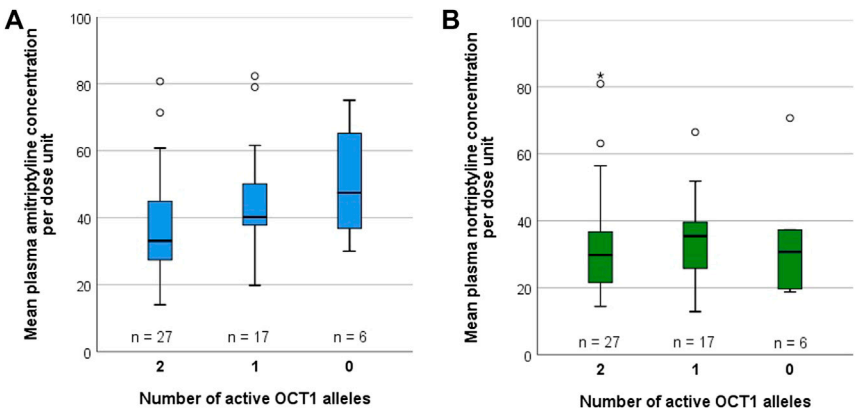


**FIGURE 6 |** Mean plasma concentrations per dose unit of **(A)** AT and **(B)** NT, stratified by OCT1 genotype, in 50 depressive disorder patients who underwent AT therapy.

**TABLE 7 |** Multiple linear regression analysis to determine the individual factors that influence mean plasma AT and NT concentrations per dose unit in depressive disorder patients.

| | Mean AT concentration per dose unit (all factors: $r$ = 0.45, $r^2$ = 0.20) | | Mean NT concentration per dose unit (all factors: $r$ = 0.68, $r^2$ = 0.47) | |
|---|---|---|---|---|
| **Individual factors** | **Coefficient** | ***p*-value** | **Coefficient** | ***p*-value** |
| OCT1 activity | −7.80 | **0.018** | −1.33 | 0.608 |
| CYP2C19 activity | −10.99 | **0.012** | 5.96 | 0.083 |
| CYP2D6 activity | −1.90 | 0.653 | −19.96 | **<0.001** |

were generally the highest, plasma IBC concentrations were reduced to 72 and 67% of the baseline IBC concentrations ($p$ = 0.001 and 0.002, paired $t$ test; **Figure 7A**).

## DISCUSSION

In this study, the effects of OCT1 polymorphism on AT and NT pharmacokinetics were investigated comprehensively in healthy volunteers and in depressive disorder patients. With their relatively high $pK_a$ values, most tricyclic antidepressants could be typical OCT1 substrates, and this hypothesis was further supported by the fact that all tested tricyclic antidepressants were moderate to strong inhibitors of OCT1 (**Figure 2**). Yet, in our two studies in healthy volunteers and patients, there was no strong and consistent effect of OCT1 on the pharmacokinetics of AT and its active metabolite NT. This indicates that non-ionic diffusion, independent of transporter activity, likely is the main mechanism of biological membrane passage or, alternatively, other transporters are involved. Transporter-mediated hepatocyte uptake could be demonstrated with saturable transport kinetics for imipramine (Hallifax and Houston, 2007), another tricyclic antidepressant with similar lipophilicity. Possible candidates might include the OCTN1 and OCTN2 transporters as well as the proton-coupled organic cation antiporter that has been described in the literature but has not yet been identified on the molecular level (Tega et al., 2021).

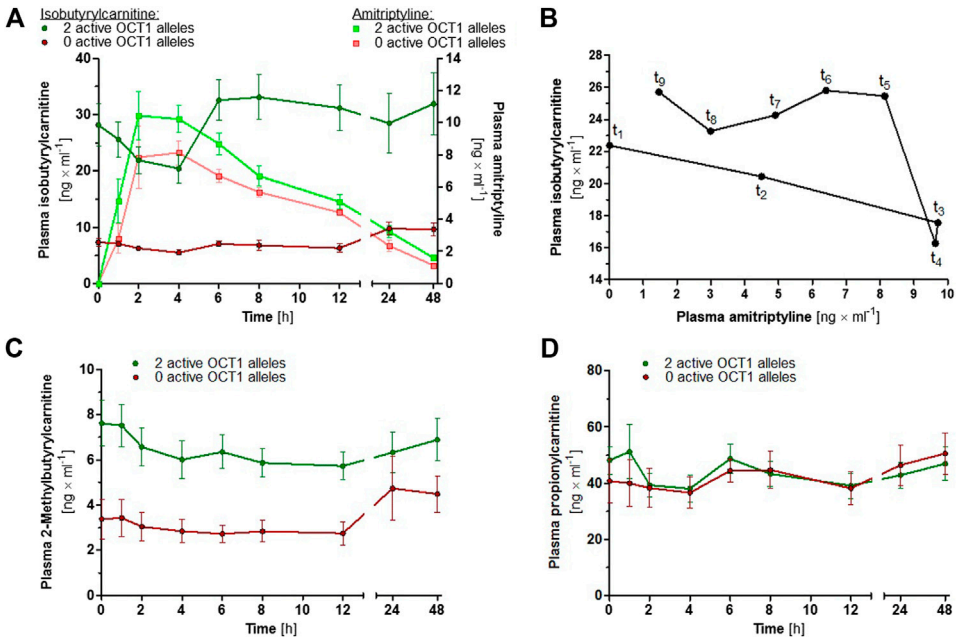


**FIGURE 7 | (A)** Mean ± SEM of IBC (dark coloured circles) and AT (light coloured squares) plasma concentrations over time, stratified by OCT1 genotype (green data points represent two active OCT1 alleles and red data points represent zero active OCT1 alleles; carriers of OCT1*2 were not included). **(B)** Hysteresis plot showing the mean plasma concentrations of AT and IBC in 13 healthy volunteers with two active OCT1 alleles. **(C)** Mean ± SEM of 2-methylbutyrylcarnitine and **(D)** propionylcarnitine plasma concentrations over time, stratified by OCT1 genotype.

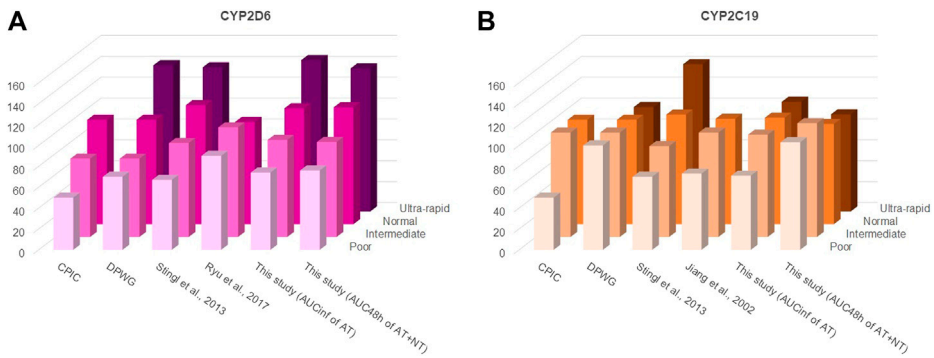


**FIGURE 8 |** Different starting dosage adjustment recommendations from the literature and based on the results of this study for **(A)** CYP2D6 and **(B)** CYP2C19. These were taken from the Clinical Pharmacogenetics Implementation Consortium (CPIC®) guideline (Hicks et al., 2017), the Dutch Pharmacogenetics Working Group (DPWG) guideline (August 2019 update), or calculated based on the formulas described by Stingl et al. (2013) and by using the AUCs determined in the respective studies. In accordance with the CPIC® and DPWG final consensus on CYP2D6 genotype to phenotype (Caudle et al., 2020), a CYP2D6 activity score of 0 was classified in this study as poor, of 0.5 and 1 as intermediate, of 1.5 and 2.0 as normal/extensive, and of >2.5 as ultra-rapid metaboliser phenotypes. For CYP2C19, an activity score of 0 was classified in this study as poor, of 1 as intermediate, of 1.5 and 2 as normal/extensive, and of >2 as ultra-rapid metaboliser phenotypes. The starting dosage adjustment recommendations are also listed in **Supplementary Table S4**. As apparent, there is a high consistency between different recommendations and the measurements from this study, particularly with regard to the CYP2D6 genotype.

While no statistically significant effects for OCT1 polymorphism were observed in healthy volunteers, a moderate trend of increasing plasma concentrations with decreasing OCT1 activity was seen for AT in depressive disorder patients. A possible reason for this discrepancy might be the differences in dose and duration. While the healthy volunteers were given a single dose of 25 mg of AT, the depressive disorder patients received a total of 150 mg per day and measurements were taken over two weeks after steady-state has been achieved. With regard to NT pharmacokinetics, both studies were concordant in that OCT1 does not appear to be a major determining factor.

The fact that only a single dose of AT was given in the study in healthy volunteers and that, accordingly, steady-state plasma concentrations were not achieved, is a potential limitation of

this study. Also, it cannot be excluded that OCT1 effects might still be observed at higher dosage. The average $C_{max}$ for AT in the healthy volunteers was 10.7 μg/L, or 0.039 μM, which is 730-fold lower than the $IC_{50}$ of 28.6 μM determined in our *in vitro* assays.

While it is apparently not necessary to take the OCT1 genotype into consideration for AT or NT dosing, CYP2C19 and CYP2D6 genotypes are highly relevant and AT or NT dosage should be adjusted accordingly (Brockmöller et al., 2000; Hicks et al., 2017). Several approaches have been proposed by different groups but their suggestions are essentially in concordance. **Figure 8** and **Supplementary Table S4** show earlier recommendations on CYP2D6 and CYP2C19 genotype-based dose adjustments by the Clinical Pharmacogenetics Implementation Consortium (CPIC®; Hicks et al., 2017), the Dutch Pharmacogenetics Working Group (DPWG; guidelines update August 2019), and based on the pharmacokinetic data from more recent clinical studies and from this study by using the calculations described in Stingl et al. (2013). The dosage adjustment recommendations based on the data from this study were similar to those calculated previously by Stingl et al. (2013), except when using the sum of the $AUC_{48\,h}$ of AT and NT for calculating adjustments for CYP2C19 poor and ultra-rapid metabolisers. This is likely due to the strong impact this enzyme has on the NT pharmacokinetics.

OCT1 is able to transport a large number of different compounds, among them many drugs, but its physiological function is not yet understood. As some endogenous acylcarnitines were shown to be OCT1 substrates, a potential physiological role of OCT1 could be the regulation of intracellular concentrations of these carnitine derivatives. It has been proposed that IBC could serve as an endogenous biomarker (Luo et al., 2020), which might be useful for further studying OCT1 activity in humans. Our results confirm its suitability, as up to five-fold differences in IBC plasma concentrations between participants with normal OCT1 activity and carriers of zero active OCT1 alleles were observed. Moreover, peak plasma concentrations of the OCT1 inhibitor AT correlated with a transient reduction in plasma IBC concentrations (**Figure 7**). The average peak plasma concentration of AT was 10.8 μg/L. With 95% plasma protein binding (Hardman et al., 2001), the peak concentration of unbound AT was 0.54 μg/L. Based on the calculations by Ahlin et al. and Ito et al. (Ito et al., 1998; Ito et al., 2002; Ahlin et al., 2008), the maximum concentration of unbound AT in the portal vein was estimated to be 745.9 μg/L or 2.69 μM. At this concentration, 23% OCT1 inhibition was achieved *in vitro*, which corresponds to the 33% decrease in IBC plasma concentration observed *in vivo*. Here, it can be concluded that IBC might indeed be a suitable endogenous OCT1 biomarker. 2-Methylbutyrylcarnitine could be considered as well, as OCT1-dependent differences were also observed, although these were less pronounced and plasma concentrations were generally lower than those of IBC. Despite the structural similarity, propionylcarnitine plasma concentrations were not affected by OCT1 genetic variation. A speculative but possible explanation for the reduction in plasma IBC concentrations at these time points could be a potential inhibition of OCT1 by high plasma AT concentrations. Alternatively, this association could be the result of complex metabolic crosstalk. As a placebo control was not part of this mainly pharmacokinetic study, possible effects due to diurnal rhythm and nutrition cannot be excluded.

In conclusion, the pharmacokinetics of AT and NT are strongly dependent on the CYP2C19 and CYP2D6 genotypes, while OCT1 polymorphism does not appear to be a major medically relevant factor. It thus remains to be elucidated which organic cation transporter(s) are relevant for intestinal absorption, hepatic uptake, and passage across the blood-brain barrier.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the University of Göttingen and the Technical University of Munich as well as by the German Federal Institute for Drugs and Medical Devices (BfArM). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceptualisation: JB, MT; Formal analysis: JM, MR; Funding acquisition: JB, MT; Investigation: JM, MK, OJ, TO, MT, MR; Project administration: JB, MR; Resources: WS, JB; Supervision: JB; Visualisation: JM, OJ, MR; Writing – original draft: JM, JB, MR; Writing – review & editing: JB, MT, MR.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.688950/full#supplementary-material

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OPINION
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# OCT1 Polyspecificity—Friend or Foe?

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**Keywords: OCT1, SLC22A1, polyspecificity, structure-to-function relationship, ligand-transporter interaction, species differences, genetic variants**

## INTRODUCTION

Polyspecificity is one of the most characteristic features of organic cation transporter OCT1 (SLC22A1). Already upon the initial cloning it was recognized that the OCT1-mediated uptake could be inhibited by a variety of substances with different chemical structures (Gründemann et al., 1994). Following the initially identified substrate $TEA^+$, the organic cations $MPP^+$ and $ASP^+$ and endogenous compounds such as dopamine and histamine were described as substrates (Gründemann et al., 1994; Busch et al., 1996a; Busch et al., 1996b; Mehrens et al., 2000), showing that not only aliphatic, but also aromatic cations with variable structures could be OCT1 substrates. Currently, more than 150 organic cationic compounds with highly variable chemical structures, including also commonly used drugs like metformin, morphine, sumatriptan, fenoterol, and lamotrigine have been reported to be substrates of the organic cation transporter OCT1 (Wang et al., 2002; Dickens et al., 2012; Tzvetkov et al., 2013; Matthaei et al., 2016; Shen et al., 2016; Tzvetkov et al., 2018; Haberkorn et al., 2021).

However, polyspecific does not mean unspecific. We recently reported that small differences in the chemical structures of morphinan opioids lead to substantial differences in the inhibitory potency or even to the inability to interact with human OCT1 (Meyer et al., 2019).

Twenty-seven years after OCT1 discovery, the mechanisms conferring its polyspecificity are still poorly understood. Our current knowledge about the mechanisms of OCT1-mediated transport is predominantly based on mutagenesis experiments. Several amino acids have been suggested to be of key importance for substrate binding and/or translocation (Gorboulev et al., 1999; Gorboulev et al., 2005; Popp et al., 2005; Sturm et al., 2007; Volk et al., 2009). The most prominent thereof is Asp475 in transmembrane helix 11, which is generally accepted to play a key role by interacting with the positive charge of the substrate. However, OCT1 is thought to have multiple binding sites that may overlap between substrates (Gorboulev et al., 1999; Popp et al., 2005; Volk et al., 2009; Chen et al., 2017; Boxberger et al., 2018), but there is no crystal structure available, neither for OCT1, nor for any of the closely related proteins of the SLC22A family. The homology models used instead are based on distantly related transporters that share maximally 19.5% identity with human OCT1 (Koepsell, 2020). Therefore, the exact binding sites of the different OCT1 substrates remain unclear.

## OCT1 POLYSPECIFICITY AS A FOE

In many regards, the polyspecificity of OCT1 represents a disadvantage. It complicates the experimental analysis and the interpretation of the obtained results.

Firstly, OCT1 polyspecificity is a hurdle when studying the mechanisms of transport. Most of our current knowledge about OCT1 structure-to-function relationships has been obtained using $MPP^+$ or $TEA^+$ as substrates (Gorboulev et al., 1999; Gorboulev et al., 2005; Popp et al., 2005; Volk et al., 2009; Keller et al., 2019). Already for these two substrates, substrate-specific differences in the role of key amino acids were reported. While mutation of Arg440Lys affected the affinity for $TEA^+$ but not

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for $MPP^+$, mutation of Phe160Ala affected the affinity for $MPP^+$ but not for $TEA^+$ (Gorboulev et al., 2018). This may not be surprising, considering the structural differences between the two substrates. On the contrary, different ligands can be expected to interact with different amino acids of the transporter. Thus, despite their name, the so-called model substrates may be of only limited use for predictions of substrate-transporter interactions for clinically relevant ligands. Therefore, at least until we better understand the structural mechanisms of OCT1 polyspecificity, structure-to-function relationships have to be established for each substrate separately. As a practical consequence, *in vitro* data on inhibitory potencies using OCT1 model substrates may be of only limited predictive value for drug-drug-interactions (DDIs) with the actual victim drug (Koepsell, 2015, 2020; Hermann, 2021).

Secondly, in addition to the difficulties of transferring structure-to-function findings between substrates, polyspecificity aggravates the transfer of findings between species. Most of the available structure-to-function data have been obtained studying rat Oct1 (Gorboulev et al., 1999; Gorboulev et al., 2005; Popp et al., 2005; Sturm et al., 2007; Volk et al., 2009). However, human and rat OCT1 differ in 120 amino acids and each of them may potentially cause differences in OCT1 function. There is not much data directly comparing rat and human OCT1, but the affinity and the capacity of metformin and thiamine transport have been shown to differ substantially between mouse and human OCT1 (Chen et al., 2014; Meyer et al., 2020). Based on these differences, up to 11-fold higher maximal metformin concentrations may be expected in mouse than in human liver and hepatic effects of metformin in humans may be overestimated (Meyer et al., 2020). This may explain why loss of OCT1 activity in humans does not affect metformin efficacy (Zhou et al., 2009; Dujic et al., 2017) in contrast to strong effects observed in OCT1 knock-out mice (Wang et al., 2002; Wang et al., 2003).

More importantly, due to OCT1 polyspecificity, the species differences are also substrate-specific. While OCT1 inhibition results in strong differences in the uptake of ondansetron and tropisetron between mouse and human hepatocytes, no differences were observed for sumatriptan and fenoterol (Morse et al., 2020). Therefore, next to the known differences in OCT1 organ expression between the species (Gründemann et al., 1994; Schweifer and Barlow, 1996; Gorboulev et al., 1997; Green et al., 1999), differences in transport activity have to be kept in mind when interpreting existing data from animal models and cannot not be generalized among the substrates.

Thirdly, polyspecificity leads to substrate-specific effects of genetic variants in OCT1. OCT1 is genetically highly variable and common genetic variants lead to a reduction or to a loss of OCT1 function (Kerb et al., 2002; Shu et al., 2003; Seitz et al., 2015). Some of these variants have substrate-specific effects. The most common OCT1 variant in Europeans and White Americans (Seitz et al., 2015), the deletion of Met420 (*OCT1*2*), reduces the uptake of metformin, morphine, tropisetron, and O-desmethyltramadol (O-DSMT) by more than 75% (Shu et al., 2007; Tzvetkov et al., 2011; Tzvetkov et al., 2012; Tzvetkov et al., 2013), but shows normal or only slightly reduced uptake of sumatriptan, cycloguanil, and debrisoquine (Saadatmand et al., 2012; Matthaei et al., 2016; Matthaei et al., 2019). Therefore, homozygous carriers of *OCT1*2* have to be regarded as complete loss-of-function phenotypes (so-called poor OCT1 transporters) when tramadol is administrated and as fully active (extensive OCT1 transporters) when sumatriptan is administrated. Hence, individual OCT1 activity scores have to be substrate-specific and cannot be generalized. This complicates the use of OCT1 pharmacogenetics in the clinical routine and requires clinical studies for each substrate.

Finally, the polyspecificity of OCT1 questions the idea of one "ultimate" pharmacophore valid for OCT1 ligands. Indeed, the published ligand-based pharmacophore models of OCT1 differ in the number, type, and distance of their features. While the models of Bednarczyk et al. (2003), Moaddel *et al.* (Moaddel et al., 2005; Moaddel et al., 2007) and from our group (Meyer et al., 2019) show some resemblance, the model by Nies et al. (2011) shows more pronounced differences, the most striking being the absence of a positively ionizable site. This is not surprising and may simply reflect the coexistence of different binding sites in OCT1. Therefore, before we understand which ligands bind to which binding sites, it will be difficult to correctly identify chemical features necessary for interaction with OCT1.

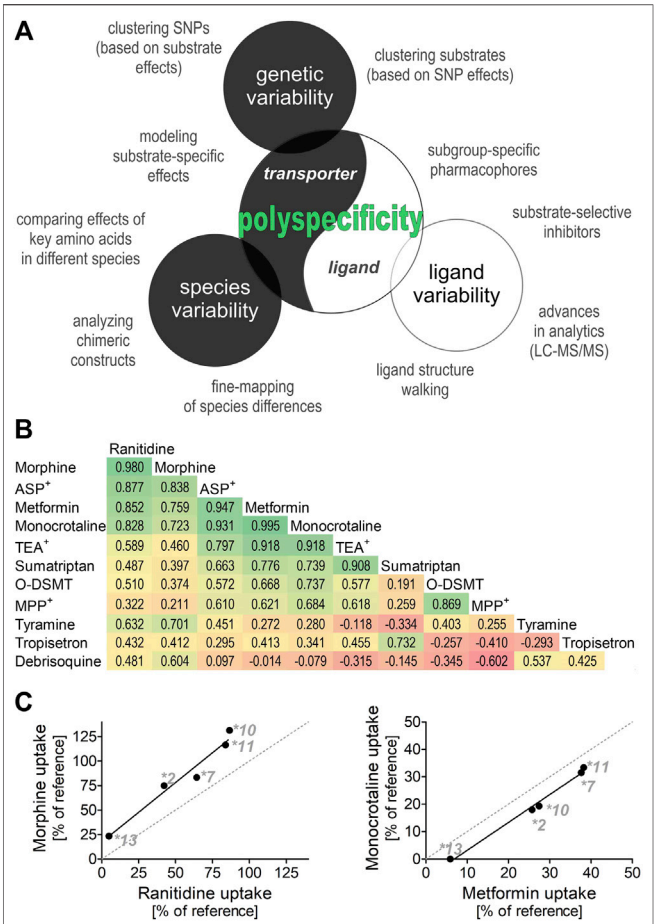
## OCT1 POLYSPECIFICITY AS A FRIEND

When utilized properly, the polyspecificity of OCT1 can also be an ally in understanding the transport mechanisms of OCT1. Many of the disadvantages listed above can be turned into experimental tools to study polyspecificity.

Firstly, the *in vitro* prediction of DDIs should be performed with more than one (victim) substrate, comparing the inhibitory potencies as has been done already for OCT2 (Hacker et al., 2015; Sandoval et al., 2018) and partially for OCT1 (Ahlin et al., 2011). This strategy has two advantages. First, using the victim drug of interest and not a model substrate enables more precisely predicting DDIs in humans. Second, and more interesting, it enables analyzing the interactions in the context of the specific substrate used. This may help identifying clusters of ligands (substrates and inhibitors) that potentially share binding positions in OCT1. In the long term, this may help to stratify ligands into groups according to similar binding properties and to generate subgroup-specific pharmacophores.

Secondly, comparing the interaction with OCT1, ligands with closely related structures may help to identify moieties that are important for the interaction. Systematic comparison of the OCT1 inhibitory potency of structurally similar morphinan opioids revealed that only minor structural changes, involving the ether linkage between C4-C5 of the morphinan ring, strongly increased the inhibitory potency for OCT1 (Meyer et al., 2019). Such systematic "ligand structure walking" may prove to be very useful to better understand the role of the ligand structure in the OCT1 transport mechanism.

All examples of experiments listed above are possible today due to technical advancement of the analytical methods. In




| | Ranitidine | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Morphine | 0.980 | Morphine | | | | | | | | | |
| ASP$^+$ | 0.877 | 0.838 | ASP$^+$ | | | | | | | | |
| Metformin | 0.852 | 0.759 | 0.947 | Metformin | | | | | | | |
| Monocrotaline | 0.828 | 0.723 | 0.931 | 0.995 | Monocrotaline | | | | | | |
| TEA$^+$ | 0.589 | 0.460 | 0.797 | 0.918 | 0.918 | TEA$^+$ | | | | | |
| Sumatriptan | 0.487 | 0.397 | 0.663 | 0.776 | 0.739 | 0.908 | Sumatriptan | | | | |
| O-DSMT | 0.510 | 0.374 | 0.572 | 0.668 | 0.737 | 0.577 | 0.191 | O-DSMT | | | |
| MPP$^+$ | 0.322 | 0.211 | 0.610 | 0.621 | 0.684 | 0.618 | 0.259 | 0.869 | MPP$^+$ | | |
| Tyramine | 0.632 | 0.701 | 0.451 | 0.272 | 0.280 | -0.118 | -0.334 | 0.403 | 0.255 | Tyramine | |
| Tropisetron | 0.432 | 0.412 | 0.295 | 0.413 | 0.341 | 0.455 | 0.732 | -0.257 | -0.410 | -0.293 | Tropisetron |
| Debrisoquine | 0.481 | 0.604 | 0.097 | -0.014 | -0.079 | -0.315 | -0.145 | -0.345 | -0.602 | 0.537 | 0.425 |

**FIGURE 1 |** Polyspecificity as a friend **(A)** Illustrates different strategies for using polyspecificity as a tool to study the mechanisms of OCT1 transport. Given are ligand-based and transporter-based approaches, including the use of species and genetic differences. **(B)** and **(C)** Summarize and analyze data of five previous studies (Tzvetkov et al., 2012; Tzvetkov et al., 2013; Seitz et al., 2015; Matthaei et al., 2016; Meyer et al., 2017) as illustration of the use of SNP effects to cluster OCT1 substrates into different subgroups. Shown are the effects of OCT1 alleles **2, *7, *10,*11,* and **13*, which are known to have strongly substrate-specific effects on transport (Seitz et al., 2015), on the OCT1-mediated uptake of 11 substrates. The pairwise correlation coefficient between the effects of different alleles are given **(B)** and the two strongest correlations are shown **(C)** O-DSMT, O-Desmethyltramadol.

contrast to the first decades of studying OCT1 where scientists were limited by the availability of radioactive OCT1 substrates, today we can use techniques such as LC-MS/MS to quantify practically any substrate of interest. The sensitivity is still not as high as in radioactive detection, but the quantification of the intracellularly accumulated substrate is highly specific. This enabled first high-throughput screens for OCT1 substrates (Hendrickx et al., 2013) and also detailed analyses of substrates with only slightly different chemical structures (Meyer et al., 2019) up to stereoselective effects of the uptake (Jensen et al., 2020).

Approaching polyspecificity from the transporter side, the species-specific differences in OCT1 transport can be used as a tool to identify domains or even single amino acids responsible for the substrate-specific effects on transport. In a proof-of-principle study we used human-mouse chimeric constructs to localize the cause for the higher affinity of mouse OCT1 for thiamine and metformin to transmembrane helices 2 and 3 (Meyer et al., 2020). For metformin, we were even able to fine-map the causal difference to the difference between Leu155 in human and Val156 in mouse OCT1. This strategy is extendable to all substrates showing species-specific differences in uptake. Furthermore, similar to the ortholog comparison, also paralogs with different substrate preferences may be compared, as has successfully been done for rat Oct1 and Oct2 (Gorboulev et al., 2005).

Similarly, substrate-specific effects of some OCT1 genetic variants may help to reveal details in the mechanism of OCT1 transport. By comparing the effects of genetic variants with substrate-specific effects on different substrates, we can identify substrates that are similarly affected and thus may share similar binding sites in the transporter. To illustrate this, we used previously published data about the effects of the substrate-specific OCT1 genetic variants *OCT1*2*, **7*, **10*, **11*, and **13* from our group (**Figures 1B**,**C** (Tzvetkov et al., 2012; Tzvetkov et al., 2013; Seitz et al., 2015; Matthaei et al., 2016; Meyer et al., 2017)). The effects on the uptake of metformin and monocrotaline, but also of morphine and ranitidine correlated very well (r of 0.995 and 0.98, respectively; **Figure 1**), suggesting at least two groups of structurally divergent substrates that may share similar binding sites in OCT1. This strategy could be used to cluster ligands into subgroups based on the impact of the substrate-specific OCT1 genetic variants. Such subgroups could be used to develop subgroup-specific pharmacophores (similar to those suggested above for analyses of substrate-specific DDIs) and to identify subgroup-specific model substrates that will facilitate the handling of OCT1 pharmacogenetics in a clinical setting.

## SUMMARY

The polyspecificity of OCT1 sets many hurdles for understanding the transport mechanisms of OCT1 and for the translation of our knowledge about OCT1 into clinical practice. However, polyspecificity may be used also as a tool, especially to reveal the mechanisms of OCT1 transport, which is an essential step for deepening our understanding of the physiological functions and potential pharmacological implications of this transporter.

## AUTHOR CONTRIBUTIONS

MM and MT analyzed the data and wrote the manuscript.

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