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„The interaction between central nervous system active substances and the human organic cation transporters 1-3 and plasma membrane monoamine transporter“

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This Master's thesis is a cumulative work as it contains the article "Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter", which was published on 30 November 2021 in the *International Journal of Molecular Sciences* and which I have worked on in parallel to the thesis.

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Author contributions

Following acknowledgements apply to the published article “Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter”, which was incorporated into this work as part of the Master’s thesis. Writing of Abstract, Zusammenfassung, Introduction and Discussion of this work as well as compilation of the therein contained Figure and Table was done by Stevan Stanković¹ and reviewed/edited by Julian Maier¹ and Harald H. Sitte¹.

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Table of contents

Abstract.....	1
Zusammenfassung	2
List of Abbreviations	3
Introduction.....	4
Discussion.....	39
References.....	48

Abstract

To date, many central nervous system active compounds have been shown to potently interact with the SLC6 neurotransmitter-sodium symporters for norepinephrine (SLC6A2; NET), dopamine (SLC6A3; DAT) and 5HT/serotonin (SLC6A4; SERT). They are known for high-affinity/low-capacity uptake of monoamines and depict essential targets for a plethora of medical and illicit substances. However, pronounced focus is being put on investigating interactions between psychoactive compounds and low-affinity/high-capacity organic cation transporters 1-3 (OCT1-3; SLC22A1-3) and plasma membrane monoamine transporter (PMAT; SLC29A4), since they show distinct overlaps in pharmacodynamic properties with SLC6 members and are proposedly co-involved in maintaining monoaminergic equilibrium in CNS. Comparing the abundance of available interaction profiles of various compounds at SLC6 proteins, a research gap concerning the amount of described substances interacting with OCTs and PMAT exists. By utilizing radiotracer-based in vitro uptake inhibition assays at stably over-expressing HEK293 cells, we herein reported data from 17 psychoactive compounds regarding their potential to inhibit substrate uptake by human isoforms of OCT1-3 and PMAT. Many compounds impeded uptake by hOCT1 and 2 in a low micromolar range, fewer interacted with hPMAT, whereas almost none showed pharmacologically relevant interactions at hOCT3. Methylphenidate and ketamine selectively interacted with hOCT1 and 2, respectively. MDMA potently inhibited all transporters but hOCT3. Enantiospecific differences as well as effects of aromatic ring substituents were analyzed. Our results accentuate the importance of investigating the rich pharmacology of hOCTs and hPMAT, but also give impetus for further research particularly on the interaction of drugs with the OCTs.

Zusammenfassung

Bis heute wurde gezeigt, dass viele Wirkstoffe, die im zentralen Nervensystem aktiv sind, potent mit den SLC6-Neurotransmitter-Natrium-Symportern für Noradrenalin (SLC6A2; NET), Dopamin (SLC6A3; DAT) und 5HT / Serotonin (SLC6A4; SERT) interagieren. Sie sind bekannt für ihre hochaffine / kapazitätsarme Aufnahme von Monoaminen und stellen wesentliche Ziele für eine Vielzahl von medizinischen und illegalen Substanzen dar. Ein vermehrter Fokus wird jedoch auf die Untersuchung von Wechselwirkungen zwischen psychoaktiven chemischen Verbindungen und niedrigaffinen / kapazitätsstarken organischen Kationentransportern 1-3 (OCT1-3; SLC22A1-3) und dem Plasmamembran-Monoamintransporter (hPMAT; SLC29A4) gelegt, da diese deutliche Überschneidungen in pharmakodynamischen Eigenschaften mit den SLC6-Mitgliedern aufweisen und ihnen eine Rolle an der Aufrechterhaltung des monoaminergen Gleichgewichts im ZNS zuteilwird. Im Vergleich zu den zahlreich vorhandenen Interaktionsprofilen verschiedener chemischer Verbindungen bei SLC6 Proteinen, besteht dennoch eine große Forschungslücke hinsichtlich der Menge beschriebener Substanzen, die mit den OCTs und PMAT interagieren. Durch die Verwendung von Radiotracer-basierten In-vitro-Aufnahmehemmungs-Assays bei stabil über-exprimierenden HEK293-Zellen berichteten wir Daten von 17 psychoaktiven Substanzen hinsichtlich ihres Potenzials, die Substrataufnahme durch die menschlichen Isoformen von OCT1-3 und PMAT zu hemmen. Viele Substanzen schränkten die Substrataufnahme durch hOCT1 und 2 im mikromolaren Bereich ein, weniger interagierten mit hPMAT, während fast keine pharmakologisch relevante Wechselwirkungen mit hOCT3 zeigte. Methylphenidat und Ketamin interagierten selektiv mit hOCT1 bzw. 2. MDMA hemmte alle Transporter, bis auf hOCT3, potent. Enantiospezifische Unterschiede sowie Effekte von aromatischen Ringsubstituenten wurden analysiert. Unsere Resultate betonen die Wichtigkeit des Untersuchens der reichen Pharmakologie von hOCTs und hPMAT, geben aber auch Impulse weitere Erforschungen von Interaktionen zwischen Medikamenten und organischen Kationentransportern.

List of Abbreviations

$^3\text{H-MPP}^+$	Tritiated 1-methyl-4-phenylpyridinium
CNS	Central nervous system
D22	Decynium-22
DDI	Drug-drug interaction
OCT1-3	Organic cation transporter 1-3
5-HT	5-hydroxytryptamine (Serotonin)
DAT	Dopamine transporter
IC ₅₀	Half maximal inhibitory concentration
MAT	Monoamine transporter
MDMA	3,4-Methylenedioxymethamphetamine (Ecstasy)
NET	Norepinephrine transporter
PMAT	Plasma membrane monoamine transporter
R- and S- α -PVP	R- and S- α -pyrrolidinovalerophenone
SERT	Serotonin transporter
SLC	Solute carrier membrane transporter
SSRI	Selective serotonin reuptake inhibitor

Introduction

Organic cation transporter 1-3 (OCT1-3; encoded by the SLC22A1-3 genes, respectively) and the human plasma membrane monoamine transporter (PMAT; SLC29A4) are facilitative transporters that belong to the solute carrier (SLC) family of eukaryotic membrane-bound transport proteins (Koepsell, Schmitt and Gorboulev, 2003; Hediger *et al.*, 2004; Schlessinger *et al.*, 2010). While the precise number is still enigmatic and debated on, current knowledge suggests that over 400 different transporters constitute the SLC family, making it the second largest group of phylogenetically related membrane proteins, following G protein-coupled receptors with over 800 identified members (Fredriksson *et al.*, 2003; Kroeze, Sheffler and Roth, 2003; Höglund *et al.*, 2011). Assignment of SLC transporters to the same specific family is principally, with just a few exceptions, performed in cases where the encoded proteins share at least 20-25% amino acid sequence identity to each other (Hediger *et al.*, 2004, 2013). Naturally, this confers the SLC families a relatively high heterogeneity when compared to other membrane protein families regarding genetic sequence similarities, e.g. the human ATP-binding cassette (ABC) transporter family or G-Protein coupled receptors (Vasiliou, Vasiliou and Nebert, 2009; Colas, Ung and Schlessinger, 2016). Currently, all SLCs are grouped in over 50 families, which again belong to 24 so-called superfamilies, as proposed by classifications based on SLC protein sequence searches against the Pfam (Protein families) database (Höglund *et al.*, 2011; Perland and Fredriksson, 2017). Through either facilitative, i.e. passive, or active transport, proteins of the SLC family move a vast number of solutes like e.g. medical compounds, important nutrients and different types of xenobiotics across the lipid membrane, implicating them in the onset of various diseases and thus rendering them prominent drug targets which carry high pharmacological importance (Jonker and Schinkel, 2004; Koepsell and Endou, 2004; Schlessinger *et al.*, 2010; César-Razquin *et al.*, 2015; Nigam, 2018). This physiological and medical relevance is underlined by the fact that approximately 10% of the human genome contains genes that directly encode for or in some way relate to transporters, of which the SLCs body a large part (Hediger *et al.*, 2013; César-Razquin *et al.*, 2015).

The SLC22 family of transporters falls into three major clades of OCTs, OCTNs (organic cation transporters novel) and OATs (organic anion transporters) and the SLC29 family comprises facilitative nucleoside/nucleotide transporters (NTs) (Young *et al.*, 2013; Nigam, 2018). Since OCT1-3 and PMAT belong to the SLC22 and SLC29 family, respectively, they are grouped under the prominent major facilitator superfamily (MFS) Pfam clan, one of a total of four SLC superfamilies that each enclose more than one SLC family, and depicts, with holding almost a

third of all SLCs, the largest member thereof (Höglund *et al.*, 2011). Recent studies classify both the SLC22 and SLC29 transporter family into the phylogenetic α -group (Höglund *et al.*, 2011). Together with the β -, γ - and δ -groups it represents one of four, and with its 13 members at the same time also the largest, major phylogenetic clusters which contain a total of 26 SLC family members (Fredriksson *et al.*, 2008; Höglund *et al.*, 2011). One important commonality of the MFS clan members is their structural fold (Quistgaard *et al.*, 2016). Current analyses propose twelve transmembrane helices to form the structural core of these transporters, subdividing them into the N- and C- domains, two structurally similar regions important for transport (Yan, 2013; Quistgaard *et al.*, 2016). However, the exact mechanism of substrate translocation has not yet been fully confirmed, and various models were established in order to explain the different transporter protein states and their involvement in moving substrates across the lipid bilayer (Law, Maloney and Wang, 2009; Yan, 2013; Colas, Ung and Schlessinger, 2016; Quistgaard *et al.*, 2016). First reported in 1966, the alternating access model has been broadly accepted for depicting SLC transport, and three types of mechanisms have since been described, intending to explain its switch between inward and outward facing conformations via an intermediate, or so-called occluded, state (Jardetzky, 1966; Colas, Ung and Schlessinger, 2016). Thus, one of three known alternating access mechanisms is accepted as the transport mechanism of proteins with an MFS fold, the so-called “Rocker-switch” model (Colas, Ung and Schlessinger, 2016). This model describes the substrate translocation by a rocker-switch type of movement of the two transporter protein domains, which work in concert and lead to global conformational changes of the protein structure (Law, Maloney and Wang, 2009). This mechanism is based on and well-studied for the lactose permease LacY from *Escherichia coli*, a transporter protein which belongs to the same MFS as OCTs and whose already explored crystal structure substantiated further modelling and deeper understanding of substrate translocation by a plethora of eukaryotic homologues (Popp *et al.*, 2005; Gorbunov *et al.*, 2008). Active transport performed by SLC family members is always coupled, divided into sym- and antiporters, i.e. exchangers, and depends on cellular energy (Hediger *et al.*, 2004). The three aforementioned organic cation transporter subtypes OCT1, OCT2, and OCT3, as well as the human plasma membrane monoamine transporter, however, hold a driving force which is solely powered by the membrane potential difference and chemical gradient, i.e. the electrochemical gradient, providing the transport energy needed to translocate organic, cationic compounds, hence classifying them as facilitative passive transporter types (Koepsell, Schmitt and Gorboulev, 2003; Koepsell, 2013; Wang, 2016). However, non-charged compounds are also transported (Koepsell, Lips and Volk, 2007). Pharmacological and biological relevance of

OCTs is highlighted by the large number of present compounds that are positively charged at physiological pH and thus depend on an organic cation transport system to be taken up into or effluxed out of cells, including exogenous substrates like medical drugs or endogenous xenobiotics (Kekuda *et al.*, 1998; Jensen *et al.*, 2021). The latter function is, in the periphery, especially taken over by the liver and the kidney, two organs that show high expressions of various eliminatory systems of xenobiotics and are well-known for their abundant expression of OCT1 and 2, respectively (Ahlin *et al.*, 2008). Strikingly, all OCTs and PMAT are, albeit with differing frequencies, located in the human blood-brain barrier, while all but OCT1 are expressed in neurons and OCT3 and PMAT are further also found in astrocytes (Koepsell, 2021).

Amongst all present described human SLC proteins, experimentally determined, high-resolution crystal structures of only six members (SLC2A1, SLC2A3, SLC4A1, SLC6A4, SLC29A1 and SLC42A3) are currently available, confining our knowledge of other SLC transporters in regards of their transport mechanisms and substrate specificities, and with them also pharmacodynamics of relevant compounds (Colas, Ung and Schlessinger, 2016; Coleman, Green and Gouaux, 2016; Bai, Moraes and Reithmeier, 2017; Wright and Lee, 2019). Concerning drug-transporter interactions, the clearly most well-described and intensely researched SLCs are members of the high-affinity and low-capacity SLC6 family, the neurotransmitter-sodium symporters that transport the prominent monoamines norepinephrine (NET; SLC6A2), dopamine (DAT; SLC6A3), and 5-hydroxytryptamine (5-HT), i.e. serotonin, (SERT; SLC6A4) and with them the plethora of drugs that interact with them (Torres, Gainetdinov and Caron, 2003; Engel, Zhou and Wang, 2004; César-Razquin *et al.*, 2015). In this respect, SLC22 and SLC29 targets have, by far, less compounds mentioned that act as e.g. inhibitors of substrate uptake against them, even though substrate similarities and, to some extent, functional overlaps with NET, DAT and SERT have been reported so far (Gebauer *et al.*, 2021; Maier, Niello, *et al.*, 2021; Maier, Rauter, *et al.*, 2021). The single most important commonality between NET, DAT and SERT, often commonly referred to as MATs (monoamine transporters), and the organic cation transporters 1-3 as well as PMAT, is their ability to interact with a high diversity of compounds by mediating their cellular uptake, but most notably monoamines, and thus maintaining an equilibrium thereof (Engel, Zhou and Wang, 2004; Grouleff *et al.*, 2015; Koepsell, 2021). In this regard, it was shown early on and confirmed several times by different groups that both the mentioned SLC22 members as well as human PMAT can uptake norepinephrine, dopamine and serotonin as a substrate, albeit with strongly varying affinities (Busch *et al.*, 1998; D. Gründemann *et al.*, 1998; Zolk *et al.*, 2009;

Zhu *et al.*, 2010; Chen *et al.*, 2014; Shirasaka *et al.*, 2017; Gebauer *et al.*, 2021). Along with enzymatic degradation, monoamine reuptake by neurotransmitter transporters of pre-synaptic neurons and adjacent glia cells plays an essential role in terminating the chemical signal evoked by presynaptic neurotransmitter release and represents a mechanism of major pharmacological relevance (Gainetdinov, Sotnikova and Caron, 2002). A plethora of nervous system active substances can act as competitive or allosteric inhibitors of such transporters, thus resulting in an increase in synaptic availability of the aforementioned neurotransmitters and consequently increase norepinephrergic, dopaminergic and/or serotonergic function within the peripheral and central nervous system (Owens, Knight and Nemeroff, 2001; Howell and Negus, 2014). This monoaminergic pathway controls physiological functions that are associated with fundamental behaviours like mood, appetite, sleep, attention, motor function and motivation (Couroussé and Gautron, 2015).

A broad number of substances, ranging from prescribed medicals to known drugs of abuse and other illicit compounds, are well-known for their interaction with NET, DAT and SERT, the hitherto best-described players related to monoamine balance in the CNS. Given their abundance and interaction with MATs, interest in describing these substances' interaction with the human OCTs and PMAT is on a constant rise, since these transporters pose, yet less well-researched, essential protagonists in the physiologically crucial monoaminergic system (Zhou, Engel and Wang, 2007; Tzvetkov *et al.*, 2011; Haenisch *et al.*, 2012; Massmann *et al.*, 2014; Mayer *et al.*, 2018; Jensen *et al.*, 2021). Because of the vast number and broad spectrum of chemical structures of organic cations and transported uncharged ions, the organic cation transporters, as well as PMAT, are defined as polyspecific or “multispecific” transporters (Ciarimboli, 2008; Wang, 2016). Furthermore, OCT1-3 are believed to be the main carriers of polyamines in humans, since, in contrast to prokaryotic and a few eukaryotic cells, no confirmed polyamine transporters have yet been found in mammalian cells (Sala-Rabanal *et al.*, 2013). Historically, the system that mediates the re-uptake of biogenic monoamines, but especially 5-HT due to its pharmacological relevance in depression treatment, as well as dopamine and norepinephrine due to their role in various psychiatric disorders was termed “uptake-1”. Over the years, it comprised the high-affinity/low-capacity Na^+ and Cl^- -dependent transporters NET, DAT and SERT, as the most efficient and best known transporters for named neurotransmitters (Jonker and Schinkel, 2004; Daws, C., 2013). However, a second, heterologous catecholamine transporting system, referred to as “uptake-2”, was gradually defined. It described a popularized alternative of uptake-1 as a potential-sensitive system, independent from Na^+ and Cl^- , which was known for its “low affinity, high capacity” uptake of

monoamine neurotransmitters. This physiological occurrence could not be merely accounted for by classical high-affinity transport (Wu *et al.*, 1998). The “uptake-2” system was first mentioned in 1965 and found in rat heart muscles, where it was shown to mediate low-affinity uptake of epinephrine and norepinephrine (Iversen, 1965). Further being detected in CNS and identified as the extraneuronal monoamine transporter (EMT; today known as OCT3) uptake-2 transport gained attention through a growing body of literature, soon suggesting it to partake in the efficacy of CNS active drugs like e.g. antidepressants. Initially, based on their predominantly peripheral tissue distribution, other organic cation transporters were excluded from the uptake-2 system, including OCT1-2 and PMAT. Today, however, these transporters are seen as part of that system, since their occurrence in CNS and physiological function qualify them for it (Wu *et al.*, 1998; Eisenhofer, 2001; Jonker and Schinkel, 2004; Daws, C., 2013; Daws, Koek and Mitchell, 2013). Since members of the OCT family are involved in the excretion of endogenous compounds, such as metabolites, or substances of exogenous origin, such as various drugs, they have overlapping substrate and inhibitor profiles (Jonker and Schinkel, 2004; Sala-Rabanal *et al.*, 2013). Substrates underlie a classical transport mechanism, where plasma membrane transporters take them up and facilitate their passage across the lipid bilayer (Perland and Fredriksson, 2017). In contrast, inhibiting substances can competitively or non-competitively bind to transporters in their outward facing conformation and thus prevent further substrate uptake (Sitte and Freissmuth, 2015). The majority of *in vitro* studies characterizing the interaction between CNS active substances and the three OCT isoforms and PMAT focused on psychoactive compounds that were already well-described in regard to their interplay with MATs (Amphoux *et al.*, 2006; Zhou, Engel and Wang, 2007; Haenisch *et al.*, 2012; Wagner *et al.*, 2017; Maier, Niello, *et al.*, 2021). This link in substrate similarity and thereof related functionality between MATs, OCTs and PMAT bases the growing literature focus on identifying various compounds as, mainly, inhibitors of OCT1-3 and PMAT and again highlights the low-affinity/high-capacity transporters' obvious role in the maintaining of monoaminergic equilibrium in the CNS (Giacomini, Huang and Tweedie, 2010; Zamek-Gliszczynski, Giacomini and Zhang, 2018a; Gasser, 2019; Daws, 2021).

Organic cation transporter 1 (SLC22A1), one of the best studied member of the SLC22 family, is located on chromosome 6q26 and consists of eleven exons and ten introns (Hayer, Bönisch and Brüss, 1999; Koepsell, Lips and Volk, 2007; Ciarimboli, 2008). Human OCT1 has a broad peripheral tissue distribution, most notably expressed in the liver, but also in various other tissues such as large and small intestine and in the kidney, where it partakes in the excretion of a wide range of exogenous drugs, xenobiotics and metabolites, and was also found, albeit with

weaker expression, in brain tissue (Gorboulev *et al.*, 1997; Koepsell, Lips and Volk, 2007; Liu *et al.*, 2016; Koepsell, 2020). The rat organic cation transporter 1 (rOCT1) was isolated from kidney tissue in 1994 and was the first identified member of the SLC22A transporter family (Gründemann *et al.*, 1994). A few years later, in 1997, the first human isoform of OCT1, hOCT1, together with hOCT2, was cloned and functionally described (Gorboulev *et al.*, 1997; Zhang, 1997). hOCT1 plays an important role in gastrointestinal absorption and hepatic elimination of a vast number of endogenous compounds and xenobiotics, but also depicts a target site for many well-described drugs (Choi and Song, 2008; Mato *et al.*, 2018; Zamek-Gliszczynski, Giacomini and Zhang, 2018b). So far, many in vivo and in vitro studies have reported OCT1 to be important for the pharmacokinetics of various organic cationic drugs and their response in patients, OCT1 is often described as a (possible) mediator of many drug-drug interactions (DDIs), an issue of increasing importance for pharmacotherapy (Boxberger, Hagenbuch and Lampe, 2014; Zhou, Zeng and Shu, 2021). In this sense, OCT1 is site of action for inhibiting (perpetrating) drugs which decrease hepatic clearance of substrate drugs (Zamek-Gliszczynski, Giacomini and Zhang, 2018a). Due to known effects of single nucleotide polymorphisms of OCT1 on interindividual variations in drug response, attention has been paid to OCT1 genetics, intending to further investigate its impact on reduced therapeutical, or even adverse, effects of a plethora of medical drugs (Amphoux *et al.*, 2006; Ahlin *et al.*, 2008, 2011; Al-Abdulla *et al.*, 2019; Zhou, Zeng and Shu, 2021). One popular and very well-described example of drug action being affected by either genetically altered or functionally hindered hOCT1 is the changed distribution of metformin, a prominent and widely prescribed antidiabetic and cationic drug that is normally transported across the hepatocyte membrane into the liver (Zamek-Gliszczynski, Giacomini and Zhang, 2018b). Studies revealed that both adjuvant use of OCT1 inhibiting drugs as well as presence of reduced-function alleles highly increased metformin intolerance in patients, underlining the transporters' key role in hepatic metformin uptake (Dujic *et al.*, 2015; Stage *et al.*, 2016; Zhou, Zeng and Shu, 2021). This stresses the fact that OCT1 regulation of drug metabolism partakes in mediation of gastrointestinal (GI) side effects, since genetic variants and inhibiting drugs can cause accumulation of OCT1 substrates in the intestines and thus, in the worst cases, result in treatment discontinuation due to adverse events (Tarasova *et al.*, 2012; Dujic *et al.*, 2015; Stage *et al.*, 2016). Earlier studies have demonstrated elimination of biogenic amines like norepinephrine, dopamine and 5-HT by rOCT1 expressed in HEK293 cells (Busch *et al.*, 1996). The putative role of OCT1 mediated hepatic inactivation of circulating monoamines was further confirmed, albeit showing differing transport activities of OCT1 towards different

catecholamines (Breidert *et al.*, 1998). In liver, hOCT1 was later found to be a major 5-HT uptake transporter with significantly higher affinities in comparison to the weaker expressed “uptake-2” members OCT3 and PMAT. Commonly prescribed CNS active drugs like e.g. antidepressants inhibit OCT1-mediated 5-HT uptake (Boxberger, Hagenbuch and Lampe, 2014). In brain, OCT1 was found weakly expressed in rat choroid plexus (CP) (Choudhuri *et al.*, 2003), murine hippocampus (Schmitt *et al.*, 2003; Baganz *et al.*, 2008), murine and human choroid plexus (Duan and Wang, 2013), and rat, murine and human brain microvascular endothelial (BMEC) (Friedrich *et al.*, 2001; Lin *et al.*, 2010) as well as cerebral microvessel endothelial (CMEC) cells (Sekhar *et al.*, 2017, 2019) of the blood-brain barrier. So far, no hOCT1 expression in neurons or glia cells was reported (Koepsell, 2021). This rather restricted expression in CNS explains why in vivo studies with OCT1 Knock-out mice so far focused mainly on examining the transporters' renal and hepatic function (Sweet, 2021). However, since interactions between various psychostimulant substances and hOCT1 have been broadly acknowledged by many in-vitro studies, its confirmed expression in the blood-brain barrier corroborates the common belief that OCT1 is as well important for maintaining monoaminergic equilibrium in CNS (Maier, Niello, *et al.*, 2021). Involvement of hOCT1 in various diseases and its broad effect on drug treatment is compactly summarized elsewhere (Koepsell, 2020; Daws, 2021).

Organic cation transporter 2 (SLC22A2), located on chromosome 6q26, consists of eleven exons and ten introns, shares about 70% amino acid sequence identity as well as a high proposed binding pocket similarity with hOCT1 and hence was shown to have similar affinities and ligand preferences therewith (Koehler *et al.*, 1997; Gründemann and Schömig, 2000; Sala-Rabanal *et al.*, 2013; Maier, Rauter, *et al.*, 2021). OCT2 was found to be primarily expressed in the basolateral membrane (BLM) of kidney proximal tubes, but in humans is also present in lungs, spleen, small intestine and brain (Nishimura and Naito, 2005; Koepsell, Lips and Volk, 2007; Choi and Song, 2008). In 1996, the first OCT2 mRNA expression was found in rat kidney, and reported tetraethylammonium (TEA) transport activity suggested OCT2 mediated renal organic cation transport (Okuda *et al.*, 1996). In 1997, the human OCT2 isoform was cloned and characterized (Gorboulev *et al.*, 1997; Zhang, 1997). As a renal cation transporter that arbitrates the first step in renal secretion, proposedly by facilitated transport across the basolateral membrane, OCT2 carries an important pharmacological and physiological role in the absorption and excretion of endogenous and exogenous compounds and toxins (Fujita *et al.*, 2006; Saito, 2010). OCT2 association with pharmacokinetics of important drugs was shown e.g. by studies with genetic variants, which confirmed OCT2 to be involved in renal elimination

of the above-mentioned antidiabetic drug metformin or found it to be of critical importance for cisplatin toxicity (Chen *et al.*, 2009; Filipski *et al.*, 2009). Renal accumulation of the latter can be inhibited by the medical compounds cimetidine and verapamil, by which its nephrotoxic effect gets reduced and thus serves as an example for the importance of OCT2 mediated DDIs (Hacker *et al.*, 2015). Along with OCT1, OCT2 is considered as a clinically relevant transporter for drug interactions in vivo and is recommended for in vitro inhibition studies by the European Medicine Agency (EMA) (EMA, 2012). rOCT2 expression in central nervous system was first shown in 1997, suggesting OCT2 as a promising medical target in the brain (Gründemann *et al.*, 1997). Early studies have put emphasis on analysing rat OCT2 expressing cells for their monoamine substrate specificity, and found that rOCT2 was capable of transporting neurotransmitters like dopamine (Dirk Gründemann *et al.*, 1998; Koepsell, Gorboulev and Arndt, 1999). So far, recognized neurotransmitters as endogenous compounds have been, amongst others, norepinephrine, dopamine, and 5-HT, implying OCT2 to partake in the monoaminergic clearance system not only at peripheral organs, but also in CNS (Dirk Gründemann *et al.*, 1998; Koepsell, Gorboulev and Arndt, 1999; Amphoux *et al.*, 2006). Busch *et al.* (1998) were the first to demonstrate the expression of a human OCT2 (hOCT2) in various brain areas and acknowledged its ability to transport NE, DA and 5-HT, proposing it as a “background transporter” for monoamine neurotransmitters and found it to have a similar localization in the well-studied rodent brain (Busch *et al.*, 1998). In their study with hOCT2 expressing *Xenopus laevis* oocytes, they reported hOCT2 inhibition by clinically relevant concentrations of amantadine and memantine, antiparkinsonian drugs that were shown, for the first time, to increase extracellular dopamine concentrations. Based on its transporting activity, substrates and presence in CNS, OCT2 was found to complement MATs as a player in monoaminergic neurotransmission (Amphoux *et al.*, 2006). OCT2 is believed to cause monoamine uptake at sites distant from locations of aminergic release, i.e. in cases of so-called volume transmission, but also compensates spill-over of monoamine neurotransmitters after e.g. blockade of high-affinity transporters by MAT inhibiting compounds (Couroussé and Gautron, 2015). This physiological relevance was underlined by an earlier in-vivo study in mice, which showed that OCT2 was important in antidepressant action and for antidepressant drugs to exert their full effects, suggesting alike mechanisms to be present in humans and further weighing investigation focus of organic cation transporters towards their role in CNS (Bacq *et al.*, 2012). Further, animal model studies have shown OCT2 to be important for the regulation of psychostimulant addiction, anxiety and depression (Bacq *et al.*, 2012; Couroussé and Gautron, 2015; Couroussé *et al.*, 2015). Based on these data, OCT2 holds potential in clarifying

some yet unaccounted aspects of interindividual variations in patients responding differently to drugs for the treatment of depression, anxiety, or PTSD (Sweet, 2021). However, a true research gap regarding *in-vivo* studies of CNS located OCT2, but also OCT1 and PMAT, still exists, rendering it difficult to assign their role in e.g. psychostimulant action (Maier, Niello, *et al.*, 2021). Involvement of hOCT2 in various diseases and its broad effect on drug treatment is compactly summarized elsewhere (Koepsell, 2020; Daws, 2021).

Organic cation transporter 3 (SLC22A3) is genetically located in close physical linkage to OCT1 and 2 on chromosome 6q27 and also consists of 11 exons and 10 introns (Gründemann and Schömig, 2000; Wieland *et al.*, 2000). OCT3 has, by far, the broadest brain tissue distribution of all organic cation transporters from the SLC22 family, ranging from (monoaminergic) neurons to the blood-brain barrier, the blood-cerebrospinal fluid barrier (BCSFB) as well as glia cells, including astrocytes (Hosoya and Tachikawa, 2011; Gasser, 2019). rOCT3 was first cloned and characterized in 1998, showing high mRNA expression in placenta and considerably higher abundance in brain than rOCT1 and 2 (Kekuda *et al.*, 1998). Shortly after, the first human isoform was detected and named extraneuronal transporter for monoamine transmitters (EMT), since it mediated their uptake at distance from their presynaptic release (D. Gründemann *et al.*, 1998). Hence, the well-known, classical neuronal uptake, i.e. high-affinity monoamine uptake by “uptake-1” transport systems by NET, DAT and SERT, located on presynaptic terminals, and the extraneuronal uptake were matched and compared in regard to their catecholaminergic substrates and sensitivity towards diverse drugs. As a member of the SLC22A family, OCT3 mediates facilitated low-affinity/high-capacity uptake of organic cations, and is best known for its role in the monoaminergic system of the human brain (Koepsell, 2021). However, due to monoamine substrate preferences and high peripheral tissue distribution in sympathetically innervated tissues, hOCT3 was, particularly in relation to the functionally strongly related and centrally expressed PMAT, assigned the role of a major uptake-2 transporter for epinephrine and NE in periphery (Duan and Wang, 2010). This nomenclature, however, differs between literature, as OCT3 is still often seen as the most important low-affinity monoamine transporter in brain. Since OCT3 shares only about 50% sequence identity with OCT1 or OCT2, this might explain differences in compound interactions and affinities (Sala-Rabanal *et al.*, 2013). OCT3, just like OCT2, mediates monoamine uptake at distance from the releasing pre-synapse and can eliminate NE, DA and 5-HT after their release (Couroussé and Gautron, 2015). To date, many *in vitro* and *in vivo* studies confirmed the interaction between hOCT3 and a vast number of compounds, but put focus mainly on psychoactive substances and CNS active drugs (Maier, Niello, *et al.*, 2021). Association studies

between OCT3 polymorphisms and substance abuse have drawn possible connections between (partial) loss of function of OCT3 and use disorders (Lazar *et al.*, 2003; Kitaichi *et al.*, 2005; Aoyama *et al.*, 2006). In an amphetamine study, amphetamine-induced locomotor activity as well as DA release was inhibited after wildtype mice were pre-treated with the non-selective OCT- & PMAT-blocker D22 (decynium-22), while it was the opposite in OCT3-deficient mice (Mayer *et al.*, 2018). Even though amphetamine is not an OCT3 substrate, the study showed further that amphetamine-triggered substrate release from the intracellular vesicular monoamine transporter 2 (VMAT2) is likely to lead to OCT3-mediated efflux. By comparing OCT3^{+/+} and OCT3^{-/-} mice, another study demonstrated that methamphetamine mediated release of dopamine by DAT was increased in the latter, deficient mutants, which further led to extracellular dopamine accumulation and thus toxicity (Cui *et al.*, 2009). Hence, a neurodegeneration modulating role was assigned to OCT3 for the first time, putting it in context of the Parkinson's disease as a contributor to neurotoxicity in the nigrostriatal dopamine pathway. Another link between OCT3 and amphetamine was shown in a recent *in vivo* study in mice, where OCT3 was demonstrated to partake in the rewarding and locomotor stimulant effects of the substance in female animals, further lending the transporter, together with PMAT and its similar effects in males, a role in sex-dependent effects of psychostimulants (Clauss, Koek and Daws, 2021). A concise summary of *in vivo* studies performed in mice, investigating the role of OCT3 in stress and addiction and sensitivity to psychoactive substances, was made elsewhere (Maier, Niello, *et al.*, 2021). However, alike OCT1 and 2, the human OCT3 and genetic variants thereof have been studied in regard of their pharmacological action of medical drugs (such as metformin and oxaliplatin, as mentioned above), but have yet to be investigated more intensely concerning their influence on drug abuse and addiction (Zhang *et al.*, 2006; Chen *et al.*, 2011).

The human plasma membrane monoamine transporter (SLC29A4) is located on chromosome 7p22.1 and was identified and first characterized in 2004 (Engel, Zhou and Wang, 2004). Before being named PMAT, the gene was previously titled human ENT4, categorized as a member of the human SLC29 equilibrative nucleoside transporter family, albeit exhibiting <20% sequence identity to any other ENT (*ibid.*). Even though it is not related to any of the OCTs or other known monoamine transporters (i.e. SLC6A members NET, DAT and SERT), it was shown early on that PMAT, unlike other SLC29 transporters, also transports organic cations and, most importantly, monoaminergic neurotransmitters, but no nucleosides or analogues thereof except for adenosine (Engel, Zhou and Wang, 2004; Wang, 2016). In relation to hOCT3, the most abundant SLC22A transporter in brain tissue, expressions of PMAT transcripts were found to

be distinctly higher in many of the investigated brain areas (Wang, 2016). In line with their preference and shown transport efficiency towards individual monoamines, hPMAT, which has a high preference for DA and 5-HT, the major centrally acting monoamines, and hOCT3, which transports epinephrine and NE most effectively, were suggested to be the major uptake-2 transporters in CNS and periphery, respectively (Duan and Wang, 2010). PMAT acts, just like OCT1-3, as a polyspecific organic cation transporter with low-affinity and high-capacity for monoaminergic neurotransmitters and was shown to have affinities towards them at an intermediate level between high-affinity MATs and the SLC22 family members (Vialou *et al.*, 2007). In general, some substrates and inhibitors of PMAT overlap with OCT1-3 and include e.g. the mentioned biogenic amines, the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) and also medical drugs (Engel and Wang, 2005). Concerning brain tissue distribution of PMAT, the main focus is put on the CP (choroid plexus) (Duan and Wang, 2013; Wang, 2016). The CP, or BCSFB (blood-cerebrospinal fluid barrier), is known to be essential for the transport of organic cations, monoamines and cationic drugs and toxins from the cerebrospinal fluid (CSF) to blood (Duan and Wang, 2013). However, since uptake of OCs at the apical membrane is mostly carrier-mediated, previous studies initially suggested hOCT2 and 3, amongst other transporters, to be involved in transport across the CP, but could not directly prove their expression (Sweet, Miller and Pritchard, 2001; Kusuhara and Sugiyama, 2004). Besides mRNA expression and protein localization studies, Duan and Wang (2013) further confirmed PMAT contribution to apical CP substrate uptake by using Slc29a4 gene knock-out mice (Duan and Wang, 2013). A clear decrease in OC and monoamine uptake in Pmat^{-/-} mice was shown, and only low contributions of OCT1-3 and SLC6 transporters to apical substrate uptake were confirmed. RT-PCR and immunohistochemical analysis revealed PMAT and OCT3 as the only low-affinity transporters expressed in human astrocytes (Yoshikawa *et al.*, 2013). Still, despite the rich evidence that links PMAT with monoamine clearance, missing *in vivo* studies continue to represent an obvious gap in research in this field, as is also the case with hOCT1 and 2 (Maier, Niello, *et al.*, 2021). However, the growing (*in vivo*) research interest in PMAT is exemplified by a recently published study of mice, which looked into the role of OCTs and PMAT in sex-dependent variations in the rewarding and locomotor stimulant effects of amphetamine, and found that OCT3 and PMAT are necessary for conditioned place preference (CPP) in females and males, respectively (Clauss, Koek and Daws, 2021). Notably, clinical relevance of PMAT was proposed in neurodegenerative and psychiatric disorders that are connected to dysregulated monoaminergic neurotransmission in brain, as e.g. in the case of Alzheimer's disease (AD)

(Sekhar *et al.*, 2019) or autism (Adamsen *et al.*, 2014). A concise review on PMAT tissue distribution and subcellular localization is present elsewhere (Wang, 2016).

To date, a vast number of CNS active compounds have been shown to interact with the human isoforms of OCT1-3 and PMAT (Giacomini, Huang and Tweedie, 2010; Koepsell, 2021; Maier, Rauter, *et al.*, 2021). Considering the commonality between SLC6 neurotransmitter-sodium symporters and OCTs and PMAT for their role in maintaining monoaminergic balance in CNS, it is thus not surprising that research is increasingly putting focus on exploring interactions between psychoactive compounds, including medications and illicit drugs, and the low-affinity/high-capacity OC transporters. Striking features of so far described hOCT and hPMAT substrates are cationic and/or neutral charge, greater sp³ character and hydrophobicity (Sala-Rabanal *et al.*, 2013; Wang, 2016). Since many known psychoactive substances comply with these characteristics, and are further structurally related to norepinephrine, dopamine, and 5-HT, OCT1-3 and PMAT (and their genetic variants) are important determinants for their pharmacokinetic fate (Jensen *et al.*, 2021). As mentioned, the majority of studies assessing the interaction between various compounds and OCTs and PMAT focused on in vitro analyses, primarily trying to recognize substances as inhibitors or substrates thereof. So far, regarding their interaction with human OCT1-3 and PMAT, studies have put emphasis on evaluating prominent psychoactive compounds like e.g. d-amphetamine, methamphetamine, MDMA and cocaine (Wu *et al.*, 1998; Amphoux *et al.*, 2006; Zhu *et al.*, 2010; Wagner *et al.*, 2017), five different α -pyrrolidinopropiophenone (α -PPP) derivatives (Maier, Rauter, *et al.*, 2021), five α -aminovalerophenone (α -PVP) derivatives (Duart-Castells *et al.*, 2021; Maier, Rauter, *et al.*, 2021) and ketamine (Amphoux *et al.*, 2006; Massmann *et al.*, 2014; Keiser, Hasan and Oswald, 2018) (for a concise comparison and summary of pharmacodynamic properties of psychoactive substances characterized by their interactions with aforementioned transporters see Maier *et al.* (2021)). In this regard, pharmaceutical drugs like e.g. bupropion (Haenisch and Bönisch, 2010; Haenisch *et al.*, 2012; Sandoval *et al.*, 2018), phenobarbital (Ahlin *et al.*, 2008) and tramadol and its metabolite *O*-Desmethyltramadol (Ahlin *et al.*, 2008; Tzvetkov *et al.*, 2011) have also been investigated. In particular, the importance of OCTs and PMAT is emphasized in the acute action of antidepressant and antipsychotic drugs, as these transporters are required for the drugs to exert their full clinical effect (Haenisch and Bönisch, 2010; Bacq *et al.*, 2012; Mayer *et al.*, 2018). Since monoamine clearance from extracellular fluids by low-affinity transporters can buffer the desired results of prominent drug classes like SSRIs (selective serotonin reuptake inhibitors), OCTs and PMAT depict promising targets in the design of improved or novel therapeutics against e.g. depression (Daws, C., 2013).

Notably, the high degree of amino acid sequence homology between OCT1 and 2 accounts for the similarity of ligands, whereas the less homologous OCT3 has a smaller pharmacodynamic overlap (Sala-Rabanal *et al.*, 2013). Nevertheless, OCT3 is a significant player in mediating psychostimulant abuse, as seen for the action of amphetamine in mice (Mayer *et al.*, 2018), and was also shown to play a role in the stress-addiction axis and anxiety in animal models and, albeit offering yet just another small step for understanding mentioned occurrences, suggesting similar mechanisms in humans (Wultsch *et al.*, 2009; Baganz *et al.*, 2010; Graf *et al.*, 2013). However, just as a high degree of amino acid sequence homology between related transporter proteins can explain their overlap in substrate and inhibitor specificities, so it has to be taken with a grain of salt when it comes to its translatability between different species, as seen for the murine, rat and human isoforms of OCT1-3 and PMAT (Maier, Niello, *et al.*, 2021). Here, the degrees of homology, albeit seeming fairly high, should perhaps not be viewed without scepticism, since they might depict the main reason for the often-observed differences in compound affinities between species. Figure 1 depicts the degrees of amino acid sequence homologies of OCT1-3 and PMAT between the three species.

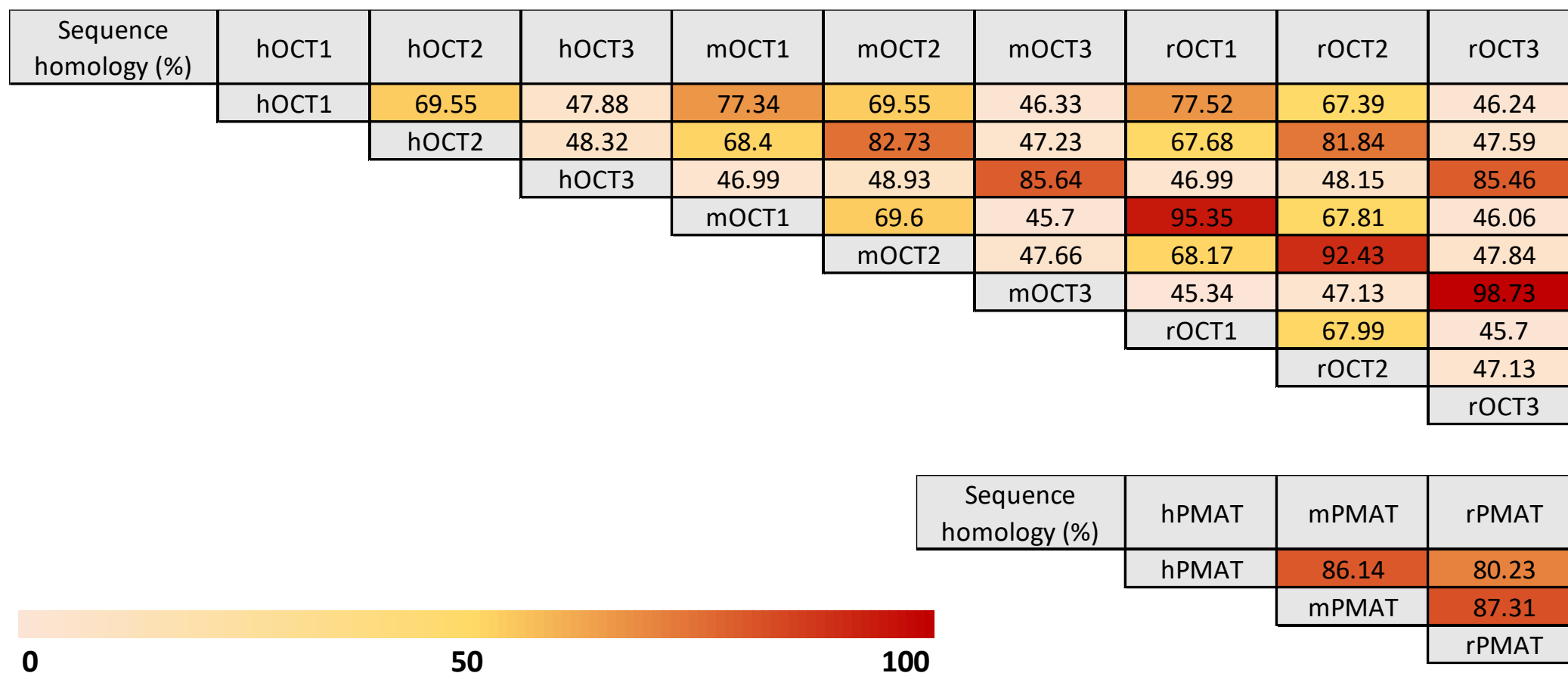


Figure 1: Heat maps portraying the degrees of amino acid sequence homology (given as %, adapted from Maier *et al.* (2021)) between the human, mouse, and rat isoforms of OCT1-3 and PMAT.

The significance of investigating drug interactions with OCTs and PMAT led to the onset of this study, aiming to establish interaction profiles between them and a total of 17 substances, most of which are known for their interplay with high-affinity transporters. The compounds stem from six different chemical and medical classes (antidepressants, antiepileptics, analgesics, new therapeutics, psychostimulants, and drugs for treatment of narcolepsy and ADHD) and were investigated regarding their potential for inhibiting substrate uptake into cells stably overexpressing human OCT1-3 and PMAT. For the herein conducted radiotracer-based uptake inhibition assays, 1,1'-diethyl-2,2'-cyanine (decynium-22; D22) was used as a control substance to assess non-specific uptake at all OCTs and PMAT. In 1992, D22 was introduced as a potent, competitive inhibitor of the human corticosterone-sensitive extraneuronal monoamine “uptake-2” transport system, which is today known as OCT3 (Russ, Sonna, *et al.*, 1992). D22 was introduced in 1993 as the first known inhibitor of renal cation uptake in LLC-PK1 cells (Schömig, Babin-Ebell and Russ, 1993) and rOCT1 inhibition in nanomolar concentrations was reported in 1994 (Gründemann *et al.*, 1994). Concurrent with their first description in 1997, D22 was confirmed as a potent uptake inhibitor at hOCT1 and 2 (Gorboulev *et al.*, 1997; Zhang, 1997), and was shown to have the highest inhibitory potency of all inhibitors tested at hPMAT, with a K_i value of 0.10 μM (Engel, Zhou and Wang, 2004). Tritiated 1-methyl-4-phenylpyridinium ($[^3\text{H}]\text{MPP}^+$) served as the radioactively labelled substrate for all aforementioned transporters, whose cellular uptake was measured and quantified via beta-scintillation counting. MPP^+ is a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic compound that is oxidized to its reactive form and known for its link to Parkinson’s Disease (Salach *et al.*, 1984). MPP^+ neurotoxicity was described in 1984, and the compound was since confirmed as a substrate of human MATs, OCTs and PMAT (Salach *et al.*, 1984; Russ, Gliese, *et al.*, 1992; Wall, Gu and Rudnick, 1995; Gorboulev *et al.*, 1997; Johnson *et al.*, 1998; Engel, Zhou and Wang, 2004). In order to set up interaction profiles of the respective compounds on hOCTs and hPMAT, IC_{50} (half maximal inhibitory concentration) values were calculated, providing key information about the inhibitory capacities of the tested substances. Since interaction profiles between psychoactive compounds and SLC6 transporters are abundantly available, we herein set sight on analogously establishing the same for low-affinity/high-capacity transporters, which are, in this regard, largely understudied. In this work, we reveal substrate uptake inhibition potencies of bupropion, S-citalopram, R-citalopram, diazepam, phenobarbital, cocaine, *d*-amphetamine, R- α -pyrrolidinovalerophenone (R- α -PVP), S- α -pyrrolidinovalerophenone (S- α -PVP), 3,4-

methylenedioxymethamphetamine (MDMA), psilocin, ketamine, and its metabolically active derivative 2-fluoro-deschloroketamine, methylphenidate, modafinil, tramadol and its metabolite *O*-desmethyl-tramadol. Table 1 underlines these substances' clinical relevance and classifies these compounds by their chemical/medical class and shows their most prevalent, known pharmacodynamic mechanism in human CNS. Since only a few of those substances have been investigated in regard of their interaction at human OCTs and PMAT, the herein presented data fills a veritable research gap and offers relevant information on these transporters' abounding pharmacology. Furthermore, some well-known, clinically relevant drugs are suggested as potential scaffolds for the development of new or adapted drugs, bringing prospects especially for patients with OCT or PMAT polymorphisms, differently impacting substrate translocation and thus pharmacodynamic fate of relevant compounds.

Category	Compound	Main pharmacodynamic mechanism	Approved or proposed uses	Source
Antidepressants	Bupropion	Inhibitor of hDAT and hNET; antagonist of nAChR	Depression, seasonal affective disorder, smoking cessation aid	Foley <i>et al.</i> (2006)
	R-citalopram	Antagonistic effect on S-citalopram at hSERT	—————	Schilström <i>et al.</i> (2011)
	S-citalopram (escitalopram)	SSRI	MDD, social and generalized anxiety disorder	Larsen <i>et al.</i> (2016), Kasper <i>et al.</i> (2005), Davidson <i>et al.</i> (2004)
Antiepileptics	Diazepam	Allosteric GABA _A receptor modulator	Anxiety, convulsive disorders, epilepsy, sedation	Calcaterra <i>et al.</i> (2014)
	Phenobarbital	Allosteric GABA _A receptor modulator	Epilepsy	Yasiry <i>et al.</i> (2012)
Psychostimulants	Cocaine	Inhibitor of hDAT, hNET and hSERT	—————	Kristensen <i>et al.</i> (2011)
	<i>d</i> -amphetamine	Releasing agent at hDAT and hNET	ADHD, narcolepsy	Sulzer <i>et al.</i> (2005), Luthy <i>et al.</i> (2015)
	R- α -PVP	Racemic compound α -PVP: inhibitor of hDAT and hNET	—————	Nóbrega <i>et al.</i> (2018)
	S- α -PVP		—————	
New therapeutics	Ketamine	NMDA receptor antagonist	Analgesic, anaesthetic	Morgan <i>et al.</i> (2012)
	2-fluoro-deschlorketamine	NMDA receptor antagonist	Anaesthetic	Moghimi <i>et al.</i> (2014)
	MDMA	Releasing agent at hDAT, hNET and hSERT	PTSD	Oeri (2020)
	psilocin	(Partial) 5-HT _{2A} agonist	—————	Kargbo (2021a, b)
Analgesics	Tramadol	μ -opioid receptor agonist	Acute and chronic pain	Kano <i>et al.</i> (2006), Smyj <i>et al.</i> (2013)
	O-desmethyltramadol	μ -opioid receptor agonist	Acute and chronic pain	Kano <i>et al.</i> (2006)
Medicals against Narcolepsy and ADHD	Methylphenidate	Inhibitor of hDAT and hNET	ADHD, narcolepsy	Challman <i>et al.</i> (2000)
	Modafinil	Inhibitor of hDAT	Narcolepsy	Minzenberg <i>et al.</i> (2008)

Table 1: Comparison table of the pharmacodynamic properties and uses of 17 herein investigated psychoactive substances.



Article

Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter

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Abstract: Many psychoactive compounds have been shown to primarily interact with high-affinity and low-capacity solute carrier 6 (SLC6) monoamine transporters for norepinephrine (NET; norepinephrine transporter), dopamine (DAT; dopamine transporter) and serotonin (SERT; serotonin transporter). Previous studies indicate an overlap between the inhibitory capacities of substances at SLC6 and SLC22 human organic cation transporters (SLC22A1–3; hOCT1–3) and the human plasma membrane monoamine transporter (SLC29A4; hPMAT), which can be classified as high-capacity, low-affinity monoamine transporters. However, interactions between central nervous system active substances, the OCTs, and the functionally-related PMAT have largely been understudied. Herein, we report data from 17 psychoactive substances interacting with the SLC6 monoamine transporters, concerning their potential to interact with the human OCT isoforms and hPMAT by utilizing radiotracer-based in vitro uptake inhibition assays at stably expressing human embryonic kidney 293 cells (HEK293) cells. Many compounds inhibit substrate uptake by hOCT1 and hOCT2 in the low micromolar range, whereas only a few substances interact with hOCT3 and hPMAT. Interestingly, methylphenidate and ketamine selectively interact with hOCT1 or hOCT2, respectively. Additionally, 3,4-methylenedioxymethamphetamine (MDMA) is a potent inhibitor of hOCT1 and 2 and hPMAT. Enantiospecific differences of R- and S- α -pyrrolidinovalerophenone (R- and S- α -PVP) and R- and S-citalopram and the effects of aromatic substituents are explored. Our results highlight the significance of investigating drug interactions with hOCTs and hPMAT, due to their role in regulating monoamine concentrations and xenobiotic clearance.

Keywords: ketamine; psilocybin; bupropion; escitalopram; diazepam; tramadol; O-desmethyltramadol; cocaine; d-amphetamine; modafinil

1. Introduction

Organic cation transporter subtypes 1–3 (OCT1–3; SLC22A1–3, respectively; OCTs) and the plasma membrane monoamine transporter (PMAT; SLC29A4) are poly-specific facilitative transporters which are involved in the uptake and elimination of various endogenous compounds, most notably monoamines, as well as of drugs, xenobiotics, and toxins [1]. In addition to the high-affinity, low-capacity solute carrier 6 (SLC6) neurotransmitter-sodium symporters of norepinephrine (norepinephrine transporter; NET; SLC6A2), dopamine (dopamine transporter; DAT; SLC6A3) and serotonin (serotonin transporter; SERT; SLC6A4), the low-affinity, high-capacity OCTs as well as PMAT are of paramount importance for the maintenance of monoaminergic equilibrium in the brain [2,3]. In addition, OCTs contribute

to protection against noxious compounds by intestinal absorption as well as hepatic and renal excretion [4–6]. Indeed, hOCT1 and hOCT2 transport numerous compounds in the small intestine, liver and kidneys, with OCT1 being primarily expressed in the liver and OCT2 predominantly acting in the kidney, while both are, together with hOCT3 and hPMAT, also found in the brain, albeit at a lower level [7]. By contrast, hOCT3 and hPMAT were found to be highly expressed in the brain, alongside their less ubiquitous occurrence in peripheral organs, such as the heart for hOCT3 [8]. Here, along with hOCT1 and 2, they participate in the regulation of the monoaminergic equilibrium, and their dysfunction has been associated with disturbance of monoaminergic pathways, leading to various psychiatric disorders and neurodegenerative diseases [9–13].

Due to their involvement in the excretion of drugs and metabolites, members of the OCT family have overlapping substrate and inhibitor profiles [5,14]. Substrates are classically transported into the cells by the transporter, while inhibitors bind to the transporter in the outward-facing conformation and prevent uptake [15]. Identification of compounds interacting with OCTs and PMAT furthers knowledge of targets and effects in the central nervous system (CNS) and, additionally, allows for the explanation of substances' observed pharmacokinetic properties. Given the plethora of CNS active substances that are well-known for their interplay with SLC6 neurotransmitter-sodium symporters (summarized as monoamine transporters; MATs), there is also a growing amount of interest in their interaction with the human OCTs as well as PMAT, since they are less well-researched potential targets in the monoaminergic system [16–21]. We have recently reported that several psychoactive substances, classified primarily as inhibitors or substrates of the MATs, equipotently interact with certain OCTs [2,22].

To further extend these observations, we screened 17 substances with CNS activity that were categorized as follows: antidepressants (bupropion, S-citalopram, R-citalopram), antiepileptics (diazepam, phenobarbital), psychostimulants (cocaine, *d*-amphetamine, R- and S- α -pyrrolidinovalerophenone (R- and S- α -PVP)), emerging therapeutics (3,4-methylenedioxymethamphetamine (MDMA), psilocin, ketamine), the ketamine derivative 2-fluoro-deschloroketamine, drugs for attention deficit hyperactivity disorder (ADHD) and narcolepsy (methylphenidate, modafinil) and analgesics (tramadol and its metabolite O-desmethyl-tramadol). 2-fluoro-deschloroketamine and O-desmethyl-tramadol can be classified as new psychoactive substances that are not yet controlled by legislation [23]. Considering the myriad of already established interaction profiles on SLC6 transporters, we tried to start an analogous process for the low-affinity, high-capacity monoamine transporters, as doing so will help to further elucidate the pharmacological interaction of compounds with OCTs and PMAT. This approach will offer more information on these transporters' rich pharmacology and potentially lead to clinical implications for patients with OCT polymorphisms impacting substrate translocation [24]. In addition, the discovery of previously unreported interactions of well-known psychoactive substances with OCTs and PMAT might inspire the usage of compounds as potential scaffolds to develop new clinically useful drugs.

2. Results

Uptake inhibition assays were performed on human embryonic kidney 293 (HEK293) cells stably expressing hOCT1, hOCT2, hOCT3 or hPMAT to assess the compounds' potency to inhibit the uptake of 1-methyl-4-phenylpyridinium ($[^3\text{H}]\text{-MPP}^+$). We found several substances to be inhibitors of substrate uptake by hOCT1, and fewer interacted with hOCT2 and hPMAT, whereas interactions with hOCT3 were exceedingly rare (see Figure 1). In summary, only 1 compound (~6%) inhibited hOCT3, 5 compounds (~29%) inhibited hPMAT, 7 compounds (~41%) inhibited hOCT2, and 11 compounds (~65%) inhibited hOCT1 with an half-maximal inhibitory concentration (IC_{50}) value lower than 100 μM (see Figure 1B).

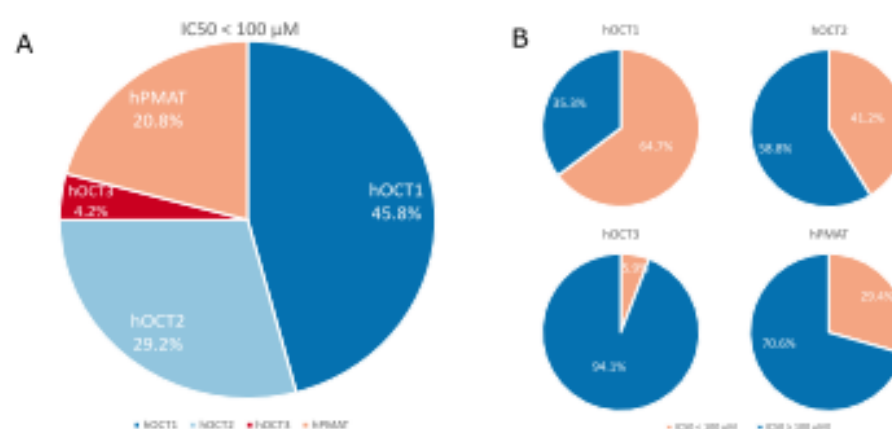


Figure 1. Graphical representation of the percentage of screened compounds inhibiting the substrate uptake of the transporters of interest with an half-maximal inhibitory concentration (IC_{50}) below 100 μ M. (A): 45.8% of compounds inhibit hOCT1 (dark blue), 29.2% inhibit hOCT2 (light blue), 20.8% hPMAT (orange) and 4.2% inhibit hOCT3 (red). (B): 11 out of 17 compounds inhibit hOCT1, 7 compounds inhibit hOCT2, 1 compound inhibit hOCT3 and 5 compounds inhibit hPMAT.

The screened antidepressants had close to identical interaction profiles on all transporters (see Figure 2) with only hOCT1 uptake being inhibited at pharmacologically relevant concentrations, with IC_{50} values between 5.11 μ M (95%-confidence interval (CI): 4.11–6.37) to 7.15 μ M (95%-CI: 5.59–9.15) (Table 1). Interestingly, R-citalopram, S-citalopram and bupropione did not interact as potently with hOCT2 and hOCT3 (see Figure 2C,D). The examined antidepressants inhibited hPMAT with low potency at high micromolar concentrations (Figure 2E).

Table 1. Heat map portraying the inhibitory potency (in μ M) of screened compounds at hOCT1–3 and hPMAT. Lower values are highlighted in red and higher in shades of blue (see legend).

Substance	Transporters			
	hOCT1	hOCT2	hOCT3	hPMAT
Bupropion	5.36			96.96
S-Citalopram	7.15			
R-Citalopram	5.11			58.32
Diazepam			44.46	29.81
Phenobarbital				
d-Amphetamine	8.39	2.21		71.77
Cocaine	6.66	27.80		
R- α -PVP	2.15	13.09		
S- α -PVP	1.07	15.02		
Ketamine		12.46		
2-Fluoro-deschloroketamin		19.18		
MDMA	1.14	2.71		7.77
Psilocin				
Tramadol	5.60			
O-Desmethylnaloxone	24.16			
Methylphenidate	0.36			
Modafinil				
Decynium-22	2.66	4.56	0.16	0.35



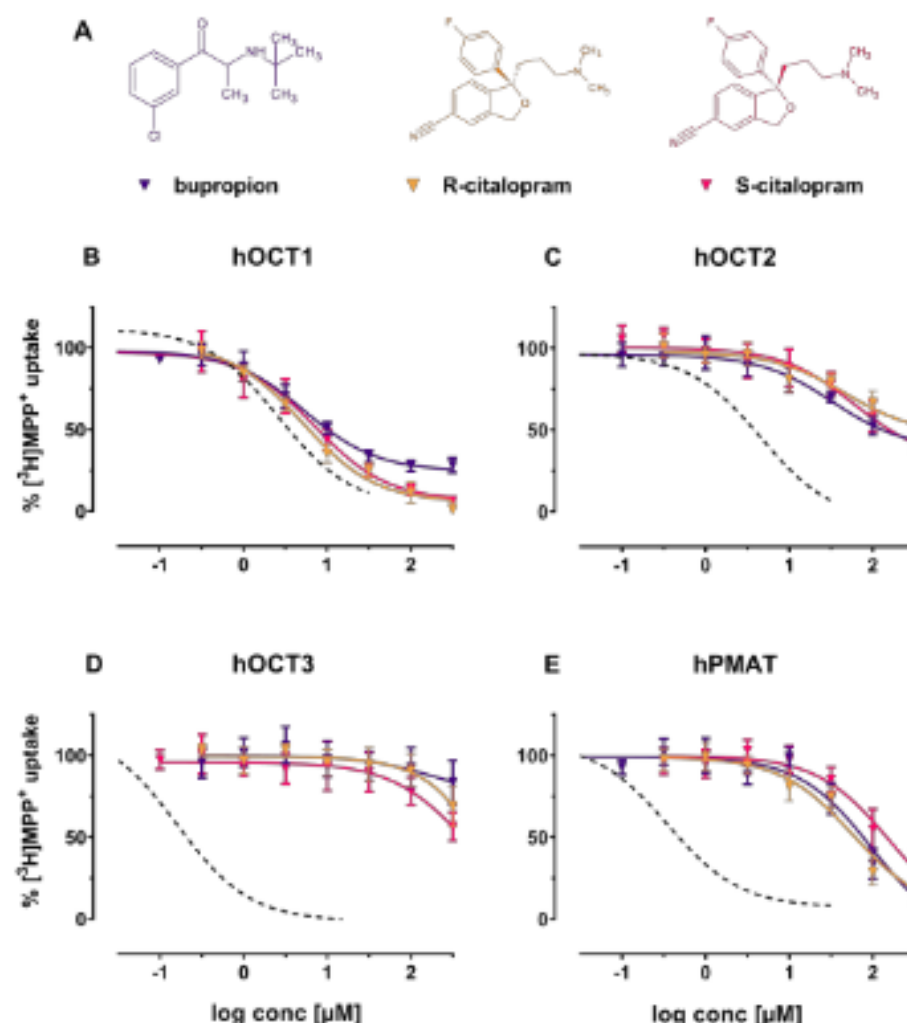


Figure 2. (A) Chemical structures of the herein investigated antidepressant compounds. From left to right: bupropion (violet), R-citalopram (yellow) and S-citalopram (red); Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP⁺ in HEK293 cells stably expressing the respective transporter.

Diazepam fully inhibited substrate uptake of all transporters (see Figure 3B–E), reaching half-maximal inhibition at 44.46 μM (95%–CI: 36.04–54.85) and 29.81 μM (95%–CI: 18.41–48.27) at hOCT3 and hPMAT, respectively, while weakly interacting with hOCT1 and hOCT2 ($IC_{50} > 100$ μM). Phenobarbital treatment induced increased uptake of substrate at increasing concentrations at hOCT1 and hOCT2, while not interacting with the other transporters at pharmacologically relevant concentrations.

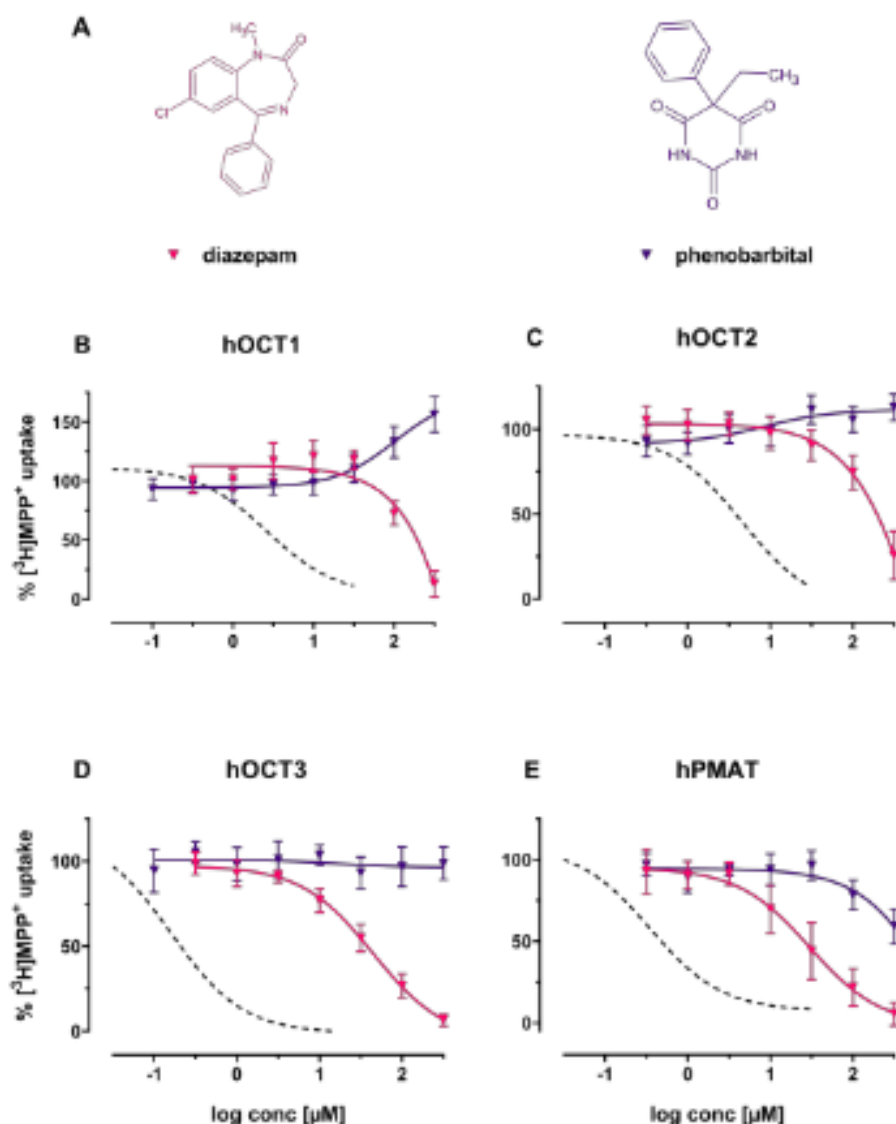


Figure 3. (A) Chemical structures of the herein investigated antiepileptics diazepam (red) and phenobarbital (violet); (B–E): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP⁺ in HEK293 cells stably expressing the respective transporter.

The psychostimulants *d*-amphetamine, cocaine, R- and S- α -PVP showed similar inhibition profiles at hOCT1 and hOCT2 (Figure 4B,C) with IC_{50} s ranging from 1.07 (95%-CI: 0.81–1.40) to 15.02 μ M (95%-CI: 11.28–20.00), with the exception of an IC_{50} of 27.80 μ M (95%-CI: 19.17–40.32) of cocaine at hOCT2 (see Figure 4C). Furthermore, no pharmacologically relevant interactions with hOCT3 and hPMAT could be detected (Figure 4D,E). R- and S- α -PVP treatment lead to increased uptake of substrate at hPMAT (Figure 4E).

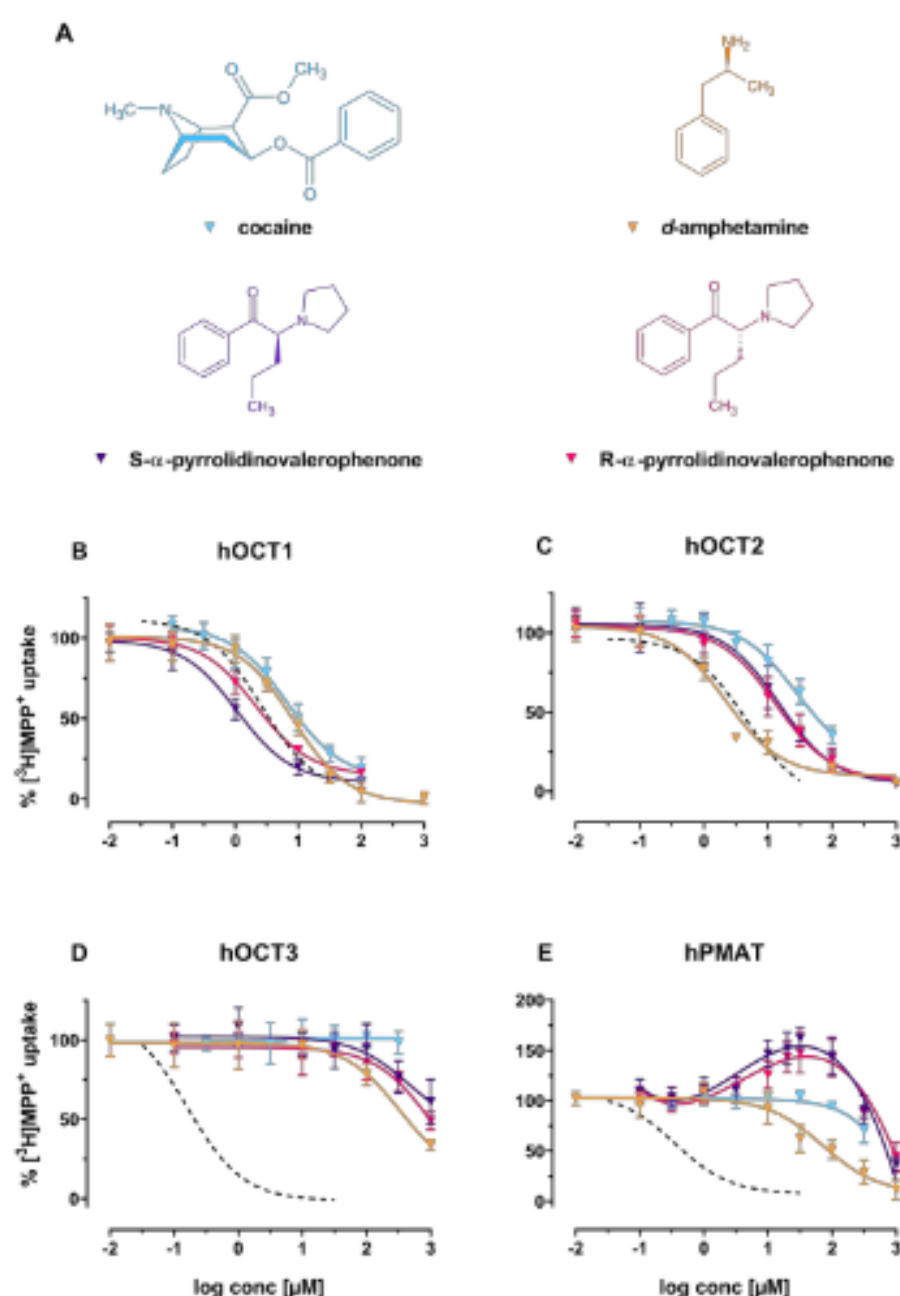


Figure 4. (A) Chemical structures of the herein investigated psychostimulants. From left to right: cocaine (blue), d-amphetamine (yellow), S-α-pyrrolidinovalerophenone (violet) and R-α-pyrrolidinovalerophenone (red); (B–E): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP⁺ in HEK293 cells stably expressing the respective transporter.

While ketamine selectively and potently inhibited substrate uptake of hOCT2, its derivative 2-fluoro-des-chloroketamine is less potent, but similarly selective (IC_{50} of 12.46 (95%-CI: 9.71–15.98) and 19.18 μM (95%-CI: 14.89–24.70), respectively). Both compounds did not fully inhibit the other transporters at pharmacologically relevant concentrations. Psilocin, the active metabolite of psilocybin, did not inhibit any of the investigated transporters, but rather caused an increase in substrate uptake in the low micromolar range at the OCTs (see Figure 5B–D). 3,4-Methylenedioxymethamphetamine (MDMA) has high

substrate uptake inhibiting potencies at hOCT1, 2 (IC_{50} of 1.14 (95%-CI: 0.90–1.44) and 2.71 μ M (95%-CI: 2.22–3.31) respectively) and hPMAT (IC_{50} of 7.77 μ M; 95%-CI: 5.92–10.21), while not inhibiting uptake by hOCT3.

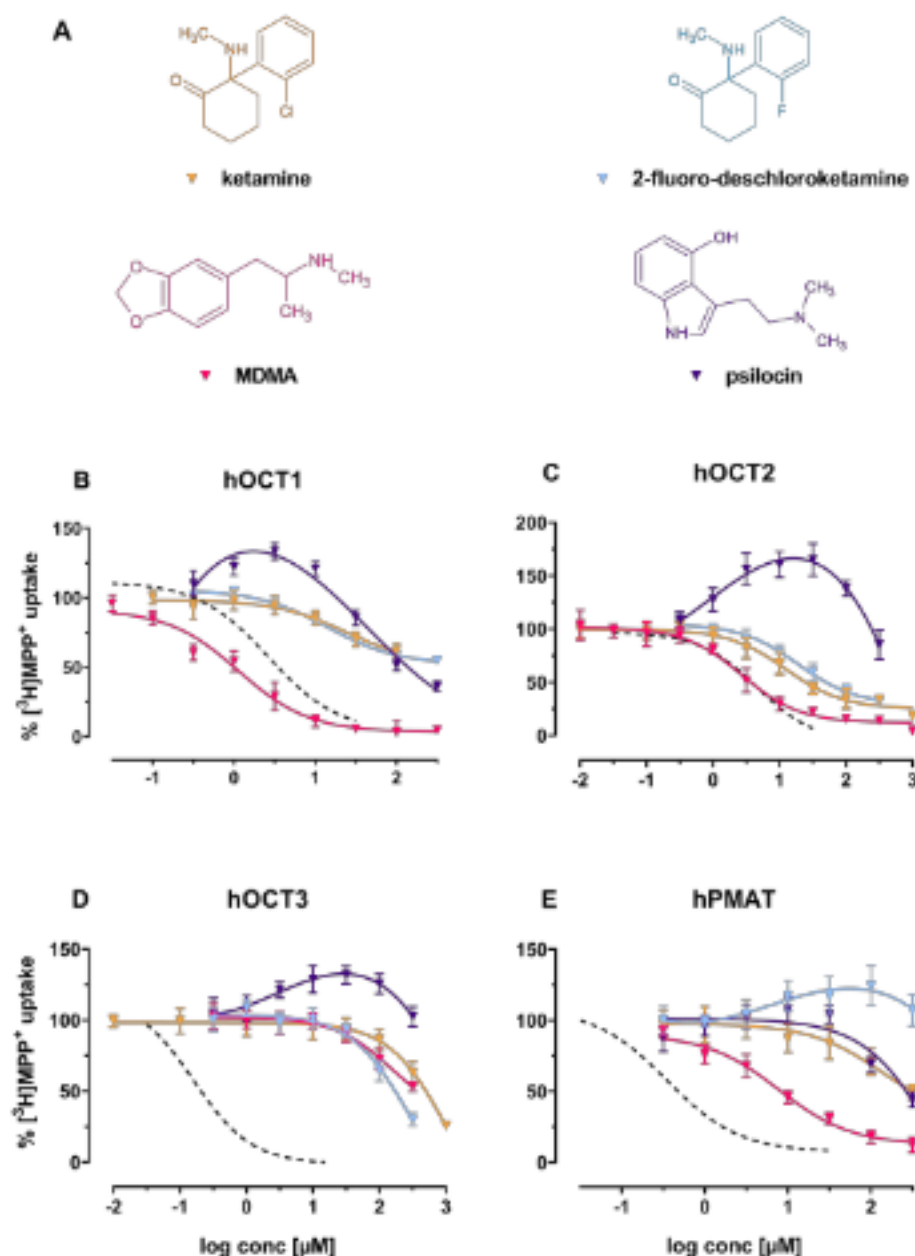


Figure 5. (A) Chemical structures of the herein investigated new therapeutics. From left to right: ketamine (yellow), 2-fluoro-deschloroketamine (blue; non-therapeutic derivative of ketamine), MDMA (red) and psilocin (violet); (B–E): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP⁺ in HEK293 cells stably expressing the respective transporter.

Tramadol and its active metabolite *O*-desmethyltramadol both inhibit hOCT1 substrate uptake at low micromolar concentrations (IC_{50} of 5.60 (95%-CI: 4.65–6.75) and 24.16 μ M (95%-CI: 19.12–30.54) respectively) (see Figure 6B). Tramadol also interacts with hOCT2 and hPMAT, but fails to fully inhibit the transporters (Figure 6C,E). No interaction of both tramadol and *O*-desmethyltramadol with hOCT3 was observed (Figure 6D).

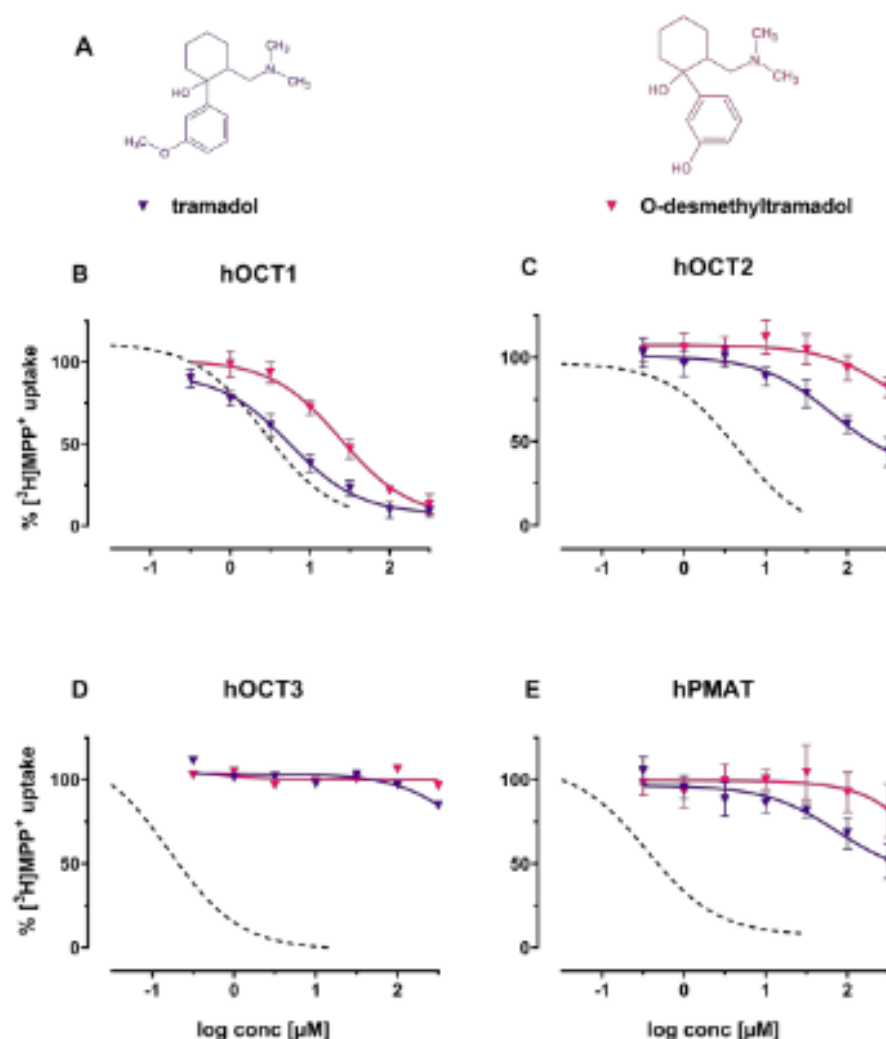


Figure 6. (A) Chemical structures of the herein investigated analgesics tramadol (violet) and *O*-desmethyltramadol (red); (B–E): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP⁺ in HEK293 cells stably expressing the respective transporter.

As seen in Figure 7B–E, modafinil did not interact with hOCTs and hPMAT at pharmacologically relevant concentrations. In contrast, methylphenidate potently interacts with hOCT1 (Figure 7B) (IC_{50} of 0.36 μ M; 95%-CI: 0.27–0.46), while not fully blocking any other investigated transporter (see Figure 7C–E).

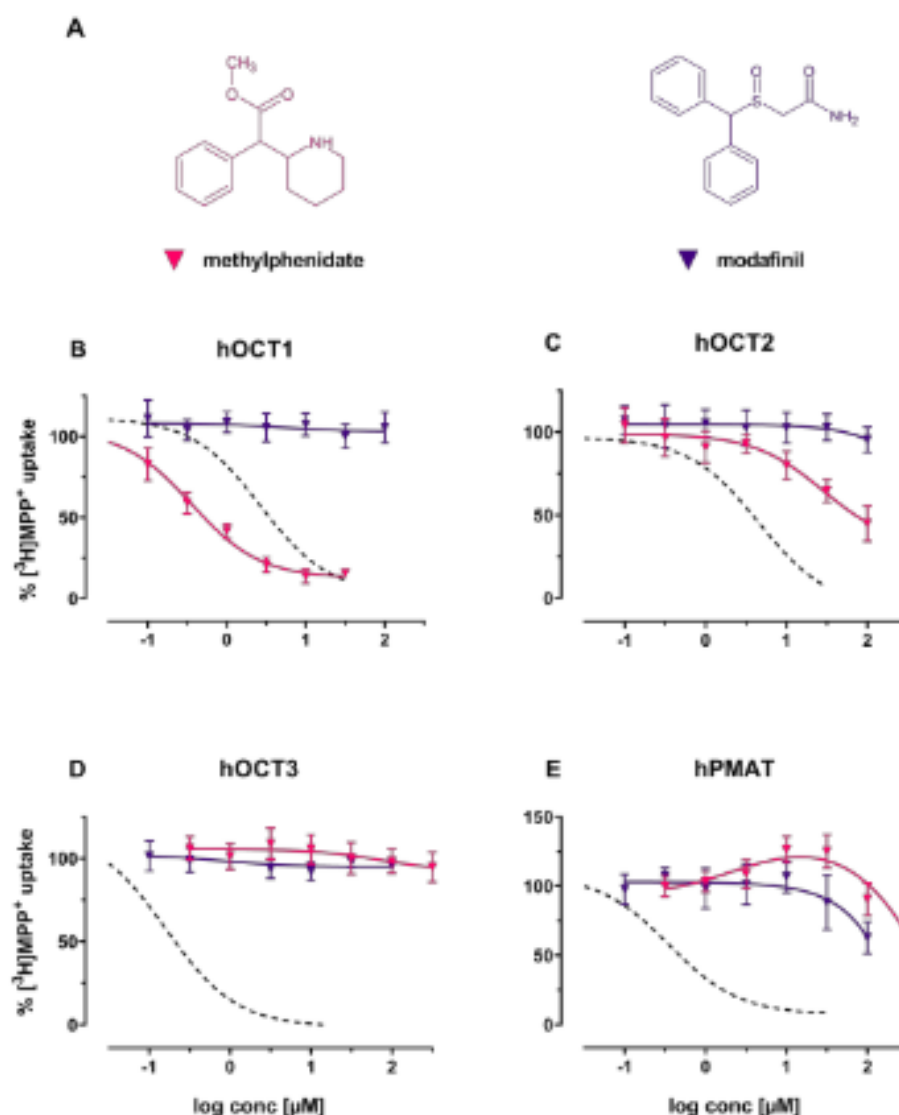


Figure 7. (A) Chemical structures of the herein investigated drugs to treat narcolepsy modafinil (violet) and ADHD methylphenidate (red); (B): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (B) hOCT1, (C) hOCT2, (D) hOCT3 and (E) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

3. Discussion

To date, many substances have been investigated concerning their interaction with the high-affinity, low-capacity SLC6 MATs due to their long history of use as clinically relevant drug targets, in addition to their well-established role in regulating the clearance of monoamines from the synaptic cleft and maintaining monoaminergic equilibrium in the CNS. However, a growing number of studies shows that the low-affinity, high-capacity organic cation transporters (OCT1–3), as well as the plasma membrane monoamine transporter (PMAT), are also distinctly involved in monoamine reuptake from the synaptic

cleft [11,25]. Furthermore, they transport and eliminate xenobiotics in the periphery, lending them pharmacological and clinical relevance [11,26–31].

Herein, we intended to systematically determine the interaction profiles of 17 psychoactive substances, including medical drugs, which have mostly been shown to interact with MATs, on hOCT1–3 and hPMAT [32–36]. The presented data thus fill a veritable research gap, since only a few of the compounds have previously been investigated concerning their interaction at the OCTs and PMAT (see Supplementary Table S1).

The first striking finding is that the various chemical classes of psychoactive compounds investigated herein predominantly interact with hOCT1, and less pronouncedly with hOCT2 and hPMAT. Previous studies have shown many medically relevant compounds, such as metformin, to interact with hOCT1, in turn influencing their pharmacokinetic fate [37]. Furthermore, polymorphisms of hOCT1 have been shown to influence substrate translocation and drug–drug interactions [38]. Overall, OCT1 polymorphisms seem to have potential clinical consequences, but further research needs to be undertaken [31]. In addition, FDA (Food and Drug Administration) and EMA (European Medicines Agency) recommend investigations of potential interactions with hOCT2 for drugs which are primarily renally excreted [39,40]. Thus, for compounds potentially interacting with hOCTs and hPMAT, the occurrence of polymorphisms in patients, differently affecting pharmacodynamic and -kinetic fates and potentially the occurrence and severity of side-effects, must be considered in future investigations and substantiated clinically.

Currently, only few selective hOCT or hPMAT inhibitors are known, which can be explained by the high degree of sequence homology between transporters [25]. In particular, hOCT1 and 2 share approximately 70% amino acid sequence identity [2,14]. It is therefore not surprising to see an overlap of compounds interacting with hOCT1 and 2. Still, we located distinct differences between these two transporters regarding some of the compounds tested. Bupropion selectively interacts with hOCT1. This contradicts results reported in previous publications [16]. Diverging data between our results and those gathered from earlier studies might be explained by differences in experimental and laboratory conditions, as well as the established cell lines across studies [41]. In addition, tramadol, its metabolite *O*-desmethyltramadol, and both citalopram enantiomers were selective hOCT1 inhibitors. Strikingly, we found methylphenidate to potently and selectively interact with hOCT1, even more potent than the unspecific OCT and PMAT inhibitor decynium-22 [1]. Furthermore, ketamine potently interacts with hOCT2, while showing little activity at other hOCTs and hPMAT. Amphoux et al. (2006) reported similar results for the OCTs but did not investigate hPMAT [26]. This highlights the interesting finding that some compounds explicitly differ in their interactions with hOCT1 and hOCT2, despite the high degree of sequence homology between the two transporters. One possible explanation for this discrepancy could be differences between substrate binding sites. Amino acid residues that are critical for substrate specificity, which are localized in regions formed by transmembrane helices crucial for substrate binding, were found to differ between OCT1 and OCT2, and might explain variations in compound interactions with those transporters [42,43]. Still, the exact molecular basis for preference of compounds for hOCT1 or 2 has not been clearly established yet [2,14,44]. While we could not solve this gap in knowledge, our investigation led to the discovery of scaffolds that can be used for the development of selective inhibitors of OCTs, expanding pharmacological and structural understanding of this transporter family.

hOCT3 shares less sequence identity (50%) with the other OCTs and it has been previously shown that many unselective inhibitors and substrates of the organic cation transporters are least efficacious at hOCT3 [14]. In our study, diazepam not only proved to be a clearly more potent inhibitor of substrate uptake at hOCT3 when compared to hOCT1 and 2, but additionally, was the only herein tested substance to exhibit any pharmacologically relevant effect on substrate uptake by hOCT3. A previous study reported an IC_{50} of 2 μ M for diazepam at hOCT3, which is much lower than the one we have measured (IC_{50} of 44.46 μ M) [18]. The discrepancy may be explained by the different tracer used,

with Massmann and colleagues having employed fluorescent 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺), while we performed uptake inhibition assays with radioactively labelled MPP⁺ [41]. In addition, diazepam relatively potently interacted with hPMAT, which was previously unreported.

In an investigation of α -pyrrolidinopropiophenone derivatives, we have previously observed that differences in aromatic ring substituents affected the hOCTs and hPMAT differently [22]. Differences in interaction with hOCT1 and 2 were marginal compared to their impact on hOCT3 and hPMAT. Here, we examined ketamine and its derivative 2-fluoro-deschloroketamine, again noticing only small differences caused by different aromatic ring substituents, although the potency to inhibit hOCT2 substrate uptake decreased for the derivative. This lack of sensitivity towards substituents of the aromatic ring differentiates hOCT1 and 2 from SLC6 MATs where, for example in the case of methcathinone analogs, fluorination of the aromatic ring improved SERT selectivity over DAT [35,45,46]. On the other hand, structural differences between tramadol and its main metabolite *O*-desmethyltramadol led to relevant changes in hOCT1 affinity, as tramadol inhibited substrate uptake of the transporter with a more than four-fold higher potency (IC_{50} of 5.60 μ M) than *O*-desmethyltramadol (IC_{50} of 24.16 μ M). Future studies need to focus on identified, potent chemical scaffolds and systematically investigate effects of substituents in larger-scale structure–activity relationship investigations.

To our knowledge, no previous studies have analyzed the effects of these compounds at human OCTs or hPMAT. Thus, we examined pharmacodynamic properties of S-citalopram and R-citalopram, as well as S- and R- α -PVP. Generally, we saw no distinct enantioselective differences in uptake inhibition between the two compounds on any transporter, emphasizing the robustness of OCTs and PMAT concerning drug–transporter interactions, which discerns them from MATs [23,47,48].

We observed the surprising phenomenon that treatment with higher concentrations of some compounds, most markedly phenobarbital, psilocin and α -PVP, led to elevated MPP⁺ uptake at hOCT1, 2 and hPMAT. Consistent with this finding is a previous study of Ahlin and colleagues, who reported increased uptake after cell treatment with high concentrations of phenobarbital [49]. One possible explanation for this striking phenomenon might include allosteric effects on the orthosteric site of the transporter. Consequently, an allosteric ligand would change the conformational dynamics of the transporter protein and thereby regulate interaction between substrates and ligand binding site [50]. In line with this phenomenon, an earlier study detailed the existence of a high-affinity binding site at OCTs, proposing it to partake in the effectivity of MPP⁺ uptake through positively affecting the transport executed by two low-affinity transporting sites [51]. However, further studies are needed to unveil the exact molecular mechanisms.

Traditionally, decynium-22 was oftentimes used as a positive control due to its high inhibitory potency at all hOCTs and hPMAT. In the present investigation, we show multiple compounds to have similar or even lower IC_{50} values. *d*-Amphetamine proved to be a highly potent hOCT1 and 2 substrate uptake inhibitor with IC_{50} values in the low micromolar range, while not interacting with hOCT3 at concentrations lower than 100 μ M, which is consistent with a previous study by Amphoux et al. (2006) [26]. Furthermore, in our experiments, MDMA was potently interacting with hOCT1 and 2, an aspect which is to some extent consistent with a previous study showing fairly low K_i values [26]. In addition, the compound potently interacted with hPMAT, an interaction which had not been previously described. The most striking finding is that methylphenidate potently and selectively inhibits substrate uptake at hOCT1 in the nanomolar range. Due to the facts that (i) similar values have been reported as IC_{50} values at the primary targets of methylphenidate, hDAT and hNET [52], and (ii) the interindividual variability concerning appropriate dosing and avoidance of toxicity is high [53], it is possible that hOCT1 polymorphisms, which have been reported to frequently occur in the general population [20], might affect the pharmacokinetic and pharmacodynamic fate of methylphenidate in vivo

in a clinically relevant manner, warranting further investigation in this particular case but also for other compounds potentially interacting with hOCT1 and hOCT2 [31].

4. Materials and Methods

4.1. Chemicals and reagents

The compounds of interest were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or LGC Standards (Teddington, UK). S- and R- α -PVP was graciously provided by the NIDA Drug Supply Program. 2-fluoro-deschloroketamine was kindly provided by S. Brandt. All other chemicals and cell culture supplies were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Sarstedt (Nuembrecht, Germany).

4.2. Cell Culture

HEK293 cells were transfected with a plasmid, encoding the transporter of interest and an N-terminal YFP-tag, carried out using jetPRIME[®] (Polyplus Transfection; VWR International GmbH, Vienna, Austria) reagent (for a 10 cm dish with $1\text{--}2 \times 10^6$ cells in 10 mL serum containing medium at 60–80% confluency: 500 μL jetPRIME[®] buffer, 10 μg DNA and 20 μL jetPRIME[®] reagent) and selection pressure maintained for two weeks [2,54,55]. Subsequently, 500,000 cells were FACS-sorted (fluorescence-activated cell sorting) according to expression level to establish cell lines stably expressing the protein of interest. Fluorescence microscopy images of cells expressing respective YFP-tagged transporters are seen in Supplementary Figure S1. Concentration dependent uptake of tritiated substrate by transiently transfected cell lines expressing hOCT1–3 and hPMAT is shown in Supplementary Figure S2. For uptake inhibition assays, cells were cultured at a subconfluent (80–90% density) state in high-glucose Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% heat-inactivated Fetal Bovine Serum (FBS) and penicillin/streptomycin (PS; $100 \text{ U} \times 100 \text{ mL}^{-1}$, each). Selection pressure was maintained by the addition of geneticin ($50 \text{ mg} \times \text{mL}^{-1}$) at 37°C and 5% CO_2 in a humidified incubator. At a density of 60,000 cells per well, HEK293 cells expressing the respective transporter were seeded onto poly-D-lysine-coated wells 24 h prior to uptake inhibition experiments in a final volume of 200 μL per well.

4.3. Uptake Inhibition Assays

Prior to the addition of the respective compounds, DMEM was removed from all wells and replaced with 200 μL of Krebs-HEPES-buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 2 mM MgSO_4 and 20 mM D-glucose, pH adjusted to 7.3) at room temperature. Cells were exposed to a pre-incubation solution containing the compound of interest in the respective concentration (dissolved in Krebs-HEPES-buffer) for 10 min with a total volume of 50 μL , which was then replaced with a 50 μL uptake solution containing 0.05 μM of tritiated 1-methyl-4-phenylpyridinium ($[^3\text{H}]\text{MPP}^+$) as substrate, and the compound of interest in respective concentrations, for 10 minutes each. Finally, uptake was terminated by washing with 200 μL of ice-cold (4°C) KHB, after which cells were lysed with 200 μL of 1% sodium dodecyl sulphate (SDS). After transferring the solution of each well into 6 mL counting vials containing 2 mL of scintillation cocktail, the vials were measured with a beta-scintillation counter for quantification of the uptake of tritiated substrate. Decynium-22 (D22) shows robust inhibitions at low micromolar concentrations at all hOCTs and at hPMAT ranging between 0.16 μM at hOCT3 and 4.56 μM at hOCT2. D22 is depicted as a reference inhibition curve (dotted line) in all Figures 2–7. The structural formula can be seen in Figure 8. To obtain specific data, non-specific uptake was assessed in the presence of 100 μM D22 and subtracted from the total data. The 100% values were defined as uptake in the absence of a tested substance, defining maximal uptake capacity. Inhibitions of the compound of interest were described as a percentage of the maximal uptake capacity. While uptake inhibition assays are an effective tool for investigating the interaction of compounds with transporters of interest, they are not able to distinguish substrates from inhibitors, per se. Thus, we cannot rule out that some of the investigated

compounds are substrates rather than inhibitors. Future studies are needed to investigate this uncertainty.

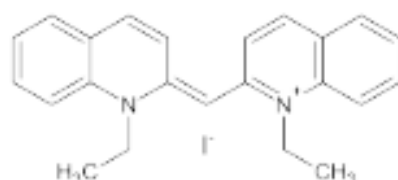


Figure 8. Chemical structure of decynium-22 (D22). D22 was used in all subsequent graphs as a highly potent transporter inhibitor for the assessment of unspecific uptake of the tritiated substrate [^3H]-MPP $^{+}$.

4.4. Data and Statistical Analysis

Half-maximal inhibitory concentration (IC_{50}) values of each substance were calculated and plotted using GraphPad Prism 8.4.3 (GraphPad Software Inc., San Diego, CA, USA). IC_{50} was determined by non-linear regression, solving the equation $Y = Bottom + (Top - Bottom) / (1 + 10^{X - LogEC_{50}})$. Data which displayed an increased uptake at higher concentrations was fit as a third-order polynomial, solving the equation: $Y = B0 + B1 * X + B2 * X^2 + B3 * X^3$. All data stem from a minimum of three separate experiments ($n \geq 3$) executed in triplicates and are shown as mean $\pm$ SD. All colors were chosen to be color-blind-friendly using a color brewer [56].

5. Conclusions

The investigation of the inhibitory potential of substances acting on the central nervous system has revealed some of them to inhibit the substrate uptake of human low-affinity organic cation transporters (OCT1–3) and PMAT in sub-micromolar or a low micromolar concentration. Bupropion and methylphenidate were found to be potent selective inhibitors of substrate uptake at hOCT1, with the latter interacting with hOCT1 more potently than the well-established OCT blocker decynium-22. MDMA was found to interact with hOCT1 and 2 more potently than decynium-22, as was *d*-amphetamine at hOCT2. Ketamine showed interaction selectivity and high uptake inhibitory potential at hOCT2. Further, tramadol, its main metabolite *O*-desmethyltramadol, and both citalopram enantiomers are selective inhibitors of the substrate uptake at hOCT1. Diazepam was the only tested compound to interact with hOCT3. We have found only small differences in the interaction caused by different aromatic ring substituents, as exemplified by ketamine and its derivative, 2-fluoro-deschloroketamine. Notably, we saw no enantioselective effect of *S*-citalopram and *R*-citalopram, or *S*- and *R*- α -PVP, accentuating the robustness of human OCTs and hPMAT. Considering the growing interest in pharmacological interaction of compounds with human OCTs and PMAT, our study provides important information regarding the complex interaction between a range of CNS-active substances and low-affinity transporters, suggesting some clinically relevant drugs as lead structures for the development of more selective inhibitors of these relatively understudied transporters [2,57].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ijms222312995/s1>.

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Supplementary Material

Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter

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Supplementary Materials

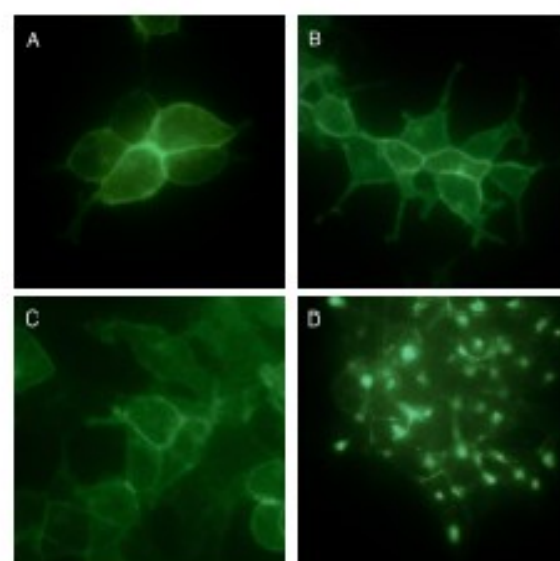


Figure S1. Microscopy images depicting cells expressing YFP-tagged transporters of interest (A): hOCT1, (B): hOCT2, (C): hOCT3 and (D): hPMAT.

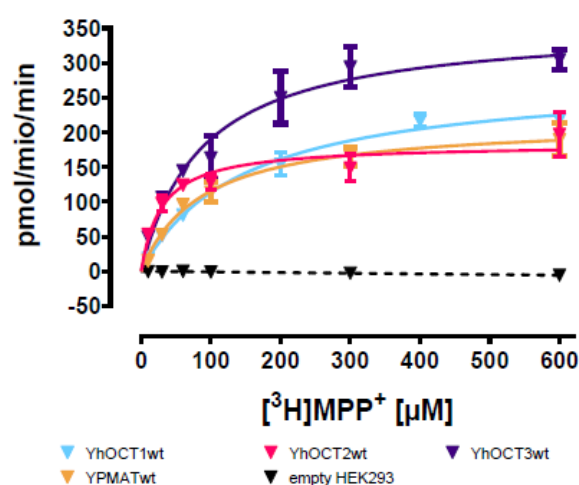


Figure S2. Representative experiments depicting concentration dependent uptake of tritiated MPP⁺ by the transporters (hOCT1 light blue, hOCT2 red, hOCT3 violet and hPMAT orange, all transiently transfected) of interest and empty HEK293 cells (dashed line) as control.

Table S1. IC₅₀ comparison table. Red boxes indicate that no respective data for comparison was found. n.s. = non-significant. Red text color: experiments were performed with ASP⁺ as substrate. Orange text color: values show the percentage of inhibition at 100 μM of the respective compound. Blue text color: Ki (μM) values were measured.

	Substance	hOCT1		hOCT2		hOCT3		hPMAT	
		IC ₅₀ literature	IC ₅₀ [μM] (95%-CI)	IC ₅₀ literature	IC ₅₀ [μM] (95%-CI)	IC ₅₀ literature	IC ₅₀ [μM] (95%-CI)	IC ₅₀ literature	IC ₅₀ [μM] (95%-CI)
Antidepressants	Bupropion	161.00 [1]	5.36 [4.19–6.85]	28.60 [1]	32.86 [21.55–50.10]	738.00 [1]	185.60 [9.36–3681]	115.00 [3]	96.96 [56.01–167.80]
	S-Citalopram		7.15 [5.59–9.15]		59.90 [34.26–104.70]		n.s.		159.80 [83.29–306.50]
	R-Citalopram		5.11 [4.11–6.37]		46.63 [25.56–85.07]		n.s.		58.32 [37.46–90.79]
	Citalopram	13.2 [1]		115.00 [1]		188.00 [1]		116.67 [4]	
Antiepileptics	Diazepam	5.0% [5]	n.s.		917.30 [229.70–3663]	2.00 [6]	44.46 [36.04–54.85]		29.81 [18.41–48.27]
	Phenobarbital	−6.0% [5]	n.s.		n.s.	40.00 (mOCT3) [6]	n.s.		1027 [39.71–26584]
Psychostimulants	d-Amphetamine	202 ± 68 [7]	8.39 [6.90–10.20]	10.5 ± 2.6 [7]	2.21 [1.70–2.87]	460 ± 140 [7]	336.6 [179.70–630.40]		71.77 [46.81–110.00]
	Cocaine	85 ± 22 [7]	6.66 [5.28–8.39]	113 ± 21 [7]	27.80 [19.17–40.32]	363 ± 56.4 [8]	n.s.		n.s.
	a-PVP	1.31 [13]		5.62 [13]		42 ± 7 [9]		13.42 [13]	
	R-α-PVP		2.15 [1.59–2.90]		13.09 [9.89–17.32]	41.5 ± 7.5 (DA)	1016 [364.90–2826]		n.s.
	S-α-PVP		1.07 [0.81–1.40]		15.02 [11.28–20.00]	24.1 ± 70 (5-HT) [10]	456.7 [144.90–1459]		n.s.
Emerging Therapeutics	Ketamine	114.5 ± 43.7 [7]	23.47 [13.34–41.37]	22.7 ± 6.3 [7]	12.46 [9.71–15.98]	225.7 ± 65 [7]	1028 [589.40–1792]		134.60 [54.70–331.20]
	2-Fluoro-deschloroketamine		14.07 [10.11–19.56]		19.18 [14.89–24.70]	440 [6]	265.5 [167.60–420.50]		n.s.
	MDMA	24.20 ± 9.20 [7]	1.14 [0.90–1.44]	1.63 ± 0.62 [7]	2.71 [2.22–3.31]	73.60 ± 50.30 [7]	153.90 [87.41–270.90]		7.77 [5.92–10.21]
	Psilocin		63.48 [44.97–89.62]		n.s.				698.20 [88.81–5488]
Analgesics	Tramadol	60.2% [5]	5.60 [4.65–6.75]		64.10 [39.58–103.80]		n.s.		74.19 [32.67–168.50]
	O-Desmethyl tramadol	172 [16]	24.16 [19.12–30.54]		371.90 [44.37–3116]		n.s.		n.s.
Drugs for Narcosis and ADHD	Methylphenidate		0.36 [0.27–0.46]		28.80 [15.11–54.90]		n.s.		n.s.
	Mofetilnifil		n.s.		n.s.		n.s.		n.s.
Control	Decynium-22		2.66 [2.049–3.44]		4.56 [3.786–5.496]		0.16 [0.1358–0.1904]		0.35 [0.2439–0.5042]

Experiments with MPP ⁺	Experiments with ASP ⁺	% inhibition at 100 μM	Ki (μM) Values	no data available
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Discussion

The first noteworthy finding is that the majority of herein tested psychoactive compounds interact with hOCT1, with some inhibiting substrate uptake at the transporter even more potently than the well-described, non-selective and potent OCT and PMAT blocker decynium-22 (D22) (Hayer-Zillgen, Brüss and Bönisch, 2002). Given its broad substrate and inhibitor profile, this observed interaction seems rather typical for OCT1 (Ahlin *et al.*, 2008; Arimany-Nardi *et al.*, 2016). In comparison, only around half of the compounds showed relevant IC₅₀ values (we saw IC₅₀ values > 100 µM as pharmacologically irrelevant) at hOCT2 and even less at hPMAT, while only one compound, besides D22, interacted with hOCT3. To date, many clinically relevant compounds were shown to interact with hOCT1 and 2, such as, for example, metformin, an antidiabetic drug whose pharmacokinetic fate is affected by hOCT1, 2 and 3 especially polymorphisms thereof (Chen *et al.*, 2009; Kwon *et al.*, 2018; Mato *et al.*, 2018). In general, previous studies have put emphasis on evaluating population specific presence of polymorphisms of these transporters, how their genetic variations influence drug absorption and distribution and, with growing focus, to what extent drug-drug interactions (DDIs) are determined by, genetically and inhibitor caused, altered transporter function (Arimany-Nardi *et al.*, 2016; Mato *et al.*, 2018; Zamek-Gliszczynski, Giacomini and Zhang, 2018a). These investigations have highlighted the importance of examining OCT polymorphisms, since they are shown to carry pharmacological and clinical consequences, albeit scientific consensus on their role of drug uptake, response and toxicity is not available yet (Goswami, 2014). Reasons for this still uncertain role of OCTs in various DDIs is that available data stems either from in vitro studies or preclinical animal studies and that, yet unresearched, contributions from other transporters most probably play an additional in the pharmacokinetic fate of compounds (Zhou, Zeng and Shu, 2021). Thus, further research needs to be undertaken to address the potential clinical consequences of OCTs and their putative substrates and inhibitors, as is e.g. recommended by FDA (Food and Drug Administration) and EMA (European Medicines Agency) in the case of hOCT2 and primarily renally excreted drugs (EMA, 2012; FDA, 2020). Hence, future investigations of compounds potently interacting with hOCTs and hPMAT must consider interindividual differences in patients carrying polymorphisms and therefor revealing different pharmacodynamic and -kinetic fates of various drugs, which can further lead to adverse effects if not clinically substantiated enough (Gorbunov *et al.*, 2008; Zolk, 2012). Also, more focus will need to be put on in vivo drug-drug interaction studies especially at the human homologues of OCT1-2 and PMAT, since these transporters have yet been almost exclusively studied in vitro (Maier, Niello, *et al.*, 2021).

Currently, from all known inhibitors of low-affinity, high-capacity monoamine transporters, only few were found to be selective at hOCT1, 2 or 3 or hPMAT (Koepsell, 2020). A possible explanation for this phenomenon can be the relatively high degree of sequence homology between transporters, as is especially the case for hOCT1 and 2, which share roughly 70% amino acid sequence identity (Sala-Rabanal *et al.*, 2013; Maier, Niello, *et al.*, 2021). The frequently shown overlap in compounds interacting with those two transporters is, to some extent, also shown in this study. However, we still found explicit differences between hOCT1 and 2 regarding IC₅₀ values of some of the compounds tested. This occurrence, on the other hand, is probably best explained with state-of-the-art knowledge about the transporters' biochemistry itself. Most SLC transporters that fall into the MFS Pfam clan share the same mode of substrate translocation by outward- and inward facing conformations via an occluded state (Colas, Ung and Schlessinger, 2016; Quistgaard *et al.*, 2016). This process, however, holds yet unexplained mechanisms, as transporters like the OCTs have cation binding regions with complex tertiary structures that may interact differently with the plethora of identified substrates and inhibitors (Nies *et al.*, 2011; Koepsell, 2018). According to that, a substrate binding region within a large binding cleft can contain an unknown number of cation binding sites, of which only a part overlaps between the OCTs (Koepsell, 2021). In line with this, it was found that amino acid residues that are essential for substrate specificity, which are localized in protein regions formed by transmembrane helices crucial for substrate binding, differ between rOCT1 and rOCT2, and thus might define variations in substance interactions with those transporters (Gorboulev *et al.*, 2005; Popp *et al.*, 2005). Further, a recent study suggested the existence of three MPP⁺ binding sites at rOCT1, with two of them acting as the familiar low-affinity binding sites that directly partake in substrate transport and the third indirectly affecting transport by allosterically acting on the other two sites (Keller *et al.*, 2019; Maier, Niello, *et al.*, 2021). However, given the inter-species contrast between rat and human isoforms of OCT1 and 2, with degrees of sequence homologies of 77.52% and 81.84%, respectively (Maier, Niello, *et al.*, 2021), they might, albeit seeming relatively low, account for essential differences in substrate specificity. By this, it becomes apparent that connections between studies with rat isoforms, as the ones mentioned before, and humans should not be drawn prematurely, which is in the same way applicable to comparisons between human and murine isoforms who share similar amino acid sequence homologies as those between humans and rats (Maier, Niello, *et al.*, 2021). In short, the exact molecular basis for affinities of compounds at hOCT1 or 2, but also at the other low-affinity transporters, has not been clearly established yet, and converging fronts of evidence underline the presence of allosteric sites targeted by inhibitors or showing atypical mechanisms

(Sala-Rabanal *et al.*, 2013; Reith *et al.*, 2015; Liu *et al.*, 2016; Niello *et al.*, 2020). Jointly, these factors provide a possible rationale why some substances selectively interact with hOCT1 but almost not at all with hOCT2, although both transporters are usually known for their broad substrate spectra, and while this study could not fill this gap in knowledge, we discovered scaffolds that can be used for the development of selective OCT inhibitors. These findings expand the pharmacological and structural understanding of the SLC22 transporter family and PMAT.

Regarding shown IC₅₀ values, differences between our results and those reported in previous studies are best explained by changed conditions concerning experiments. Ilic *et al.* (2020) compared in vitro methods for assessing drug potency at monoamine transporters, and found that changes in IC₅₀ values were due to different cell lines used as well as different methods for performing uptake inhibition assays (Ilic *et al.*, 2020). Also, results from some of the studies used for comparison of inhibition data stem from experiments where ((4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP⁺) was used as substrate, which could add to the explaining of discrepancies seen between our results and those from earlier studies. To this effect, previous reports have shown differences in inhibition potencies of compounds at hOCTs that were substrate-dependent, including ASP⁺ and MPP⁺ (Thévenod *et al.*, 2013; Hacker *et al.*, 2015; Koepsell, 2018). As one particular example, we found bupropion to selectively interact with hOCT1 (IC₅₀ = 5.36 μM, and an almost non-significant value for hPMAT), which disagrees with data from a previous publication (IC₅₀ = 160 μM at hOCT1), where the authors used, in comparison to our experiments, a different concentration of D22 for assessing non-specific uptake (30 μM instead of 100 μM), a longer pre-incubation time of 15 minutes (instead of 10) and, most importantly, a lower substrate ([³H]MPP⁺) concentration of 25 instead of 50 nM (Haenisch *et al.*, 2012). In addition to bupropion, two other antidepressants, the citalopram enantiomers (R- and S- citalopram) and the two analgesics tramadol and its metabolite *O*-desmethyltramadol were selectively inhibiting substrate uptake at hOCT1 (IC₅₀ values of 7.15 μM, 5.11 μM, 5.60 μM and 24.16 μM, respectively), while not showing any significant interaction with the other transporters. No previous studies have investigated interaction of the citalopram enantiomers with any of the hOCTs or hPMAT, but only with the racemic compound (Zhou, Engel and Wang, 2007; Haenisch *et al.*, 2012). Tramadol and *O*-desmethyltramadol, in turn, were investigated on hOCT1 with exclusively higher IC₅₀ values, but studies here either used a different substrate (ASP⁺), (Tzvetkov *et al.*, 2011) or assessed single point uptake inhibition at 100 μM (Ahlin *et al.*, 2008). Furthermore, ketamine potently interacts with hOCT2 and shows very little activity at hOCT1 or 3 or hPMAT. Amphoux *et al.*

(2006) reported similar results (as K_i values) for the OCTs, but did not investigate hPMAT (Amphoux *et al.*, 2006). Low potency of ketamine at high micromolar ranges at hOCT3 is also shown somewhere else (Massmann *et al.*, 2014). hOCT3 showed the least interaction with almost all tested compounds and interacted only with diazepam at significant IC_{50} values. This observance is concomitant with the fact that hOCT3 is more heterologous towards the other SLC22 members (Sala-Rabanal *et al.*, 2013). Also, it has been previously shown that non-selective inhibitors and substrates of OCTs are least efficacious at hOCT3, whereas many potent hOCT3 inhibitors, on the other hand, have weaker interactions with hOCT1-2 (Bönisch, 2021). Here, diazepam serves as an example for these hOCT3 specificities, since this compound not only proved to be a clearly more potent substrate uptake inhibitor at hOCT3 when compared to hOCT1 and 2, but, in addition, was the only herein tested compound to show any pharmacologically relevant interaction with the transporter. Diverging results from a previous study, where diazepam showed an IC_{50} value of 2 μ M compared to the IC_{50} of 44.46 μ M we measured, may be, again, explained by the different radioactive tracer used, with Massmann and colleagues having employed fluorescent ASP^+ instead of MPP^+ (Massmann *et al.*, 2014). Diazepam relatively potently interacted with hPMAT, which was previously unreported.

We explored enantiospecific differences of R- and S- α -pyrrolidinovalerophenone (R- and S- α -PVP) and R- and S-citalopram, and also investigated possible effects of different aromatic substituents between ketamine and 2-fluoro-deschlorketamine as well as tramadol and *O*-desmethyltramadol. Differences in aromatic ring substitutions between ketamine and 2-fluoro-deschlorketamine exhibited almost no effect on any transporter except a small decrease in uptake inhibition potency for the derivative at hOCT2, whereas tramadol and *O*-desmethyltramadol both remained non-potent at hOCT3 and hPMAT. Affinities towards both hOCT1 and 2 decreased for the derivative, with tramadol inhibiting substrate uptake at hOCT1 with an four-fold higher potency than *O*-desmethyltramadol (IC_{50} s of 5.60 and 24.16 μ M, respectively). This observed ratio is in line with an earlier investigation of the same compounds on hOCT1, where tramadol and *O*-desmethyltramadol inhibited ASP^+ uptake with IC_{50} values of 30.3 and 172 μ M, respectively (Tzvetkov *et al.*, 2011). A previous study about the characterization of interactions between five α -pyrrolidinopropiophenone (α -PPP) derivatives and human MATs, OCTs and PMAT observed that different substitutions on these substances' aromatic rings indeed affected both high- and low affinity transporter classes (Maier, Rauter, *et al.*, 2021). There, differences in interaction with hOCT1 and 2 were shown to be less pronounced compared to their effect on hOCT3 and hPMAT, which might just again be accounted for by these transporters' higher homology between each other. However, the here

shown, low sensitivity of OCTs and PMAT towards differing aromatic ring substituents sets them apart from SLC6 transporters, where, for example, substituted cathinones exhibited pronounced effects and led to differences in selectivity for one MAT over another (Rickli, Hoener and Liechti, 2015; Eshleman *et al.*, 2017; Niello *et al.*, 2019). Concerning R- and S- α -PVP and R- and S-citalopram, we found no enantioselective differences in uptake inhibition within each pair of enantiomeric compounds on any transporter. To our knowledge, no previous studies have analyzed these compounds at hOCTs or hPMAT, but our results emphasize their robustness regarding drug-transporter interactions. This again distinguishes them from MATs in many cases, as latter are, for example, well-known targets of subtly chemically modified versions of pharmacologically heterogeneous psychoactive compounds (Baumann *et al.*, 2018; Maier *et al.*, 2018; Niello *et al.*, 2021). No high-resolution crystal structures of any low-affinity transporter are available, and large-scale structure-activity relationship (SAR) analyses have yet to be undertaken to further identify and confirm potent chemical scaffolds for hOCTs and hPMAT (Maier, Niello, *et al.*, 2021). Effects of both differing substituents as well as enantiomeric compounds, amongst other chemical characteristics, need to be further investigated, since criteria for OCT and PMAT substrates and inhibitors still seem highly understudied. For what is known today, at least one positive charge at physiological pH and a high degree of hydrophobicity are major physicochemical requirements for OCT and PMAT substrates/inhibitors (Bednarczyk *et al.*, 2003; Engel and Wang, 2005; Sala-Rabanal *et al.*, 2013), but only little research has detailed further chemical criteria, as was e.g. done with increasing alkyl chain lengths in n-tetraalkylammonium compounds which increased compound hydrophobicity and thus inhibition potency at hOCT1 (Bednarczyk *et al.*, 2003; Sala-Rabanal *et al.*, 2013), or phenylalkylamine derivatives, which showed increased inhibitory potency at hPMAT with increasing distance between the aromatic ring and the N-atom (Ho *et al.*, 2011). Latter was corroborated by a study with α -PPP and α -PVP, which demonstrated an increased carbon side chain length to result in a higher inhibitory potency (Maier, Rauter, *et al.*, 2021). Parallel to ligand properties themselves, an increasing body of literature emphasizes the heterogeneity in binding sites available in neurotransmitter transporters (Niello *et al.*, 2020). In general, proteins may possess one or more ligand binding sites that differ from the inherent orthosteric site, which are basically known as secondary or allosteric sites (Leach, Sexton and Christopoulos, 2007; Changeux and Christopoulos, 2016). These protein sites, that are distant from the central binding site of the large binding cleft, are proposedly able to modulate the function and activity of the orthosteric site after ligand binding, either by directly transmitting a positive or negative effect on different parts of the transporter, or by, and this has yet to be

further confirmed, inducing a change in the physicochemical kinetics by increasing or decreasing the energetic barrier, which has to be overcome for the enzymatic reaction to happen (Motlagh *et al.*, 2014; Niello *et al.*, 2020).

This “allosterism” might also be of relevance for the interaction between psychoactive substances and low-affinity transporters, as it provides a possible explanation for a surprising phenomenon that we observed, namely that treatment with higher concentrations of some compounds led to elevated MPP⁺ uptake, as seen most markedly for phenobarbital at hOCT1 and 2, both α -PVP enantiomers at hPMAT and psilocin at all three hOCTs. Consistent with our finding are results from a previous investigation of Ahlin and colleagues, who reported increased substrate uptake at hOCT1 after cell treatment with a 100 μ M concentration of phenobarbital, but also generally showed negative percentage values for inhibitory potencies of many other compounds (Ahlin *et al.*, 2008). In their study, they examined the chemical space of a high number of registered oral drugs and found that some molecular properties, like e.g. higher dipole moments and a higher amount of hydrogen bonds, negatively correlated to OCT1 inhibition. Be that as it may, until this phenomenon is fully cleared up, what remains is to look at it in the context of the aforementioned allosteric mechanisms these compounds may have on transporter action. Consequently, some compounds might not bind to the orthosteric site but rather to topographically distinct positions and act as allosteric ligands, by which they would change the conformational dynamics of the protein and ultimately increase the intrinsic transport rate (Niello *et al.*, 2020). Moreover, the above-mentioned existence of a third, high-affinity binding site at OCTs could be a target for such allosteric ligands, since it was proposed to partake in the effectivity of substrate uptake through positively affecting transport dynamics of the low-affinity sites (Keller *et al.*, 2019). What is more, high-affinity cation binding sites carrying mutations of amino acids in key positions essential for transport could go through a functional shift and, contrary to their possible positive effect in wild-types, become high-affinity inhibition sites, partially or totally competing with ligands bound to the low-affinity sites and thus easing the way for adverse drug reactions in patients (Gorbunov *et al.*, 2008; Sala-Rabanal *et al.*, 2013). This mechanism would show once more how much of an importance OCT polymorphisms and investigations on ligand-transporter interactions depict for adapted drug therapy.

Conventionally, D22 was many times used as a standard positive control in uptake inhibition assays, as in this case for the assessment of non-specific uptake into cells, due to its high substrate uptake inhibitory potency at all hOCTs and hPMAT (Hayer-Zillgen, Brüss and Bönisch, 2002; Engel, Zhou and Wang, 2004). In our present investigation, however, we have

found numerous compounds to have similar or even lower IC₅₀ values. d-Amphetamine and MDMA both interacted with hOCT1 and 2 in a low micromolar range, while the latter also highly potently interacted with hPMAT. d-Amphetamine inhibited uptake at hOCT1 with an IC₅₀ of 8.39 µM, which was amongst the lowest 25% of measured IC₅₀ values, whereas its IC₅₀ at hOCT2 (2.21 µM) was even lower than that of D22 (4.56 µM). Similarly, MDMA inhibited substrate uptake at both OCT1 and 2 more potently than D22. These findings, concerning interaction profiles of d-amphetamine and MDMA at hOCT1 and 2 are, to some extent, consistent with an earlier investigation (Amphoux *et al.*, 2006).

One noteworthy finding from that study, which is also distinctly shown here, is that d-amphetamine and other psychostimulants only weakly or not at all interact with hOCT3. This often observed low affinity of psychostimulants towards OCT3 is insofar interesting, as (i) OCT3 in rats was localised in brain regions that are proven to constitute anatomical substrates for the response to various psychostimulants, (ii) in humans, OCT3 was often recognized as a putative important protagonist in the monoaminergic clearance system, a mechanism very well-known to be strongly affected by numerous psychostimulants and (iii) related to that, OCT3 expression was found in NE, DA and serotonergic projection regions, all of which are target sites for the action of psychostimulants (Berke and Hyman, 2000; Gasser *et al.*, 2009; Sitte and Freissmuth, 2010; Barr *et al.*, 2013; Mayer *et al.*, 2018). Accordingly, one would expect hOCT3 to be a, more or less, relevant target of those psychoactive compounds that potently interact with NET, DAT and SERT, which apparently is not the case, as was also shown by other groups (Jensen *et al.*, 2021). Additionally, a lot of data is present on the fact that dopamine and serotonin have only poor substrate affinities towards hOCT3, an aspect which further blurs the otherwise undeclared functionality of OCT3 in the central nervous system and was recently confirmed by a study about substrate overlaps between human MATs and OCTs (Gebauer *et al.*, 2021). This discrepancy in substrate spectra might provide one necessary part of the explanation for why some SLC6 affine drugs do not interact with hOCT3. However, previous in vivo studies with knockout mice have, in turn, shown that OCT3 does play a role in the behavioural effects of some psychoactive drugs, assigning the transporter a role in the increase of sensitivity towards psychostimulants (Kitaichi *et al.*, 2005; Vialou *et al.*, 2008; Mayer *et al.*, 2018). In this regard, it was indicated that psychostimulant-evoked substrate release, including MPP⁺, is (partially) mediated by OCT3, suggesting a bidirectional nature of the transporter (Gasser, Lowry and Orchinik, 2006; Mayer *et al.*, 2018). This efflux mechanism has been theoretically backed for OCTs in general, since the concerted action of a sufficiently high outwardly-directed cation gradient in depolarized cells and the absence of extracellular cations

could provide the driving force for it, as was e.g. theorized for hOCT2-mediated efflux of acetylcholine (Lips *et al.*, 2005; Koepsell, 2018). This proposed mechanism, however, remains hypothetical, as it has not yet been examined more closely, let alone fully understood.

Taking into account that the biggest share of studies investigating interactions between various compounds and OCTs and PMAT in vitro have put focus on psychoactive substances, which, as mentioned, show the clearly weakest inhibitory potential at hOCT3, it is evident that a different chemical scaffold might be more eligible for exploring compounds that potently interact with this transporter. The probably most striking finding here is that methylphenidate, known under the brand name Ritalin, selectively and highly potently inhibits substrate uptake at hOCT1 in a nanomolar range, with an IC₅₀ value of 0.36 µM, the second lowest of all IC₅₀ values we measured, just after D22 at hOCT3. Now when we consider that this value is similar to the nanomolar IC₅₀ values that have been reported at this drugs' primary targets, hNET and hDAT (Luethi *et al.*, 2018), and that the observed interindividual variabilities in patients concerning dose-response relationships and avoidance of toxicity are relatively high (Kimko, Cross and Abernethy, 1999), it is probable that altered hOCT1 function, caused by its polymorphisms, affects the pharmacokinetic and pharmacodynamic fate of methylphenidate in vivo, impeding a favourable response to this medical drug. As mentioned, hOCT1 polymorphisms are reportedly frequent in the general population (Tzvetkov *et al.*, 2011), which once again accentuates their relevance for public health and academia. Thus, this particular case of methylphenidate and hOCT1 is yet just another example for the importance of investigating this transporters' polymorphisms, and is in the same way applicable to the large number of other compounds potently interacting with the other hOCTs and hPMAT, warranting further investigations in this field of research (Goswami, 2014).

We herein presented substrate uptake inhibition data that provide important information for and could help elucidate the interaction between a range of prominent CNS-active compounds and hOCTs and hPMAT. However, it seems appropriate to mention that one needs to be cautious about relying entirely on such data, as are in this case the measured IC₅₀ values, since those cannot tell whether a substance is just a competing substrate or an actual inhibitor (Bönisch, 2021). Further, in this field there is the well-known need for distinguishing competitive from non-competitive inhibition when dealing with inhibition data (Liu *et al.*, 2016), which, as well, forms a true desideratum in pharmacological research. One possible way for addressing these issues would be the establishment of advanced computational models for understanding the molecular mechanisms of hOCT- and hPMAT inhibition and transport, as was seen for many other transporter types (Matsson and Bergström, 2015). Developments that would facilitate

future efforts for such modelling would be the expansion of datasets of confirmed transporter substrates, ligand-transporter inhibition (yet again characterized by the underlying mechanisms of inhibition, i.e. competitive vs. allosteric, indirect inhibition) and, most importantly, high-resolution structures of the respective transporters in relevant states of the transport cycle and with co-crystallized inhibitors to back up data generated by, as in this case, in vitro uptake inhibition assays (*ibid.*). Latter constitutes a general gap in research for many transporter proteins, let alone this understudied group of low-affinity cation transporters (Keller *et al.*, 2019; Koepsell, 2020). Also, modelling of transporters as multispecific as the SLC22 family and hPMAT is principally more difficult, as they handle structurally much more diverse compounds than other transporter families (Liu *et al.*, 2016) and would thus need more and detailed pharmacokinetic data for such models to function. The fact that the herein investigated transporters, but most notably hOCT1 and 2, are highly polymorphic and known to carry mutations relatively often just adds to this issue, but at the same time ascribes our presented data an all the greater importance. This study succeeded at elucidating the pharmacological interaction between several central nervous system active drugs at the human organic cation transporters 1-3 and the human plasma membrane monoamine transporter. Our results contribute to the advance in this relatively young but promising field of research and introduce some prominent, clinically relevant compounds as potential scaffolds for the development of new or adapted medical drugs. This might give impetus for further research to investigate these transporters' abounding pharmacology and builds groundwork for exploring substrate translocation and thus pharmacodynamic fate of relevant compounds especially in patients with OCT and/or PMAT polymorphisms, to whom an individualized drug therapy will represent a major clinical progress.

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