Biomedicine & Pharmacotherapy 161 (2023) 114454

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Combined and independent effects of OCT1 and CYP2D6 on the cellular disposition of drugs

Lukas Gebauer *, Christof Dücker, Ole Jensen, Jürgen Brockmöller

Institute of Clinical Pharmacology, University Medical Center Göttingen, D-37075 Göttingen, Germany

ARTICLE INFO

Keywords:
Organic cation transporter
Cytochrome P450 2D6
Drug transport
Drug metabolism
Drug-drug interactions

ABSTRACT

The organic cation transporter 1 (OCT1) mediates the cell uptake and cytochrome P450 2D6 (CYP2D6) the metabolism of many cationic substrates. Activities of OCT1 and CYP2D6 are affected by enormous genetic variation and frequent drug-drug interactions. Single or combined deficiency of OCT1 and CYP2D6 might result in dramatic differences in systemic exposure, adverse drug reactions, and efficacy. Thus, one should know what drugs are affected to what extent by OCT1, CYP2D6 or both. Here, we compiled all data on CYP2D6 and OCT1 drug substrates. Among 246 CYP2D6 substrates and 132 OCT1 substrates, we identified 31 shared substrates. In OCT1 and CYP2D6 single and double-transfected cells, we studied which, OCT1 or CYP2D6, is more critical for a given drug and whether there are additive, antagonistic or synergistic effects. In general, OCT1 substrates were more hydrophilic than CYP2D6 substrates and smaller in size. Inhibition studies showed unexpectedly pronounced inhibition of substrate depletion by shared OCT1/CYP2D6 inhibitors. In conclusion, there is a distinct overlap in the OCT1/CYP2D6 substrate and inhibitor spectra, so *in vivo* pharmacokinetics and -dynamics of shared substrates may be significantly affected by frequent OCT1- and CYP2D6-polymorphisms and by comedication with shared inhibitors.

1. Introduction

Membrane transport and metabolism are the major determinants of the pharmacokinetics of most drugs, and both processes are also essential in endogenous metabolism. More than 200 different solute carriers (SLC) mediate cell influx or cell efflux transport of more hydrophilic substances, and efflux transport of more lipophilic drugs and other substances is mediated by ATP-binding cassette (ABC) transporters [1]. Membrane transport and metabolism must be well coordinated to present the substrates to the (mostly intracellularly localized) drug-metabolizing enzymes and to avoid cellular overload with substrates or metabolites. For instance, cytochrome P450 3A enzymes and the efflux transporter MDR1 (ABCB1) overlap extensively in their substrates and in their transcriptional regulation as well [2]. While MDR1 and CYP3A4 mostly handle amphiphilic or hydrophobic substrates, the OATP1B1 and CYP2C9 cooperate in handling negatively charged drugs like torsemide or fluvastatin [3,4].

Here we analyzed one particularly interesting transporter-enzyme interaction relevant to organic cations. About 30 % of all drugs are organic cations positively charged at typical pH in the human body [5,6]. If these organic cations are relatively hydrophilic ($logD_{pH7.4} < 1.5$), they cannot efficiently diffuse through cell membranes. Thus, for their efficient intestinal absorption or uptake into the liver prior to biotransformation, organic cations require transport via an organic cation transporter like the organic cation transporter 1 (OCT1). A notable feature of OCT1 is its high inherited variation in most human populations.

An important phase-I drug-metabolizing enzyme with a corresponding preference for positively charged substrates and an even more considerable genetic variation is cytochrome P450 2D6 (CYP2D6). This enzyme was discovered more than 50 years ago as debrisoquine and sparteine hydroxylase. CYP2D6 is involved in the metabolism of about

Abbreviations: COMT, Catechol-O-methyltransferase; CYP2D6, cytochrome P450 2D6; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks buffered salt solution; HPLC-MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry; MAO, monoamine oxidase; OCT1, organic cation transporter 1; qRT-PCR, quantitative real-time PCR; SMILES, simplified molecular-input line-entry system; SULTs, sulfotransferases; UDPGTs, uridine 5′-diphospho-glucuronosyltransferases.

* Correspondence to: Institute of Clinical Pharmacology, University Medical Center Göttingen, Georg-August University, Robert-Koch-Str. 40, 37075 Göttingen, Germany.

E-mail address: lukas.gebauer@med.uni-goettingen.de (L. Gebauer).

https://doi.org/10.1016/j.biopha.2023.114454
Received 14 December 2022; Received in revised form 20 February 2023; Accepted 26 February 2023
Available online 3 March 2023

25 % of all approved drugs [7]. A typical structural requirement of CYP2D6 substrates is a lipophilic moiety (typically an aromatic residue) oxidized by CYP2D6. This lipophilic moiety is about 5–10 Å apart from the positive charge [8–10]. However, several well-known CYP2D6 substrates like metoprolol or prajmaline do not follow that rule.

As introduced above, there is extensive inherited genomic variation in CYP2D6. That variation in most human populations ranges from complete deficiency to ultra-high activity [11–13]. This genetic variation can result in decreased or increased metabolic activation or inactivation of drugs, resulting in significant variation in drug efficacy or adverse reactions [12,14–18].

While one genetically polymorphic transporter or enzyme may be responsible for high inter-individual differences in systemic exposure to drugs, combined interactions between two polymorphic proteins involved sequentially in the disposition of a drug or a toxin may result in even more considerable differences in systemic exposure. Recently, the combined effects of OCT1 and CYP2D6 have been confirmed for sparteine and debrisoquine [19,20].

In the present study, we wanted to characterize the overlap between OCT1 and CYP2D6 substrates for the known substrates of both proteins. With this data, we wanted to find physicochemical and structural determinants of overlap and divergence between the OCT1 and CYP2D6 substrate spectra. Moreover, for those substances which are reasonably good substrates of both, we wanted to assess how shared substrates might be affected by combined the combined action OCT1 and CYP2D6. Finally, based on published inhibition data, we expanded this comparison toward shared and specific inhibitors of OCT1 and CYP2D6.

2. Methods

2.1. Database of CYP2D6 and OCT1 substrates

Based on reviews and databases [17,21–26], we identified substrates of CYP2D6, and for every listed CYP2D6 substrate, we searched for confirmation in the primary literature (see Supplementary Table 1). Prescribing information for professionals was also considered as valid data. To find shared substrates, we integrated those confirmed CYP2D6 substrates in a database on *in vitro* OCT1 transport [27–33]. To characterize the extent of overlap between OCT1 and CYP2D6 inhibitors, we included data on inhibition of OCT1-mediated ASP^+ uptake [29,34,35] and bioassay data from PubChem [36] on CYP2D6 inhibition (bio assay record AID891 [37]).

2.2. In vitro uptake experiments in OCT1-overexpressing cells

All experiments were carried out in HEK293 cells stably transfected with the empty expression vector, OCT1, CYP2D6, or both. Cells were regularly cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL). Cell lines were kept in culture for no longer than 30 passages. An empty-vector transfected control cell line and the OCT1-overexpressing cells were generated using the Flp-In system (Thermo Fisher Scientific, Darmstadt, Germany) as described previously [20]. The CYP2D6-transfected cell line was generated according to the standard protocol of the Flp-In system provided by the manufacturer. For the creation of the OCT1-CYP2D6-double transfected cell line, a modified version of the Flp-In system based on a second expression vector was used [38]. The detailed protocols for the generation and validation of the CYP2D6 and the CYP2D6-OCT1 double-transfected cell lines are described in the supplementary methods section.

For transport experiments, 300,000 HEK293 cells were plated 48 h ahead of the experiment in poly-d-lysine precoated 24-well plates. Prior to substrate addition, the cells were washed once with 1 mL prewarmed (37 °C) HBSS+ (Hanks buffered salt solution, supplemented with 10 mM HEPES). After this, cells were incubated for 2 min with 2.5 µM test substrate dissolved in prewarmed HBSS+. Transport was stopped by adding 1 mL ice-cold HBSS+, and cells were washed two times with ice-cold HBSS+ before cell lysis was performed with 80 % acetonitrile containing an appropriate internal standard for eventual analysis by LC-MS/MS. In addition, two wells per cell line were lysed using RIPA buffer for subsequent protein quantification. This was later used to normalise the uptake data to account for variation in the density of seeded cells. Protein content was quantified by comparison to a standard curve of bovine serum albumin (Sigma-Aldrich, Darmstadt, Germany) in a bichinonic acid assay [39]. Transporter-mediated uptake was then quantified as uptake ratio into OCT1-overexpressing cells over empty-vector control cells. As widely used, substances with an uptake ratio above 2.0 were considered OCT1 substrates [23]. However, we must be aware that any minor but statistically significant uptake ratio indicates OCT1 activity with the respective substrate. To evaluate a possible OCT1 relevance with minor uptake ratios, we also tested substrates with a ratio between 1.5 and 2.0 in the 90-minutes substrate depletion assays.

2.3. In vitro uptake and metabolism experiments in OCT1-CYP2D6 double-transfected cells

For uptake and metabolism experiments, 300,000 HEK293 cells were plated on poly-D-lysine coated 24-well plates 48 h before the experiment. The experiment was initiated by washing the cells once with 1 mL prewarmed HBSS+. Subsequently, cells were incubated for 90 min with 1 µM substrate dissolved in DMEM supplemented with 20 mM HEPES at 37 °C in a humidified atmosphere inside the cell culture incubator. After incubation, the supernatant was collected and centrifuged to remove detached cells (400g, 5 min, rt). The cells were washed and lysed before quantification of intracellular concentrations was done as described for the 2-minutes uptake experiments above. The cellular supernatant was precipitated with acetonitrile/methanol (ratio 9:1) containing an internal standard for subsequent LC-MS/MS analysis. For the single-timepoint substrate depletion screening, the extracellular (medium) substrate leftover after 90 min of incubation was quantified relative to the empty-vector transfected control cell line. For the time-dependent experiments, absolute quantification of intra- and extracellular substance concentration was done by comparison to standard curves with known concentrations.

2.4. HPLC-MS/MS concentration analyses

Intracellular as well as extracellular drug concentrations were quantified by HPLC-MS/MS analysis. The HPLC system was composed of a Shimadzu Nexera HPLC system with a LC-30AD pump, a SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A controller (all Shimadzu, Kyoto, Japan). Separation was performed on a SPP RP-Amide column (4.6 × 100 mm inner dimension with 2.7 µm particle size, PerkinElmer, Waltham, MA) with a corresponding C18 guard column. The aqueous mobile phase contained additionally 0.1 % (v/v) formic acid and organic additive (acetonitrile/methanol (6:1), both LGC Standards, Wesel, Germany) ranging from 3 % to 50 % (v/v) depending on the substrate. Isocractic chromatography was carried out with a flow rate of 0.3 or 0.4 mL/min and an oven temperature of 40 °C. Subsequent detection was done by an API4000 tandem mass spectrometer (AB SCIEX, Darmstadt, Germany) operating in the multiple reaction monitoring mode. Peak detection and quantification was achieved using the Analyst 1.6.2 software (AB SCIEX). MS detection parameters as well as analyte-specific mobile phases are listed in Table S3.

2.5. Calculations and software

The database was set up via DB Browser for SQLite, structural files were downloaded from PubChem and were managed using DataWarrior [36,40], logD and charge at pH 7.4 were calculated with cxcalc (ChemAxon, Budapest), all other chemical descriptors were calculated

using RDKit [41]. Numerical and structural data were joined and analyzed in python using pandas [42] and was plotted using matplotlib [43]. The crystallography of CYP2D6-bound thioridazine was downloaded from the Protein Data Bank (PDB ID: 4wnw [9]) and visualized using pymol.

The drop of substrate concentrations and significance of the effects of OCT1 and CYP2D6, as well as the significance of possible interaction (i.e., super-additive or antagonistic effects of the combination of both), was analyzed by multiple linear regression analysis according to $v = a * CYP2D6 + b * OCT1 + c * CYP2D6 \times OCT1$ with a, b, and c denoting the relative contributions of the three factors and v the concentration of the substrate after 90 min incubation. CYP2D6×OCT1 denotes the interaction (synergistic or antagonistic) if existent.

3. Results

We first identified all CYP2D6 substrates currently known according to an extensive literature search. Then, we analyzed the overlap between CYP2D6 and OCT1 substrates. OCT1 substrates were defined as those with an uptake ratio above 2 in OCT1 overexpressing cells over empty vector-transfected cells. Then, we present substrate depletion data with OCT1 and CYP2D6 overexpressing cells indicating the relative contribution of OCT1 versus CYP2D6. These analyses should also indicate super-additive or antagonistic interactions between transporter and enzyme in the assays with OCT1-CYP2D6 single and double transfected cells. Finally, we present data on the combined inhibition of OCT1 and CYP2D6 by selected presumably specific or shared inhibitors.

For 246 substrates, the contribution of CYP2D6 to biotransformation was documented in the original references (Table S1). *In vivo* data from clinical pharmacogenetic or drug-drug interaction studies were considered reliable evidence for the CYP2D6 contribution to the metabolism of the respective substance. However, most literature evidence was based on in-vitro studies using liver microsomes or microsomes purified from single CYP-transfected cell lines. Any data showing that CYP2D6 is capable of metabolizing a substance was recorded regardless of the quantitative contribution of CYP2D6-mediated metabolism to the overall metabolism or the clinical pharmacokinetic data.

To show the overlap of OCT1 and CYP2D6 substrates, we combined the list of CYP2D6 substrates with the list of OCT1 substrates and OCT1 non-substrates. For the latter, we used already published data on OCT1 transport, and screened 108 additional substances for OCT1 transport (Table S4). In this screening, we identified the known CYP2D6 substrates aclidinium, almotriptan, and umeclidinium as good OCT1 substrates. Based on all new experimental data and all published data, 414 substances were tested for OCT1 transport, and 132 of these were significantly transported by OCT1 by applying a cut-off of a 2-fold higher uptake in OCT1 overexpressing cells compared to EV-control cells. For 127 substances, the activity information were available for both OCT1 and CYP2D6 (Fig. 1). And of those 127, 31 were shared substrates of OCT1 and CYP2D6, whereas 16 and 80 were exclusive substrates of OCT1 and CYP2D6, respectively. The remaining 220 substances were either OCT1 or CYP2D6 substrates, but no experimentally confirmed assignment concerning the other protein was available. However, almost all of those CYP2D6 substrates for which no OCT1 transport data are available (Table S1) are most likely not substrates of OCT1 because they are too lipophilic to have a relevant net transport by OCT1. Thus, most substances which remained unclassified concerning OCT1 (Fig. 2) are very unlikely to be substrates of OCT1. On the other side, most OCT1 substrates not characterized for CYP2D6 appeared very unlikely to be CYP2D6 substrates based on their molecular structures. The complete list of all substrates, including substrates with only minor OCT1-mediated transport (ratio below 2.0) is given in the supplement.

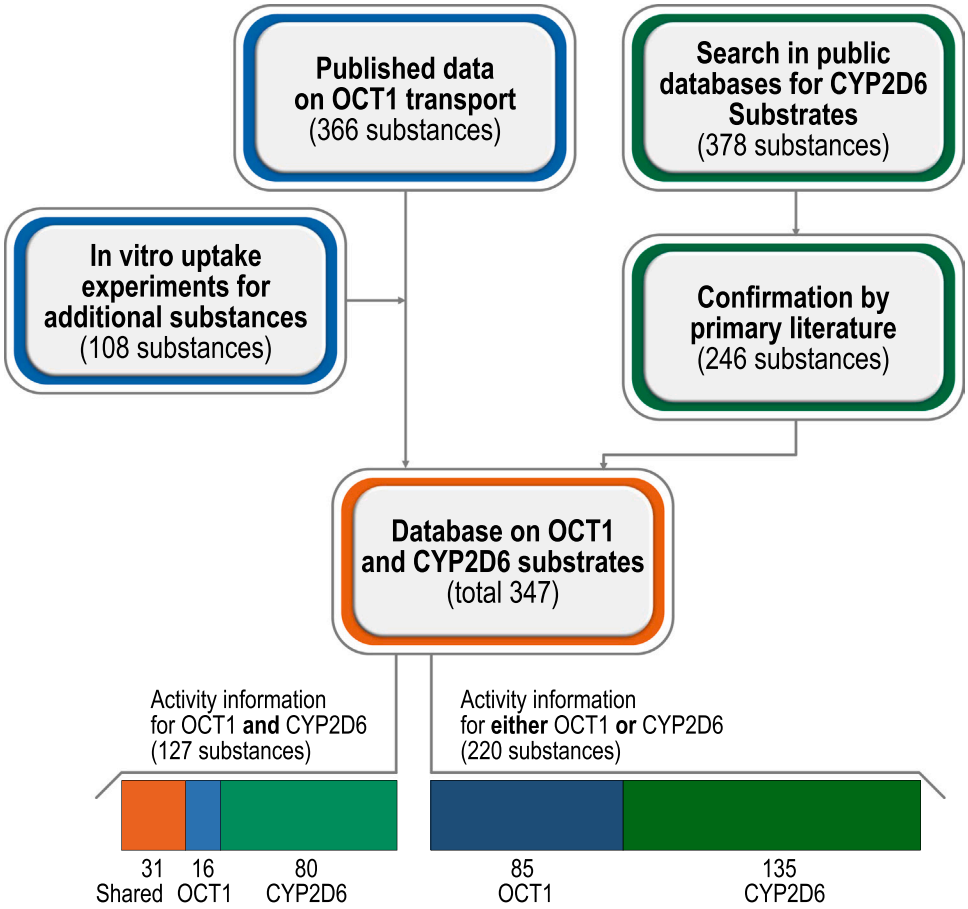

Fig. 1. Identification of OCT1 and CYP2D6 substrates. For identifying OCT1 substrates, 108 substances were tested for OCT1 transport, and the uptake data were combined with published data. This resulted in transport data for 414 different substances. Of these, 132 were considered as OCT1 substrates when applying a cut-off of two-fold increased uptake in OCT1-overexpressing cells over control cells. For CYP2D6, putative substrates were identified by searching public databases, but finally, only those were considered as CYP2D6 substrates which were confirmed by primary literature (Table S1). The discrepancy in why 132 substrates were listed in databases without confirmation could not be elucidated in all cases, but in several cases, it may be due to a mix-up of inhibition versus substrate properties. The resulting database consists of entries for 347 different substances. For 127 out of those, activity information for OCT1 and CYP2D6 was available. The remaining 220 substances were either OCT1 or CYP2D6 substrates, but no information for the respective other protein was available.

CYP2D6	OCT1	Name	CYP2D6	OCT1	Name	CYP2D6	OCT1	Name	CYP2D6	OCT1	Name
1	1	Acebutolol	1	0	Clonidine	1	0	Imatinib	1	0	Propafenone
1	0	Acetaminophen	1	0	Clozapine	1	0	Imipramine	1	0	Propranolol
1	1	Aclidinium	1	0	Codeine	1	0	Ketamine	1	1	Ranitidine
1	1	Almotriptan	1	0	m-CPP	1	0	Loperamide	0	1	Rizatriptan
1	0	Alprenolol	1	1	Debrisoquine	1	0	Maprotiline	1	1	Rucaparib
0	1	Amifampridine	1	0	Delavirdine	1	1	MDMA	1	0	Sertraline
1	0	Amiodarone	1	0	Deprenyl	0	1	Metformin	0	1	Sotalol
0	1	Amisulprite	1	0	Desipramine	1	0	Methamphetamine	1	1	Sparteine
1	0	Amitriptyline	1	1	Desvenlafaxine	1	0	Metoclopramide	0	1	Sumatriptan
1	0	Amphetamine	1	0	Dextromethorphan	1	0	Metoprolol	0	1	Synephrine
0	1	Atenolol	1	0	Diltiazem	1	1	Mexiletine	1	0	Tamoxifen
1	0	Atomoxetine	1	0	Diphenhydramine	0	1	Milnacipran	1	1	Tamsulosin
1	1	Berberine	0	1	Disopyramide	1	0	Mirabegron	1	0	Tapentadol
1	0	Betaxolol	1	1	DMPP	0	1	Octopamine	1	0	Timolol
1	0	Bicifadine	1	0	Domperidone	1	0	Ondansetron	1	0	Tolterodine
1	0	Biperiden	1	0	Doxepine	1	0	Oxycodone	1	0	Tramadol
1	0	Brofaromine	1	0	Eletriptan	1	1	Oxyphenonium	1	0	Trimipramine
1	0	Bunitrolol	1	1	Fenfluramine	1	0	Palonosetron	1	1	Tropisetron
1	0	Buspirone	1	0	Flecainide	1	0	Paroxetine	0	1	Tryptamine
1	0	Captropril	1	0	Fluoxetine	1	0	Pentamidine	1	1	Tyramine
1	1	Carteolol	1	0	Fluphenazine	1	0	Perphenazine	1	0	Ulotaront
1	0	Carvedilol	1	0	Fluvoxamine	1	1	Phenformin	1	1	Umeclidinium
1	0	Cevimeline	1	1	Formoterol	1	0	Pinoline	1	0	Venlafaxine
1	0	Chloroquine	1	1	Frovatriptan	1	0	PMA	1	0	Viloxazine
1	0	Chlorpheniramine	1	0	Galanthamine	1	0	PMMA	1	1	Zolmitriptan
1	0	Chlorpromazin	1	1	Glycopyrrolate	1	1	Procainamide	1	0	Zotepine
1	0	Citalopram	1	0	Haloperidol	1	0	Prochlorperazine			
1	0	Clemastine	1	1	Harmaline	0	1	Proguanil			
1	0	Clomipramine	1	0	Hydrocodone	1	0	Promethazine			

Fig. 2. Substrates of CYP2D6, or OCT1 or of both in alphabetical order. Drug metabolites were excluded from this overview. Substrates are marked with a '1' whereas non-substrates indicated by a '0'. OCT1 substrates are additionally highlighted in blue and CYP2D6 substrates in green. We included only the substances where the status for both proteins was known. The complete list is provided in Table S1. Abbreviations: m-CPP, meta-Chlorophenylpiperazine; DMPP, Dimethylphenylpiperazinium; MDMA, 3,4-Methyl enedioxy methamphetamine; PMA, para-Methoxyamphetamine; PMMA, para-Methoxymethylamphetamine.

As illustrated in Fig. 3, dual substrates of OCT1 and CYP2D6 were distributed over the entire range of OCT1 transport activities. The CYP2D6 substrates showing the highest OCT1 transport were glycopyrrolate and dimethylpiperazinium. One-third of moderate and poor substrates of OCT1 were also CYP2D6 substrates, whereas excellent OCT1 substrates or non-substrates were less frequently CYP2D6 substrates (Fig. 3). In this figure, we also showed the transport ratios below unity. However here, we did not further analyse the mechanisms behind that, which might in some instances be the random scatter, but in other instances, OCT1-mediated efflux transport.

Fig. 2 illustrates that most substances were either OCT1- or CYP2D6 exclusive substrates. To further analyze the reasons for that, we compared chemical descriptors between substrates of OCT1 and substrates of CYP2D6. As shown in Fig. 4A, the main chemical difference between OCT1 and CYP2D6 substrates is their polarity. OCT1 substrates are, on average, by more than two orders of magnitude more hydrophilic than CYP2D6 substrates. Additionally, OCT1 substrates are smaller and more flexible, as indicated by lower molecular weight and a lower intramolecular ring count, respectively (Fig. 4B+C). Also, other properties related to the $logD_{pH7.4}$ value, such as the topological surface area and the number of H-bond donors, illustrate that OCT1 substrates are more polar than CYP2D6 substrates (Fig. 4D–F). Not unexpectedly, the shared substrates of both proteins were characterized by an intermediate polarity as they tended to be more lipophilic than the specific OCT1 substrates but still clearly more hydrophilic than the exclusive CYP2D6 substrates. Besides this, our analysis confirmed that both proteins mainly accept substrates which carry at least one positive charge at pH 7.4 (Fig. 4G). Overall, only 4 and 49 substrates of OCT1 or CYP2D6, respectively, were uncharged at the physiological pH of 7.4. The surprisingly high number of uncharged CYP2D6 substrates is partially caused by zwitterionic substances and substances, which are characterized by a basic pKa value close to but below 7.4. Both cases are counted here as uncharged molecules.

3.1. Depletion screening in single- and double-transfected cells

To test whether OCT1 and CYP2D6 indeed contribute to the respective substances's cellular disposition in a quantitatively relevant extent, we performed drug depletion screening using a relatively low substrate concentration of 1 μM and an extended incubation time of 90 min. With this experimental setup, we analyzed 47 substances (Fig. 5, Table 1). Those substances included all shared OCT1 and CYP2D6 substrates and additionally known CYP2D6 substrates which showed only weak OCT1 transport (uptake ratio 1.5–2.0). Moreover, for comparison, we tested sumatriptan and amitriptyline as clinically relevant OCT1 or CYP2D6 index substrates, respectively.

For 9 substances (19 %), we observed combined effects of OCT1 and CYP2D6. However, for 11 (23 %) and 19 (40 %) substances, substrate depletion was only mediated by OCT1 or CYP2D6 alone, respectively. Exemplarily, substrate depletion of phenformin, umeclidinium and zolmitriptan was mediated only by OCT1 (Fig. 5A). Substrate depletion of carteolol, mexiletine, and viloxazine was mediated only by the action

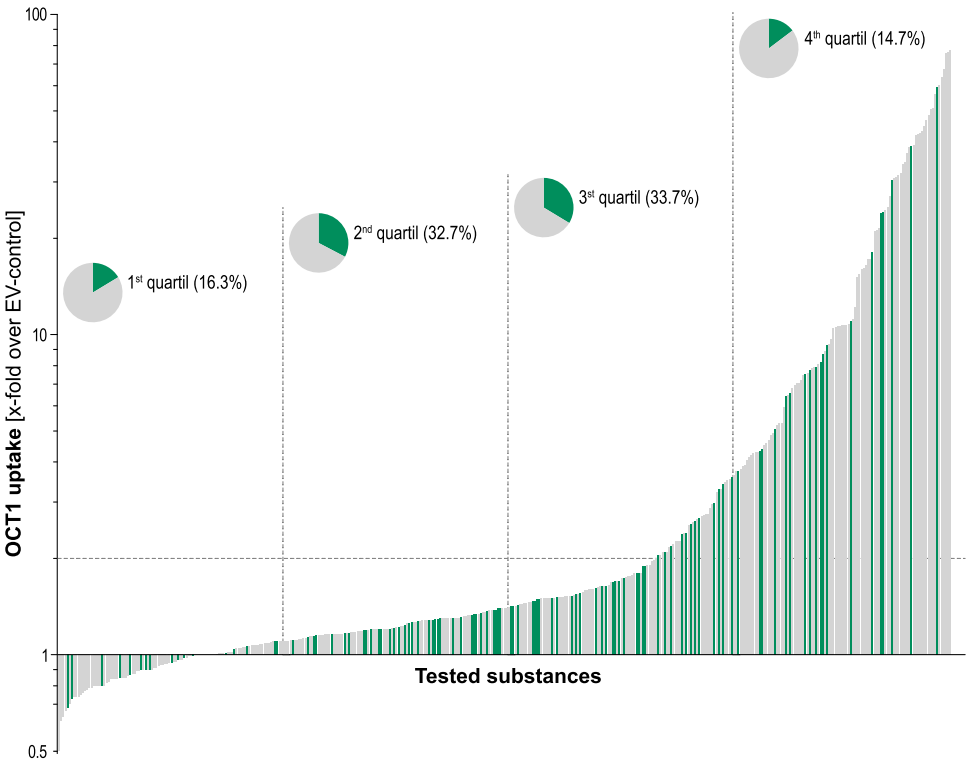

Fig. 3. OCT1 uptake activities. All data on OCT1 uptake activity (in total 414 substances) are shown in ascending order with increasing OCT1 activity; green bars indicate substances that were substrates of CYP2D6 based on our literature search. As can be seen, combined substrates were found with all OCT1 activities but were particularly frequent associated with very low to moderate OCT1 activities. The horizontal, dashed grey lines indicate the threshold applied in this study to define OCT1 substrates. For each quartile of the transport data, the proportion of literature-based CYP2D6 substrates is given in the corresponding pie charts. Concerning the classification and the numbers presented in the manuscript, please note that only good substrates of OCT1 with ratios above 2.0 were counted as OCT1 substrates.

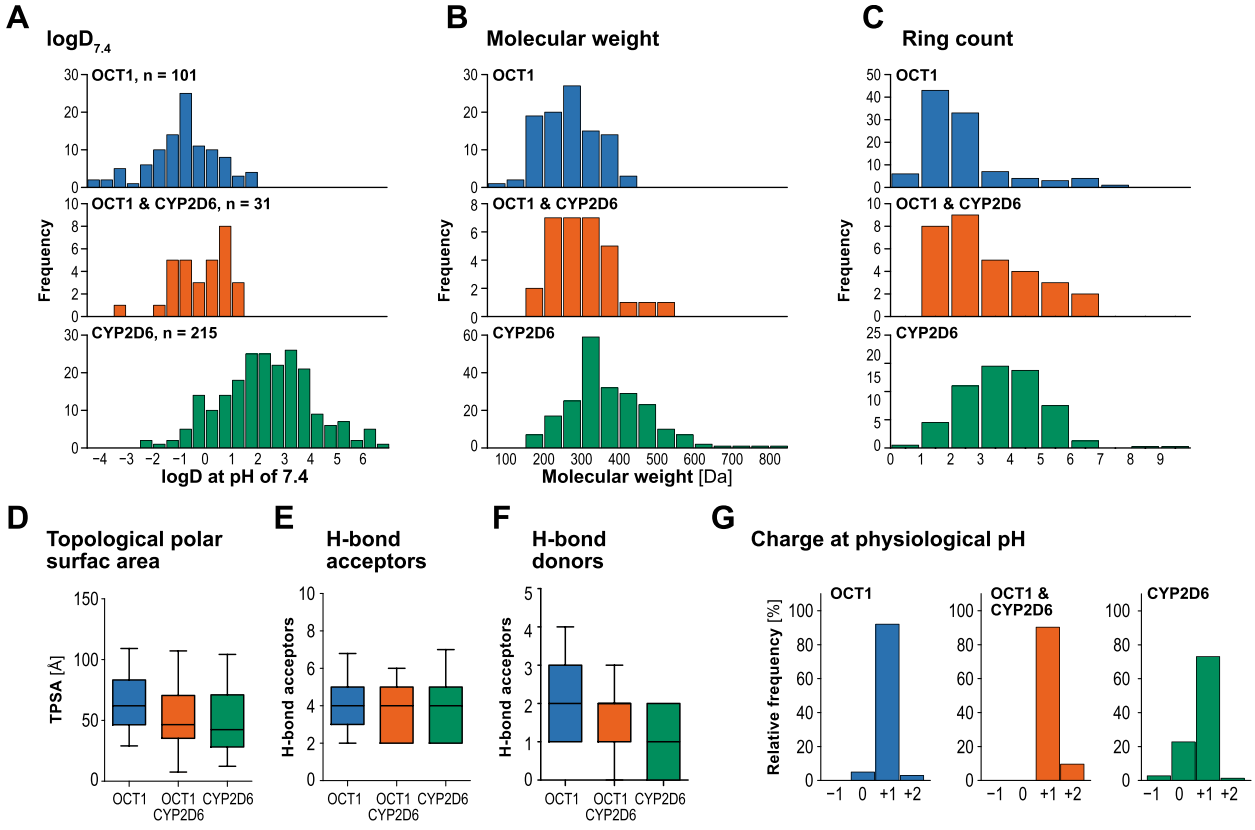

Fig. 4. Comparison of OCT1 and CYP2D6 substrates. We compared $logD_{pH7.4}$ values at physiologicial pH (A), molecular weight (B), the number of rings (C), the topological polar surface area (D), the number of H-bond acceptors (E) and donors (F), and the charge of the major molecular form at physiological pH (G). $logD_{pH7.4}$ values and the charge at pH 7.4 were calculated with cxcalc (ChemAxon, Budapest), whereas the other properties were calculated using the chemistry module of DataWarrior [40]. As seen, the majority of CYP2D6 substrates had a $logD_{pH7.4}$ value above 1.0, while most OCT1 substrates were more hydrophilic. Also, the molecular weight of typical OCT1 substrates was smaller, and the numbers of H-bound donors were significantly lower in OCT1 substrates.

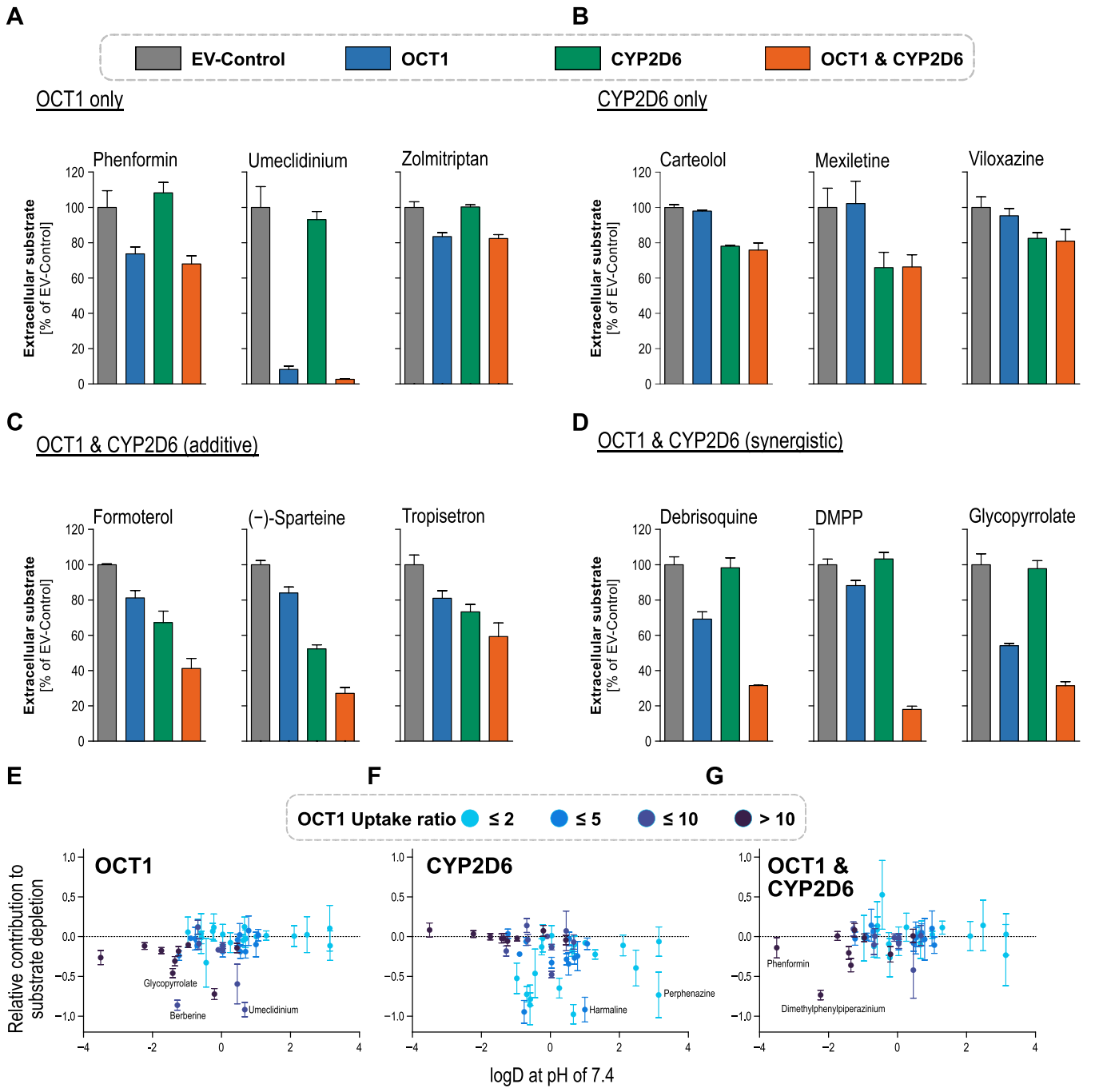

Fig. 5. Substrate-depletion screening of OCT1 and CYP2D6 substrates. HEK293 cells overexpressing OCT1, CYP2D6 and both OCT1/CYP2D6 and empty-vector transfected control cells were incubated with 1 μM test substance for 90 min. After this, the amount left over in the cellular supernatant was quantified by LC-MS/MS analysis. Results are shown as mean ± SEM of at least three independent experiments. Values were normalized to the EV-control cells. We observed only OCT1 (A) or only CYP2D6 (B) mediated effects. Substrates affected by both showed either a simple additive (C) or a synergistic effect (D) of OCT1 and CYP2D6. Data were also analyzed by multiple linear regression analysis to identify significant contributions of OCT1, CYP2D6 or both to substrate depletion (Table 1). The obtained coefficients are shown in relation to the polarity of the investigated substances as represented by the $logDpH_{7.4}$ values (E-G).

of CYP2D6 (Fig. 5B). Out of the 9 substances with a combined OCT1 and CYP2D6 effect, 4 showed a synergistic effect of both proteins (Table1). Berberine, formoterol, both sparteine enantiomers and tropisetron, showed additive effects of OCT1 and CYP2D6 (Fig. 5C). Synergistic (more than simple additive) effects of OCT1 and CYP2D6 were observed for debrisoquine, dimethylphenylpiperazinium, glycopyrrolate and oxyphenonium (Fig. 5D). Interestingly, relevant effects of OCT1 were mainly observed for substances with $logD_{pH7.4}$ values below 0 (Fig. 5E). Although there was no strong quantitative correlation (Fig. S2; $r = 0.36$), there was a good agreement between the uptake ratios, which are usually determined with short incubation periods of 2 min, and the here presented effects of OCT1 after 90 min of incubation. Notably, substances with low uptake ratios (1.5 – 2.0) also showed no relevant OCT1 effect after 90 min of incubation. For CYP2D6, a relevant contribution to substrate depletion was observed for more lipophilic substances, which might be explained by the fact that only lipophilic substances can passively enter the cell efficiently (Fig. 5F). Remarkably, more than additive effects of OCT1 and CYP2D6 were only observed for highly polar substances (Fig. 5G).

Table 1
Substrate depletion of tested OCT1/CYP2D6 substrates.

Drug	$logD_{pH7.4}$	OCT1 Uptake ratio	Remaining extracellular substrate, mean ± SEM [relative to EV-control in %]								
			OCT1			CYP2D6			OCT1 & CYP2D6		
No nominal net effects											
Acebutolol	− 0.68	5.20	111.8	±	2.6	113.9	±	4.5	115.5	±	6.1
Almotriptan	− 0.65	8.20	91.1	±	3.8	96.1	±	5.3	87.6	±	5.9
Cevimeline	− 0.22	1.89	111.5	±	12.9	97.7	±	12.1	82.8	±	13.6
Desvenlafaxine	1.07	3.28	101.8	±	5.9	91.0	±	3.4	82.1	±	4.9
Hydromorphone	0.04	2.00	102.7	±	8.9	101.2	±	9.4	97.8	±	7.0
Ketamine	3.15	1.49	88.6	±	15.0	93.7	±	14.7	85.1	±	7.9
Mirabegron	0.70	1.70	97.2	±	7.4	89.7	±	6.4	91.0	±	9.6
Ondansetron	2.11	1.61	97.5	±	7.7	90.6	±	7.6	89.9	±	7.5
OCT1 only effect											
Aclidinium	0.46	7.76	**40.5**	**±**	**10.0***	107.2	±	33.4	5.6	±	1.8
Edrophonium	− 0.96	42.6	**89.7**	**±**	**0.6****	97.9	±	3.0	86.2	±	2.8
Frovatriptan	− 1.74	30.3	**82.4**	**±**	**3.1****	99.8	±	0.0	83.0	±	2.0
Phenformin	− 3.50	24.0	**73.7**	**±**	**3.9***	108.2	±	5.9	68.0	±	4.7
Procainamide	− 0.70	4.41	**89.3**	**±**	**3.3***	95.6	±	1.2	90.6	±	2.4
Ranitidine	0.45	18.1	**85.7**	**±**	**4.3***	95.7	±	3.8	82.2	±	4.9
Rucaparib	0.55	2.61	**81.1**	**±**	**3.3***	97.2	±	9.2	77.8	±	5.4
Sumatriptan	− 1.24	24.4	**81.7**	**±**	**5.6***	94.3	±	3.5	84.5	±	4.5
para-Tyramine	− 1.23	2.98	**76.1**	**±**	**5.9****	103.6	±	2.7	77.1	±	4.0
Umeclidinium	0.68	7.52	**8.3**	**±**	**1.8*****	93.1	±	4.5	2.7	±	0.3
Zolmitriptan	− 0.09	9.27	**83.4**	**±**	**2.3*****	100.3	±	1.2	82.4	±	2.2
CYP2D6 only effect											
Amitriptyline	2.48	1.21	103.4	±	8.2	**57.2**	**±**	**3.8***	70.6	±	12.1
Bicifidine	− 0.68	1.64	112.2	±	10.7	**27.5**	**±**	**3.1*****	27.5	±	0.5
Broforamine	0.26	1.51	92.4	±	8.2	**35.3**	**±**	**0.5*****	39.6	±	9.2
Bunitrolol	− 0.58	1.64	98.9	±	8.3	**21.3**	**±**	**7.1*****	33.8	±	2.1
Carteolol	− 0.89	3.41	98.0	±	0.4	**78.1**	**±**	**0.5*****	75.9	±	4.0
meta-Chlorophenyl-piperazine	0.67	1.56	112.4	±	14.6	**2.4**	**±**	**0.4*****	4.5	±	0.4
Dexfenfluramine	0.79	2.04	107.5	±	12.0	75.5	±	12.0	79.7	±	4.0
Flecainide	1.01	1.70	100.0	±	3.0	**92.2**	**±**	**1.9***	94.5	±	2.5
Harmaline	1.00	2.39	90.1	±	21.8	**8.3**	**±**	**0.5*****	8.7	±	0.7
MDMA	− 0.76	2.40	97.4	±	11.2	**5.4**	**±**	**0.6*****	17.2	±	0.6
Methylamphetamine	− 0.44	1.50	67.3	±	19.4	53.7	±	12.9	73.6	±	18.7
Metoclopramide	− 0.25	1.80	106.7	±	6.5	87.1	±	6.4	84.6	±	7.4
Mexiletine	0.53	4.31	102.2	±	12.7	**65.9**	**±**	**8.7***	66.3	±	6.9
Perphenazine	3.14	1.80	110.5	±	25.7	**26.8**	**±**	**16.2***	0.140	±	0.072
PMMA	− 0.59	1.70	103.9	±	10.3	**14.2**	**±**	**1.9****	26.8	±	3.0
Remoxipride	1.30	1.53	101.3	±	2.5	**77.6**	**±**	**1.4****	90.2	±	6.1
Tamsulosin	0.47	2.56	96.1	±	2.6	**71.5**	**±**	**5.1*****	59.2	±	7.3
Timolol	− 0.97	1.54	105.6	±	18.6	**47.6**	**±**	**6.7***	51.3	±	0.3
Viloxazine	0.63	1.48	95.2	±	4.2	**82.3**	**±**	**3.4***	80.9	±	6.6
OCT1 and CYP2D6 additive effects											
Berberine	− 1.28	8.67	**13.8**	**±**	**1.8*****	**81.8**	**±**	**4.3****	5.3	±	0.6
Formoterol	0.04	2.67	**81.2**	**±**	**4.2****	**67.2**	**±**	**6.4*****	41.3	±	5.6
(−)-Sparteine	0.04	6.43	**84.0**	**±**	**3.4****	**52.3**	**±**	**2.3*****	27.2	±	3.3
(+)-Sparteine	0.04	7.94	**88.9**	**±**	**1.7***	**86.7**	**±**	**2.5****	74.4	±	0.4
Tropisetron	0.70	2.86	**81.0**	**±**	**4.2***	**73.3**	**±**	**4.2****	59.3	±	7.8
OCT1/CYP2D6 synergistic effects											
Debrisoquine	− 1.35	11.1	**69.2**	**±**	**4.1*****	98.2	±	5.6	**31.5**	**±**	**0.2****
Dimethylphenylpiperazinium	− 2.23	38.9	**88.2**	**±**	**2.9***	103.2	±	3.8	**18.0**	**±**	**1.8*****
Glycopyrrolate	− 1.41	59.1	**54.1**	**±**	**1.3*****	97.7	±	4.5	**31.4**	**±**	**2.3***
Oxyphenonium	− 0.20	24.2	**27.8**	**±**	**3.9*****	107.3	±	5.6	**12.8**	**±**	**2.9***

The drop of substrate concentrations and significance of the effects of OCT1 and CYP2D6 were analyzed by multiple linear regression analysis. Statistical significant factors are printed in bold and significance is indicated by asterix with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: MDMA, 3,4-Methyl enedioxy methamphetamine; PMMA, para-Methoxy-*N*-methylamphetamine.

3.2. Time-dependent depletion in double-transfected cells

For more detailed transport kinetic analysis, we analyzed the time-dependence of substrate depletion in single and double-transfected cells. This was performed with debrisoquine, (−)-sparteine, (+)-sparteine (pachycarpine), and amitriptyline. For debrisoquine, we also analyzed the formation of the metabolite 4-hydroxy-debrisoquine, and for amitriptyline, the formation of its three metabolites nortriptyline, 10-hydroxy amitriptyline and 10-hydroxy nortriptyline.

The experiments with debrisoquine confirmed the necessity for active uptake as a prerequisite for efficient biotransformation (Fig. 6A). Notably, depletion of the hydrophilic debrisoquine in the cell supernatant of CYP2D6-single transfected cells did not significantly differ from the empty-vector control cells. Moreover, after 90 min of incubation, the intracellular concentration of 4-hydroxydebrisoquine was 66-fold higher in the OCT1/CYP2D6-overexpressing cells as compared to the single CYP2D6-transfected ones. As 4-hydroxy-debrisoquine is even about tenfold more hydrophilic than debrisoquine, the emergence of 4-hydroxy-debrisoquine in the cell culture medium suggest OCT1-mediated outward transport of the metabolite (Fig. 6A). For sparteine, the substrate depletion in the cellular supernatant of CYP2D6 single-transfected cells was much stronger as compared to debrisoquine. This corresponds to its higher lipophilicity (sparteine $logDpH_{7.4}$ 0.04 versus −1.35 for debrisoquine) and thereby enhanced diffusion. Pachycarpine, the (+)-enantiomer to sparteine, showed a weaker depletion. That was apparently due to the stereoselectivity of CYP2D6 metabolism, but not

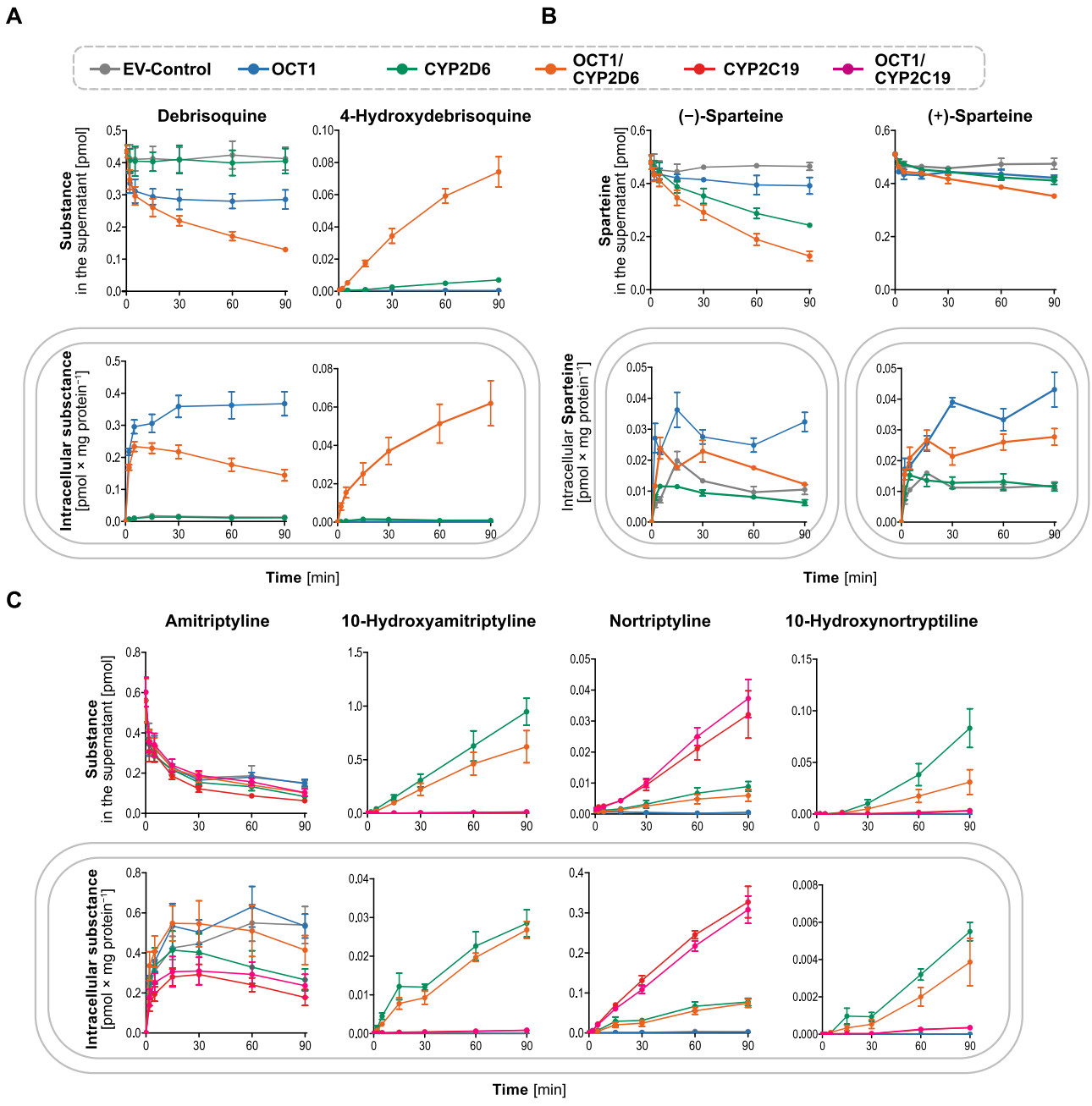

Fig. 6. Time-dependent uptake and metabolism of debrisoquine (A), two sparteine enantiomers (B) and amitriptyline (C). HEK293 cells overexpressing OCT1, CYP2D6, OCT1/CYP2D6, CYP2C19 or OCT1/CYP2C19 as well as empty vector (EV)-transfected controls were incubated with 1 μM substance over a time period of 90 min. Intracellular and extracellular drug and metabolite concentrations were quantified after 2, 5, 15, 30, 60 and 90 min. Data is presented as mean ± SEM of three independent experiments.

OCT1-mediated transport (Fig. 6B). Amitriptyline was tested in this system to elucidate if double transfection might reveal OCT1 mediated net influx transport of such hydrophobic drugs. Amitriptyline is metabolized to nortriptyline via CYP2C19, and CYP2D6 metabolizes both to either 10-hydroxy-amitriptyline or 10-hydroxy-nortriptyline. As seen in Fig. 6C, the joint effects of OCT1/CYP2D6 or OCT1/CYP2C19 did not exceed effects in single-transfected cells only expressing CYP2D6 or CYP2C19.

3.3. Shared inhibitors of OCT1 and CYP2D6

Drug-drug interactions may be due to interactions at membrane transport and interactions at intracellular biotransformation. Thus, we studied combined effects of transport and metabolic interactions using the cell system described here. Generally, one is interested in differentiating drug-drug interactions based on inhibition of transport versus inhibition of drug metabolism. With substances inhibiting both, membrane transport and metabolism, it is an interesting question whether there are antagonistic, additive or more-than-additive effects.

Concerning substrate properties, we identified polarity as the major difference between OCT1 and CYP2D6 substrates. According to earlier observations, OCT1 inhibitors are generally more lipophilic than its substrates. By comparison of OCT1 and CYP2D6 inhibitors, we observed indeed no major differences in the $\log D_{pH7.4}$ (Fig. S3).

Sparteine was chosen as a test substrate for the inhibition studies because it showed OCT1-mediated uptake, but also sufficient uptake in cells not expressing OCT1 (Fig. 7), so inhibition of OCT1 alone and inhibition of CYP2D6 alone could be shown. We used fluoxetine,

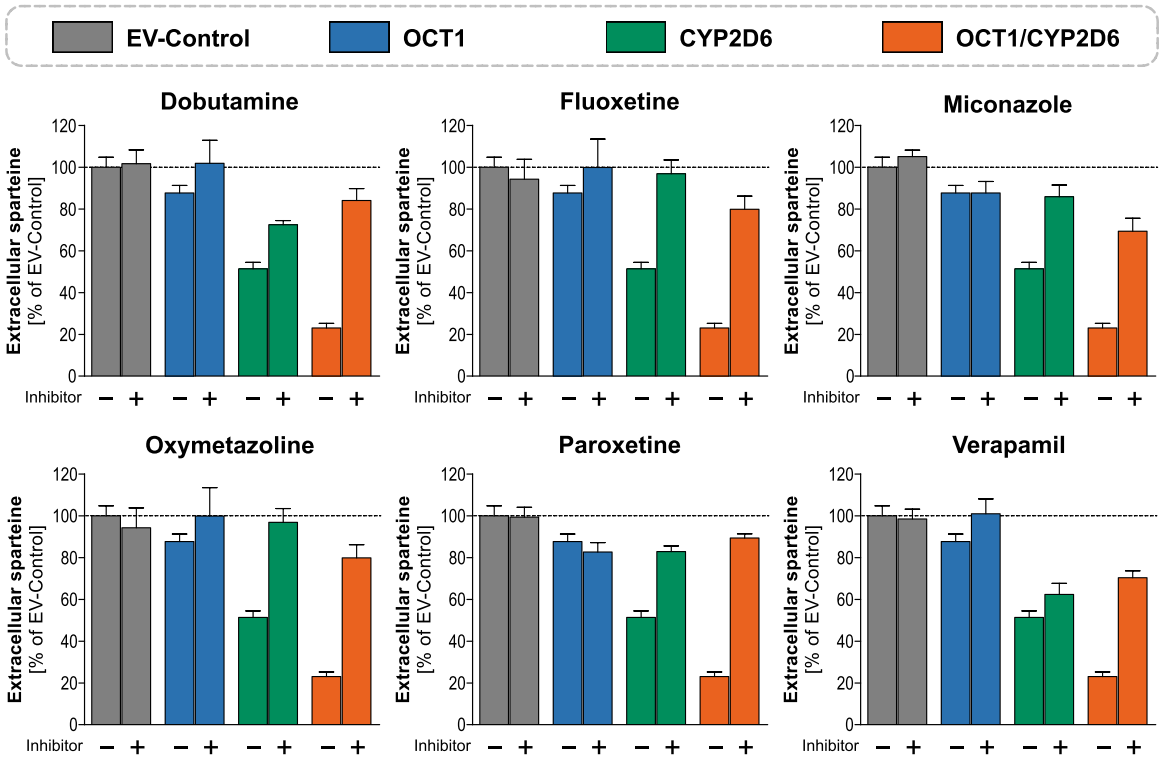

Fig. 7. Exploration of the double-transfected cell system for inhibition screening. HEK293 cells overexpressing OCT1, CYP2D6 or OCT1/CYP2D6, as well as empty vector (EV)-transfected controls, were incubated with 1 μM sparteine in the presence (+) or absence (−) of 20 μM inhibitor for 90 min. Sparteine, which was found to be additively dependent on both, OCT1 and CYP2D6, was used here as a probe drug. Extracellular sparteine was quantified after the incubation period and was normalized to the leftover in the supernatant of the empty-vector transfected control cells. Data are presented as mean ± SEM of three independent experiments.

oxymetazoline, paroxetine and verapamil as presumably shared inhibitors whereas dobutamine and miconazole as OCT1 or CYP-specific inhibitors, respectively. As sparteine was chosen because of its sufficient passive uptake for showing inhibition effects in CYP2D6-only expressing cells, it was not surprising that OCT1 inhibition effects were not significant. Nevertheless, all substances except miconazole and paroxetine showed reduced extracellular probe-substrate depletion by OCT1 (Fig. 7). CYP2D6 was inhibited most strongly by fluoxetine and oxymetazoline. The effects of all six inhibitors were always most strongly in the OCT1&CYP2D6 double-transfected cells.

4. Discussion and conclusions

OCT1 and CYP2D6 substrates share many molecular features like a positively charged nitrogen, an aromatic ring, and a molecular weight between 150 and 600. Therefore, we expected substantial overlap between OCT1 and CYP2D6 substrates. However, only 31 (12.6 %) of the 246 CYP2D6 substrates were also OCT1 substrates. Lipophilicity is the most frequent distinguishing feature between OCT1 and CYP2D6 substrates. Because of the high frequency of functionally relevant genetic variation in both genes, particularly the relatively small subset of drugs affected by both OCT1 and CYP2D6 may cause problems in individual dosing. One might even speculate that double-affected drugs are relatively rare because double-affected drugs are often less well tolerated. However, there is no proof for that hypothesis, and thus far, the medical relevance of combined OCT1 and CYP2D6 deficiency has only rarely been studied clinically.

By coincidence, both drugs, which resulted in the discovery of the CYP2D6 polymorphism, sparteine, and debrisoquine, are also affected by the OCT1 polymorphism. These two drugs are nowadays clinically irrelevant, but other joint OCT1/CYP2D6 substrates are most relevant. For instance, formoterol is among the most frequently used drugs in asthma and COPD. The combined effects of OCT1 and CYP2D6 polymorphisms may affect the systemic cardiovascular adverse effects of formoterol, mediating systemic blood concentrations, but this has not yet been studied clinically. A relatively large proportion of pulmonary drugs, both adrenergic drugs and anticholinergic drugs, are substrates of OCT1 or combined substrates of OCT1 and CYP2D6 (Fig. 8). The antiemetic drug tropisetron was already identified earlier as a substrate of OCT1 and CYP2D6 [44]. Clinically, the effect of OCT1 polymorphisms on tropisetron pharmacokinetics was moderate only. That may correspond to the only moderate OCT1-dependent substrate depletion consistent with only minor effects seen in OCT1 knock-out mice [45]. However, the combined activities of OCT1 and CYP2D6 resulted in 50 % substrate depletion (Table 1).

Another area where several substrates of both OCT1 and CYP2D6, are applied in drug therapy is neurology. Here, two anti-migraine triptans are affected by both OCT1 and CYP2D6. Fenfluramine was initially used as an appetite suppressant but exhibited numerous adverse events, including drug-induced valvular heart disease [46]. One may hypothesise that disposition to this blood-concentration-dependent adverse event is related to polymorphisms in OCT1 or CYP2D6 or even more to double-deficiency, but that was not yet analysed. In the original formulation, fenfluramine was combined with phentermine, but phentermine is only a weak OCT1 substrate (uptake ratio of 1.9). Nowadays, fenfluramine is used only in relatively rare specific types of epilepsy. The commonly circulated effect of CYP2D6 on phenformin [47] could not be confirmed and also the CYP2D6 effect on zolmitriptan [48] could not be confirmed here (Table 1). However, this may be compatible with the fact that CYP2D6 is not mediating the main pathway of elimination and substrate depletion assays are not very sensitive in detecting minor metabolic pathways.

The only double-substrate for which thus far genetic polymorphisms in OCT1 and CYP2D6 have been studied clinically, was tamsulosin, a drug frequently used in benign prostatic hyperplasia. Clinically, significant effects of OCT1 and CYP2D6 polymorphisms on pharmacokinetic

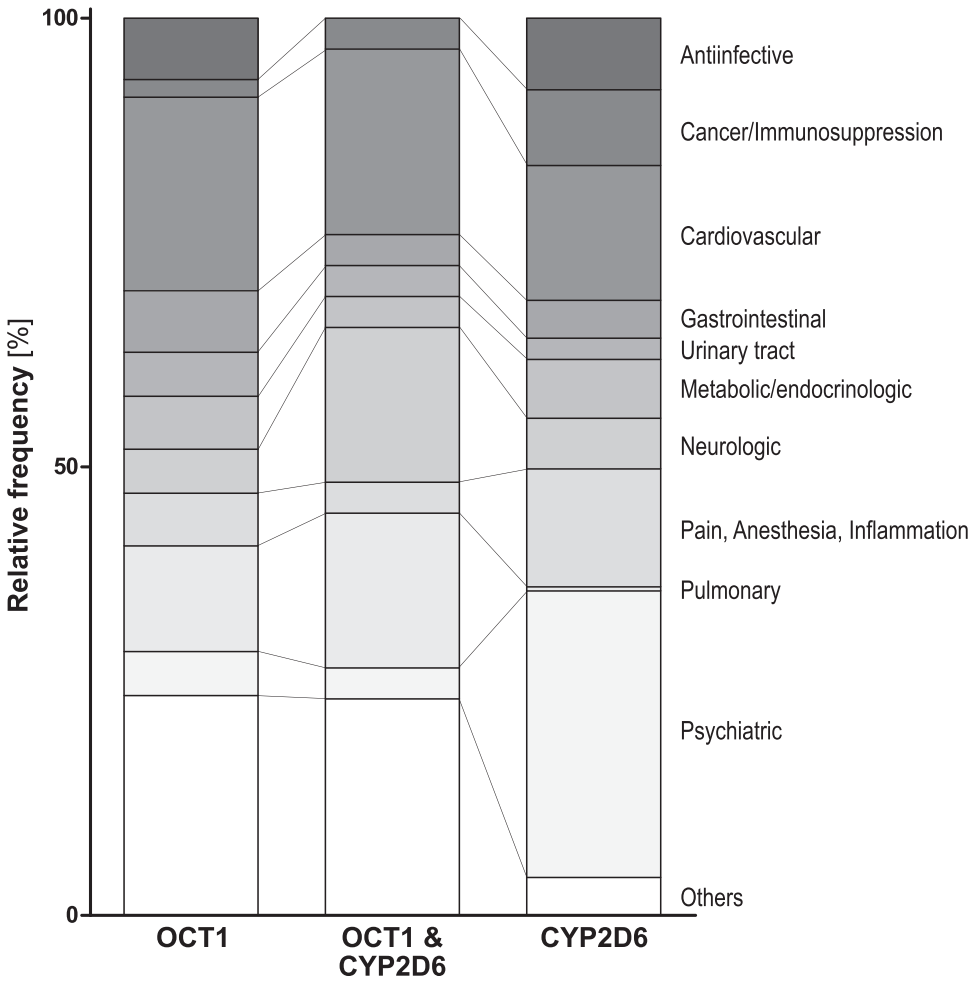

Fig. 8. Proportion of OCT1 and CYP2D6 substrates in the different fields of drug therapy. The left and right columns contain the exclusive OCT1 and CYP2D6 substrates and whereas the shared substrates are represented by the middle column. Singly drugs are listed in Fig. 2 and supplementary table S1. As seen, combined OCT1 and CYP2D6 substrates are particularly used in pulmonary, neurological and cardiovascular disorders. On the other hand, in psychiatric and pain treatment, drugs were preferentially only CYP2D6 substrates.

parameters were found [49]. With our double-transfected cell system, only the CYP2D6 effect on tamsulosin depletion was statistically significant, but nominally, also the OCT1 effect could be seen (Table 1) which supports the idea that this assay can correctly predict the clinical reality.

The non-shared OCT1 and CYP2D6 substrates differed a little in their size and molecular flexibility, as indicated by molecular weight and the ring count (Fig. 4). However, the major difference is polarity. Whereas almost all OCT1 substrates are relatively hydrophilic with a $logD_{pH7.4}$ below 1.5, many CYP2D6 substrates are highly lipophilic. Lower lipophilicity in OCT1 substrates might be viewed as experimental artifact because more lipophilic substances will show higher passive diffusion that may mask active transport. Additionally, it is possible that the *in vitro* assay is less sensitive to active transport of lipophilic compounds. Indeed, using other experimental conditions than we used, transport of similarly lipophilic substances could be demonstrated [50]. On the other side, preference for relatively hydrophilic substrates might simply be a property of OCT1. The known upper limit of good OCT1 substrates was about 400 Dalton (Fig. S4, see also [51]), whereas numerous CYP2D6 substrates had a molecular weight above 400 Dalton. According to the earlier distinction as so-called type-I and type-II organic cation transport processes, many of the organic cations larger than 400 Dalton were suggested to be substrates of OATPs [52,53] However, this hypothesis about organic cations as substrates of OATPs is not yet confirmed by a larger substrate screening.

An obvious question is whether the similarities in the substrate spectrum correspond to similarities in the molecular structure. As shown in the supplementary data (Fig. S5), CYP2D6 has a relatively large hydrophobic binding pocket capable of receiving larger hydrophobic moieties like the phenothiazine structure of thioridazine. CYP2D6 substrates, which are also accepted by OCT1 such as debrisoquine, only have smaller hydrophobic residues but may still similarly well fit into the hydrophobic binding pocket of CYP2D6 as illustrated in Fig. S5.

As shown previously [29], OCT1 inhibitors are much more lipophilic than its substrates. One explanation for that might be that with increasing lipophilicity of OCT1 substrates their affinity increases. This reduces the ability to dissociate rapidly from the transporter once switched to the intracellular site. Indeed, lipophilicity of OCT1 substrates seems to correlate with OCT1 inhibition (Fig. S6). While the correlation was not especially strong ($r = 0.44$), it nevertheless supports the hypothesis. One starting point of the present investigation was the hypothesis, that there might be a common OCT1/CYP2D6-substrate pharmacophore, but with the data obtained in the present investigation we have to conclude that it was not unexpected that increasing lipophilicity changes compounds towards better CYP2D6 substrates and towards OCT1 inhibitors instead of OCT1 substrates. Increasing hydrophilicity on the other site improves OCT1 transport but reduces CYP2D6 metabolism corresponding to the common conception that CYP enzymes make lipophilic substances more hydrophilic. As OCT1 can also mediate drug efflux with a few selected substrates [54], efflux transport of CYP2D6-hydrophilized metabolites might be a relevant OCT1′s function as suggested by the 4-OH-debrisoquine data presented here.

An interesting observation by use of the double transfected cells was the phenomenon of interactions, i.e. the combined effect of both factors was different (mostly more) than expected from single effects of OCT1 and CYP2D6, respectively. To elucidate the mechanisms behind such

findings might require additional experiments. One mechanism behind the interaction of the three examples illustrated in Fig. 5 (debrisoquine, dimethylphenylpiperazinium, and glycopyrrolate) may obviously be the very poor membrane diffusion of quite hydrophilic substances. Theoretically, with 90-minutes incubations in transport experiments, one would expect significant efflux transport once the gradient between extra- and intra-cellular concentration has declined. Because intracellular metabolism would maintain that gradient, one might expect even more interactions. However, at least no immediately apparent effects of this type were found (Fig. 5, Table 1). This would support the concept that OCT1 does indeed not efficiently transport hydrophobic substrates and rarely acts as an efflux transporter [54]. Intracellular metabolism, as a possible factor reducing back-transport to the extracellular side of the cell, was also our motivation to perform the experiments with amitriptyline. However, also in the double-transfected cells, no OCT1 effect could be seen. This may indicate that hydrophobic drugs like amitriptyline and nortriptyline are indeed no OCT1 substrates [14] (Fig. 6) or that metabolism was too slow to modify the concentration-gradient in a relevant manner. From the data presented in Fig. 6, one would expect that the CYP2D6 genetic polymorphism is relevant for debrisoquine only in carriers of active OCT1. However – and that is not reflected in the double transfected cell system – debrisoquine is also a good substrate of OCT2 and OCT3 [55]. Thus, with debrisoquine other transporters may mediate membrane transport in OCT1 deficient subjects.

Although about 15 % of OCT1 substrates undergoes some metabolism by CYP2D6, the majority of OCT1 substrates, especially most of the substances showing very high rates of transport, were not CYP2D6 substrates (Fig. 2). Since several small and polar OCT1 substrates are primary amines and in addition, several also carry hydroxyl groups, it is not surprising that several of these are metabolized by monoamine oxidases (MAO) and by the catechol-O-methyl transferase (Fig. 9). These include monoamine neurotransmitter-related substances such as phenylephrine and synephrine. Because MAO is expressed in many cell types beyond the liver, the hepatic OCT1 may often not be rate-limiting for metabolic inactivation by MAO. However, with the MOA-substrate sumatriptan, a clear pharmacokinetic effect of OCT1 could be seen [56].

Alternative metabolic pathways of primary amines or hydroxylated substances are glucuronidation and sulfation. A large fraction, including several beta-agonists such as fenoterol, pirbuterol and salbutamol, undergo direct conjugation to glucuronides or sulphated metabolites without prior oxidation by CYP enzymes. Finally, the fraction of highly polar OCT1 substrates is excreted unchanged without metabolism. These include, for instance, the antidiabetic drugs imeglemin and metformin. Among the cytochrome-P450 enzymes, CYP2D6 seems to be most commonly involved in the metabolism of OCT1 substrates but also CYP1A2, – 2C19, and – 3A4 are involved. CYP1A2 and CYP2C19 are also primarily hepatic expressed, so it is not unlikely that OCT1 is also a rate-limiting transport protein for some CYP1A2 and CYP2C19 substrates.

Although we used relatively low substrate concentrations, extrapolation to the mostly even lower concentrations seen in human patients may not be valid. However, as seen in the example of fenoterol, the *in vitro* to *in vivo* extrapolation was very good [57].

In conclusion, there is some overlap between OCT1 and CYP2D6 substrates. However, the majority of CYP2D6 substrates are too hydrophobic to be good OCT1 substrates, and the majority of OCT1 substrates undergo other metabolic pathways or are not metabolized at all. Nevertheless, about 15 % of OCT1 substrates also affected by CYP2D6 may cause particular problems in persons with a genetic deficiency in both proteins, which should be studied clinically. As a first step in this direction, the 90-minutes substrate depletion assay with single and double transfected cells described here may be suitable to filter out the drugs where really both OCT1 and CYP2D6 do play a relevant role. The assay indicated if there are strong additive or complex more-than-additive interactions. Additionally, OCT1 and CYP2D6 activities are affected by single and shared inhibitors, and this may even aggravate interindividual variation. So far, there are only a few clinically widely relevant overlapping substrates of OCT1 and CYP2D6. However, there might be more in the future, and those could be relevantly affected either by reduced-function variants or by the many clinically relevant common inhibitors of OCT1 and CYP2D6. The clinical pharmacokinetic pendant to those effects seen by double action (Fig. 5) and double inhibition (Fig. 7) deserves further studies in humans looking at both the effects of genetic variation and the effects of inhibitors in OCT1 and CYP2D6. As illustrated by the present data, studies in double transfected model cell systems with transporters and metabolizing enzymes may be a valuable additional tool in preclinical drug development.

CRediT authorship contribution statement

Lukas Gebauer: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing, Visualization. **Christof Dücker:** Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Ole Jensen:** Investigation, Methodology. **Jürgen Brockmöller:** Conceptualization, Funding acquisition, Project administration, Resources, Writing –

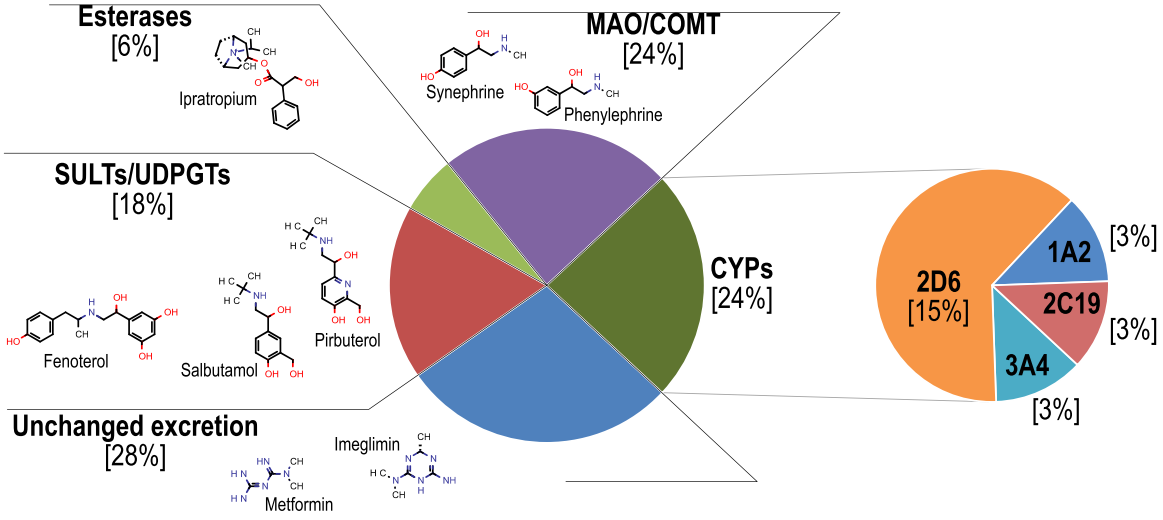

Fig. 9. Metabolism of OCT1 substrates with high in-vitro transport rates. This figure is based on available data on the metabolism of OCT1 substrates characterized by an uptake ratio of at least three (Table S1). COMT, Catechol-O-methyltransferase; MAO, monoamine oxidase; SULTs, sulfotransferases; UDPGTs, Uridine 5′-diphospho-glucuronosyltransferase.

original draft, Writing – review & editing.

Conflict of Interest Statement

The authors declare that they do not have any competing financial interests or any other conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgements

This project was funded in part by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 437446827. We thankfully acknowledge Ellen Bruns for her support with HPLC-MS/MS measurements. We thank ChemAxon, Budapest, Hungary, for generously providing the academic license for cxcalc™. Furthermore, we want to thank everyone who contributed to the open-source software that was used, including RDKit, Matplotlib, and pandas.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114454.

References

[1] M.D. Pizzagalli, A. Bensimon, G. Superti-Furga, A guide to plasma membrane solute carrier proteins, FEBS J. 288 (9) (2021) 2784–2835.
[2] V.J. Wacher, C.Y. Wu, L.Z. Benet, Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy, Mol. Carcinog. 13 (3) (1995) 129–134.
[3] S.V. Vormfelde, M.R. Toliat, M. Schirmer, I. Meineke, P. Nürnberg, J. Brockmöller, The polymorphisms Asn130Asp and Val174Ala in OATP1B1 and the CYP2C9 allele *3 independently affect torsemide pharmacokinetics and pharmacodynamics, Clin. Pharmacol. Ther. 83 (6) (2008) 815–817.
[4] J. Kirchheiner, D. Kudlicz, C. Meisel, S. Bauer, I. Meineke, I. Roots, J. Brockmöller, Influence of CYP2C9 polymorphisms on the pharmacokinetics and cholesterol-lowering activity of (−)-3S,5R-fluvastatin and (+)-3R,5S-fluvastatin in healthy volunteers, Clin. Pharmacol. Ther. 74 (2) (2003) 186–194.
[5] D. Douguet, Data sets representative of the structures and experimental properties of FDA-approved drugs, ACS Med. Chem. Lett. 9 (3) (2018) 204–209.
[6] P.S. Charifson, W.P. Walters, Acidic and basic drugs in medicinal chemistry: a perspective, J. Med Chem. 57 (23) (2014) 9701–9717.
[7] G.R. Wilkinson, Drug metabolism and variability among patients in drug response, N. Engl. J. Med. 352 (21) (2005) 2211–2221.
[8] P. Rowland, F.E. Blaney, M.G. Smyth, J.J. Jones, V.R. Leydon, A.K. Oxbrow, C. J. Lewis, M.G. Tennant, S. Modi, D.S. Eggleston, R.J. Chenery, A.M. Bridges, Crystal structure of human cytochrome P450 2D6, J. Biol. Chem. 281 (11) (2006) 7614–7622.
[9] A. Wang, C.D. Stout, Q. Zhang, E.F. Johnson, Contributions of ionic interactions and protein dynamics to cytochrome P450 2D6 (CYP2D6) substrate and inhibitor binding, J. Biol. Chem. 290 (8) (2015) 5092–5104.
[10] S.W. Ellis, G.P. Hayhurst, G. Smith, T. Lightfoot, M.M. Wong, A.P. Simula, M. J. Ackland, M.J. Sternberg, M.S. Lennard, G.T. Tucker, et al., Evidence that aspartic acid 301 is a critical substrate-contact residue in the active site of cytochrome P450 2D6, J. Biol. Chem. 270 (49) (1995) 29055–29058.
[11] U.M. Zanger, J. Fischer, S. Raimundo, T. Stuven, B.O. Evert, M. Schwab, M. Eichelbaum, Comprehensive analysis of the genetic factors determining expression and function of hepatic CYP2D6, Pharmacogenetics 11 (7) (2001) 573–585.
[12] U.M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation, Pharm. Ther. 138 (1) (2013) 103–141.
[13] J. Kirchheiner, H. Schmidt, M. Tzvetkov, J.T. Keulen, J. Lötsch, I. Roots, J. Brockmöller, Pharmacokinetics of codeine and its metabolite morphine in ultra-rapid metabolizers due to CYP2D6 duplication, Pharm. J. 7 (4) (2007) 257–265.
[14] J. Matthaei, J. Brockmöller, W. Steimer, K. Pischa, S. Leucht, M. Kullmann, O. Jensen, T. Ouethy, M.V. Tzvetkov, M. Rafehi, Effects of genetic polymorphism in CYP2D6, CYP2C19, and the organic cation transporter OCT1 on amitriptyline pharmacokinetics in healthy volunteers and depressive disorder patients, Front. Pharmacol. 12 (2021), 688950.
[15] J. Matthaei, J. Brockmöller, M.V. Tzvetkov, D. Sehrt, C. Sachse-Seeboth, J. B. Hjelmborg, S. Möller, U. Halekoh, U. Hofmann, M. Schwab, R. Kerb, Heritability of metoprolol and torsemide pharmacokinetics, Clin. Pharmacol. Ther. 98 (6) (2015) 611–621.
[16] M. Ingelman-Sundberg, Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity, Pharm. J. 5 (1) (2005) 6–13.
[17] C. Dücker, J. Brockmöller, How precise is quantitative prediction of pharmacokinetic effects due to drug-drug interactions and genotype from in vitro data? A comprehensive analysis on the example CYP2D6 and CYP2C19 substrates, Pharm. Ther. 217 (2021), 107629.
[18] J. Kirchheiner, K. Nickchen, M. Bauer, M.L. Wong, J. Licinio, I. Roots, J. Brockmöller, Pharmacogenetics of antidepressants and antipsychotics: the contribution of allelic variations to the phenotype of drug response, Mol. Psychiatry 9 (5) (2004) 442–473.
[19] C. Neul, U. Hofmann, E. Schaeffeler, S. Winter, K. Klein, K.M. Giacomini, M. Eichelbaum, M. Schwab, A.T. Nies, Characterization of cytochrome P450 (CYP) 2D6 drugs as substrates of human organic cation transporters and multidrug and toxin extrusion proteins, Br. J. Pharmacol. 178 (6) (2021) 1459–1474.
[20] A.R. Saadatmand, S. Tadjerpisheh, J. Brockmöller, M.V. Tzvetkov, The prototypic pharmacogenetic drug debrisoquine is a substrate of the genetically polymorphic organic cation transporter OCT1, Biochem Pharm. 83 (10) (2012) 1427–1434.
[21] D.S. Wishart, Y.D. Feunang, A.C. Guo, E.J. Lo, A. Marcu, J.R. Grant, T. Sajed, D. Johnson, C. Li, Z. Sayeeda, N. Assempour, I. Iynkkaran, Y. Liu, A. Maciejewski, N. Gale, A. Wilson, L. Chin, R. Cummings, D. Le, A. Pon, C. Knox, M. Wilson, DrugBank 5.0: a major update to the DrugBank database for 2018, Nucleic Acids Res. 46 (D1) (2018) D1074–D1082.
[22] M. Kanehisa, M. Furumichi, Y. Sato, M. Ishiguro-Watanabe, M. Tanabe, KEGG: integrating viruses and cellular organisms, Nucleic Acids Res. 49 (D1) (2021) D545–D551.
[23] FDA, In vitro drug interaction studies - cytochrome P450 enzyme- and transporter-mediated drug interactions, 2020. ⟨https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm⟩.
[24] Flockhart D.A., Thacker, D., McDonald, C., Desta, Z. The Flockhart Cytochrome P450 Drug-Drug Interaction Table. Division of Clinical Pharmacology, Indiana University School of Medicine (Updated 2021). https://drug-interactions.medicine.iu.edu. Accessed 02/07/2022.
[25] S. Rendic, Summary of information on human CYP enzymes: human P450 metabolism data, Drug Metab. Rev. 34 (1–2) (2002) 83–448.
[26] H. Buzkova, K. Pechandova, O. Slanar, F. Perlik, Genetic polymorphism of cytochrome P450 and methods for its determination, Prague Med. Rep. 107 (4) (2006) 383–393.
[27] M.J. Meyer, V.E. Neumann, H.R. Friesacher, B. Zdrazil, J. Brockmöller, M. V. Tzvetkov, Opioids as substrates and inhibitors of the genetically highly variable organic cation transporter OCT1, J. Med Chem. 62 (21) (2019) 9890–9905.
[28] O. Jensen, J. Brockmöller, C. Dücker, Identification of novel high-affinity substrates of OCT1 using machine learning-guided virtual screening and experimental validation, J. Med Chem. 64 (5) (2021) 2762–2776.
[29] O. Jensen, L. Gebauer, J. Brockmöller, C. Dücker, Relationships between inhibition, transport and enhanced transport via the organic cation transporter 1, Int. J. Mol. Sci. 23 (4) (2022).
[30] R. Hendrickx, J.G. Johansson, C. Lohmann, R.M. Jenvert, A. Blomgren, L. Borjesson, L. Gustavsson, Identification of novel substrates and structure-activity relationship of cellular uptake mediated by human organic cation transporters 1 and 2, J. Med Chem. 56 (18) (2013) 7232–7242.
[31] L. Gebauer, O. Jensen, M. Neif, J. Brockmöller, C. Dücker, Overlap and specificity in the substrate spectra of human monoamine transporters and organic cation transporters 1, 2, and 3, Int. J. Mol. Sci. (2021).
[32] O. Jensen, M. Rafehi, L. Gebauer, J. Brockmöller, Cellular uptake of psychostimulants - are high- and low-affinity organic cation transporters drug traffickers? Front. Pharmacol. 11 (2020), 609811.
[33] Y.-a Bi, C. Costales, S. Mathialagan, M. West, S. Eatemadpour, S. Lazzaro, L. Tylaska, R.J. Scialis, H. Zhang, J. Umland, E. Kimoto, D.A. Tess, B. Feng, L. M. Tremaine, M.V.S. Varma, A.D. Rodrigues, Quantitative contribution of six major transporters to the hepatic uptake of drugs: "SLC-phenotyping" using primary human hepatocytes, J. Pharmacol. Exp. Ther. 370 (1) (2019) 72.
[34] G. Ahlin, J. Karlsson, J.M. Pedersen, L. Gustavsson, R. Larsson, P. Matsson, U. Norinder, C.A. Bergström, P. Artursson, Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1, J. Med Chem. 51 (19) (2008) 5932–5942.
[35] E.C. Chen, N. Khuri, X. Liang, A. Stecula, H.C. Chien, S.W. Yee, Y. Huang, A. Sali, K. M. Giacomini, Discovery of competitive and noncompetitive ligands of the organic cation transporter 1 (OCT1; SLC22A1), J. Med Chem. 60 (7) (2017) 2685–2696.
[36] S. Kim, J. Chen, T. Cheng, A. Gindulyte, J. He, S. He, Q. Li, B.A. Shoemaker, P. A. Thiessen, B. Yu, L. Zaslavsky, J. Zhang, E.E. Bolton, PubChem in 2021: new data content and improved web interfaces, Nucleic Acids Res. 49 (D1) (2021) D1388–d1395.
[37] H. Veith, N. Southall, R. Huang, T. James, D. Fayne, N. Artemenko, M. Shen, J. Inglese, C.P. Austin, D.G. Lloyd, D.S. Auld, Comprehensive characterization of cytochrome P450 isozyme selectivity across chemical libraries, Nat. Biotechnol. 27 (11) (2009) 1050–1055.
[38] O. Jensen, S. Ansari, L. Gebauer, S.F. Müller, K. Lowjaga, J. Geyer, M.V. Tzvetkov, J. Brockmöller, A double-Flp-in method for stable overexpression of two genes, Sci. Rep. 10 (1) (2020) 14018.
[39] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M. D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1) (1985) 76–85.

[40] T. Sander, J. Freyss, M. von Korff, C. Rufener, DataWarrior: an open-source program for chemistry aware data visualization and analysis, J. Chem. Inf. Model. 55 (2) (2015) 460–473.
[41] G. Landrum, RDKit: open-source cheminformatics software, 2019. ⟨https://www.rdkit.org/⟩.
[42] W. McKinney, Data structures for statistical computing in python, Proceedings of the 9th Python in Science Conference 445 (2010) 51–56.
[43] J.D. Hunter, Matplotlib: a 2D graphics environment, Comput. Sci. Eng. 9 (3) (2007) 90–95.
[44] M.V. Tzvetkov, A.R. Saadatmand, K. Bokelmann, I. Meineke, R. Kaiser, J. Brockmöller, Effects of OCT1 polymorphisms on the cellular uptake, plasma concentrations and efficacy of the 5-HT(3) antagonists tropisetron and ondansetron, Pharm. J. 12 (1) (2012) 22–29.
[45] B.L. Morse, A. Kolur, L.R. Hudson, A.T. Hogan, L.H. Chen, R.M. Brackman, G. A. Sawada, J.K. Fallon, P.C. Smith, K.M. Hillgren, Pharmacokinetics of organic cation transporter 1 (OCT1) substrates in Oct1/2 knockout mice and species difference in hepatic OCT1-mediated uptake, Drug Metab. Dispos. 48 (2) (2020) 93.
[46] S. Bhattacharyya, A.H. Schapira, D.P. Mikhailidis, J. Davar, Drug-induced fibrotic valvular heart disease, Lancet (Lond., Engl.) 374 (9689) (2009) 577–585.
[47] N.S. Oates, R.R. Shah, J.R. Idle, R.L. Smith, Genetic polymorphism of phenformin 4-hydroxylation, Clin. Pharmacol. Ther. 32 (1) (1982) 81–89.
[48] L. Han, Y. Jia, Y. Zhao, C. Sun, M. Zhao, Y. Peng, J. Zheng, Metabolic activation of zolmitriptan mediated by CYP2D6, Xenobiotica; Fate Foreign Compd. Biol. Syst. 51 (11) (2021) 1292–1302.
[49] G. Villapalos-García, P. Zubiaur, M. Navares-Gómez, M. Saiz-Rodríguez, G. Mejía-Abril, S. Martín-Vílchez, M. Román, D. Ochoa, F. Abad-Santos, Effects of cytochrome P450 and transporter polymorphisms on the bioavailability and safety of dutasteride and tamsulosin, Front. Pharmacol. 12 (2021), 718281.
[50] D. Hallifax, J.B. Houston, Saturable uptake of lipophilic amine drugs into isolated hepatocytes: mechanisms and consequences for quantitative clearance prediction, Drug Metab. Dispos.: Biol. Fate Chem. 35 (8) (2007) 1325–1332.
[51] K.-E.M. Redeker, O. Jensen, L. Gebauer, M.J. Meyer-Tönnies, J. Brockmöller, Atypical substrates of the organic cation transporter 1, Biomolecules (2022).
[52] J.E. Van Montfoort, M. Müller, G.M. Groothuis, D.K. Meijer, H. Koepsell, P. J. Meier, Comparison of "type I" and "type II" organic cation transport by organic cation transporters and organic anion-transporting polypeptides, J. Pharmacol. Exp. Ther. 298 (1) (2001) 110–115.
[53] P. Chandra, K.L.R. Brouwer, The complexities of hepatic drug transport: current knowledge and emerging concepts, Pharm. Res. 21 (5) (2004) 719–735.
[54] O. Jensen, J. Matthaei, H.G. Klemp, M.J. Meyer, J. Brockmöller, M.V. Tzvetkov, Isobutyrylcarnitine as a biomarker of OCT1 activity and interspecies differences in its membrane transport, Front. Pharmacol. 12 (2021), 674559.
[55] L. Gebauer, O. Jensen, J. Brockmöller, C. Dücker, Substrates and inhibitors of the organic cation transporter 3 and comparison with OCT1 and OCT2, J. Med. Chem. 65 (18) (2022) 12403–12416.
[56] J. Matthaei, D. Kuron, F. Faltraco, T. Knoch, J.N. Dos Santos Pereira, M. Abu Abed, T. Prukop, J. Brockmöller, M.V. Tzvetkov, OCT1 mediates hepatic uptake of sumatriptan and loss-of-function OCT1 polymorphisms affect sumatriptan pharmacokinetics, Clin. Pharmacol. Ther. 99 (6) (2016) 633–641.
[57] M.V. Tzvetkov, J. Matthaei, S. Pojar, F. Faltraco, S. Vogler, T. Prukop, T. Seitz, J. Brockmöller, Increased systemic exposure and stronger cardiovascular and metabolic adverse reactions to fenoterol in individuals with heritable OCT1 deficiency, Clin. Pharmacol. Ther. 103 (5) (2018) 868–878.