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The prototypic pharmacogenetic drug debrisoquine is a substrate of the genetically polymorphic organic cation transporter OCT1

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ABSTRACT

Debrisoquine is a probe drug for *in vivo* phenotyping of human CYP2D6 metabolic activity. However, debrisoquine is positively charged under physiological conditions and it is unclear how it enters the hepatocytes to undergo CYP2D6 metabolism. We analysed whether debrisoquine is a substrate of the hepatic organic cation transporter OCT1 and whether drug–drug interactions at OCT1, or polymorphisms in *OCT1* gene, affect debrisoquine uptake.

Debrisoquine showed low carrier-independent membrane permeability (P_e of 0.01×10^{-6} cm/s in artificial PAMPA membranes) and strongly inhibited the uptake of the model OCT1 substrate MPP^+ (IC_{50} of 6.2 ± 0.8 μM). Debrisoquine uptake was significantly increased in HEK293 cells overexpressing OCT1 compared to control cells. The OCT1-mediated uptake of debrisoquine followed Michaelis–Menten kinetics (K_M of 5.9 ± 1.5 μM and V_{max} of 41.9 ± 4.5 pmol/min/mg protein) and was inhibited by known OCT1 inhibitors and by commonly used drugs. OCT1-mediated debrisoquine uptake was reduced or missing in cells expressing loss-of-function OCT1 isoforms. Deletion of Met420 or substitution of Arg61Cys or Gly401Ser reduced V_{max} by 48, 63 and 91%, respectively, but did not affect the K_M. The OCT1 isoforms carrying Cys88Arg or Gly465Arg substitutions completely lacked OCT1-mediated debrisoquine uptake.

In conclusion, debrisoquine is a substrate of OCT1 and has the potential to be used as a phenotyping marker for OCT1 activity. Moreover, variations in debrisoquine metabolic phenotypes and their associations with diseases may be due not only to genetic variations *CYP2D6*, but also in *OCT1*.

1. Introduction

Debrisoquine (3,4-dihydro-1H-isoquinoline-2-carboxamidine sulfate), formally used as an antihypertensive drug, marked a milestone in the history of pharmacogenetics. In the 1970s and early 1980s, pioneer works by Robert Smith and colleagues described variations in debrisoquine metabolism as one of the first monogenetic autosomal recessive traits in pharmacology [1–3]. The cytochrome P450 isoform CYP2D6 was identified as the major enzyme catalyzing debrisoquine hydroxylation in humans [4,5] and debrisoquine, together with sparteine, played an important role in revealing the genetic variability of CYP2D6 [4,6–11].

As a sympatholytic antihypertensive drug, debrisoquine has a relatively poor benefit-risk ratio and is no longer available for clinical use in most countries. However, debrisoquine remains a useful probe drug for CYP2D6 and is part of a number of drug cocktails designed to phenotype cytochrome P450 activity *in vivo* [12–16].

Although we know more than 80 CYP2D6 alleles today (http://www.cypalleles.ki.se/cyp2d6.htm, accessed on November 21, 2011), genetic variability in CYP2D6 still can only partially explain the inter-individual variability in debrisoquine hydroxylation. While a lack of active *CYP2D6* alleles successfully predicts lack of debrisoquine hydroxylation, high unexplained variability in debrisoquine hydroxylation exists among carriers of the same *CYP2D6* genotype if one or more active CYP2D6 alleles are present [17,18]. Furthermore, clinical phenotypes like susceptibility to lung cancer were significantly associated with individual debrisoquine hydroxylation rates, but less consistently with *CYP2D6* genotype [19,20]. All these suggest that genetic variability in other steps relevant for debrisoquine pharmacokinetics may affect individual debrisoquine hydroxylation rates.

It is not clear how debrisoquine penetrates the hepatocellular membrane before undergoing metabolism by CYP2D6. Debrisoquine contains a guanidine moiety that is easily protonated. With a pK_a value of 11.95, more than 99.99% of debrisoquine molecules are positively charged at the physiological pH of 7.4 (Fig. 1). Therefore, debrisoquine will probably only poorly penetrate cell membranes by passive diffusion and will likely

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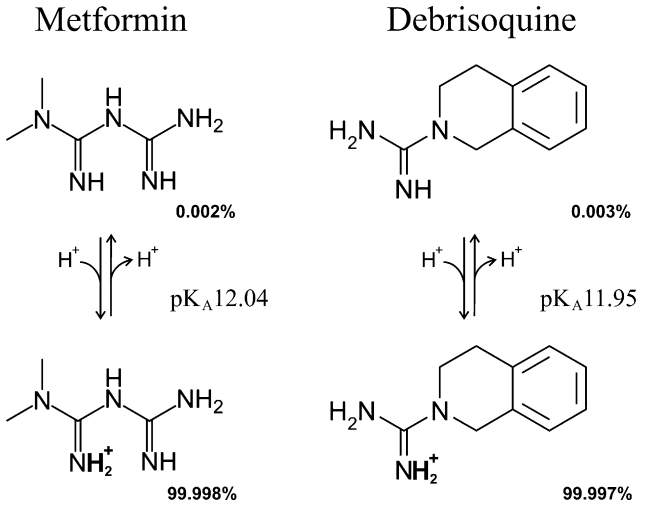

Fig. 1. Equilibrium between uncharged and positively charged forms of the drugs: comparison between metformin and debrisoquine. The structure of the protonated forms and the pK_a values were obtained using ADMET Predictor™ (Simulations Plus, Lancaster, USA). The percentages represent the proportion of protonated (i.e. positively charged) and non-protonated (i.e. uncharged) drugs at physiological pH (7.4).

require carrier-mediated membrane transport for its cellular uptake. In this respect debrisoquine shows some similarities with the antidiabetic drug metformin. Metformin is a well known substrate of organic cation transporters [21–24] and recently a number of other guanidine-containing molecules were reported to interact with organic cation transporters [25]. Therefore organic cation transporters may play an important role for the cellular uptake of debrisoquine.

The organic cation transporter 1 (OCT1; alternative gene name *SLC22A1*) is by far the most strongly expressed organic cation transporter in the sinusoidal membrane of the human liver [21,26,27]. Furthermore, OCT1 was shown to be essential for the uptake and activity of metformin in the liver [22]. Similar to *CYP2D6*, *OCT1* is highly genetically polymorphic. Nine percent of Caucasians are compound homozygous carriers of one of the five common coding polymorphisms (Arg61Cys, Cys88Arg, Gly401Ser, Gly465Arg and deletion of Met420) and therefore have reduced ability to take up drugs such as metformin, tropisetron or O-desmethyltramadol in the liver [22,28,29].

We hypothesize that the organic cation transporter OCT1 is involved in debrisoquine uptake into the liver, and that genetic polymorphisms in *OCT1* may contribute to the high inter-individual variability in debrisoquine hydroxylation rates. In this study we tested whether debrisoquine is a substrate of OCT1 and whether drug–drug interactions or genetic polymorphisms in the *OCT1* gene may affect cellular uptake of debrisoquine.

2. Materials and methods

2.1. Materials

^{3}H-labeled 1-methyl-4-phenylpyridinium (MPP^+, methyl-^{3}H, 80 Ci/mmol) was obtained from Hartmann Analytics (Braunschweig, Germany). Debrisoquine, 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+), MPP^+, tetra-N-butylammonium (TBA^+), tropisetron, ondansetron, tramadol, venlafaxine, paroxetine, sodium acetate, sodium hydroxide, sodium dodecyl sulfate and poly D-lysine (molecular weight of 1000–5000, #P0296) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Acetonitrile was obtained from Merck (Darmstadt, Germany).

The T-REx™ cells (HEK293 cells genetically engineered to be used as hosts for targeted chromosomal integration), *E. coli* TOP10 cells, the Flp-In™ system, Dulbecco's Modified Eagle Medium (DMEM), Hank's Buffered Salt Solution (HBSS), fetal calf serum and the antibiotics penicillin, streptomycin and hygromycine B were obtained from Invitrogen (Karlsruhe, Germany). FuGene6 transfection reagent was obtained from Roche (Mannheim, Germany).

The kits for DNA and RNA extraction (DNeasy® Blood and Tissue Kit and RNeasy® Plus Mini Kit, respectively) were obtained from Qiagen (Hilden, Germany). The SuperScript® II reverse transcriptase was obtained from Invitrogen. Gene expression measurements were performed using pre-designed TaqMan® gene expression assays with ID-number Hs00427550_m1 for OCT1 and ID-number 4326322E for TATA-box binding protein TBP, obtained from Applied Biosystems (Darmstadt, Germany). The pcDNA3::hOCT1 plasmid that we use to clone the human OCT1 was kind gift from Herman Koepsell and Valentin Gorboulev (University of Würzburg, Würzburg, Germany). PAMPA 96-well plates were obtained from Gentest (part of BD Biosciences, Heidelberg, Germany). The restriction endonuclease DpnI was obtained from NEBiolabs (Frankfurt am Main, Germany); the other restriction nucleases that were used in this study were obtained from Fermentas (St. Leon-Rot, Germany). KOD Hot Start DNA Polymerase was obtained from Merck.

2.2. Parallel artificial membrane permeability assay

The parallel artificial membrane permeability assay (PAMPA) was performed using pre-coated PAMPA 96-well plates according to the manufacturer's instructions (Gentest, BD Biosciences). Briefly, 300 μl of increasing concentrations of debrisoquine in phosphate buffered saline (PBS, pH 7.4) were pipetted into the donor wells and 200 μl of PBS (pH 7.4) into the acceptor wells. After incubation at room temperature for 5 h, the debrisoquine concentrations in both donor and acceptor wells were measured by HPLC as described below. Membrane permeability (P_e) was calculated as follows:

$$P_e = -\ln[1 - C_A/(1 - (0.3 * C_D + 0.2 * C_A)/0.3 * C_0)]/45$$

where C_0 is the initial concentration in the donor well and C_D and C_A are the end concentrations in the donor and acceptor wells, respectively.

2.3. Construction and validation of OCT1-overexpressing cell lines

Cell lines stably overexpressing human OCT1 were generated by targeted integration of *OCT1* cDNA into the genome of T-REx™ cells (HEK293 cells genetically engineered to be used as hosts for targeted chromosomal integration). First, the OCT1 overexpressing chromosomal integration vector pcDNA5.1::OCT1 was generated. For this purpose, the OCT1 open reading frame was cut out from the pcDNA3::hOCT1 using *Hind*III and *Eco*RV restriction enzymes, purified from a 0.8% agarose gel, and ligated into the pcDNA5.1 plasmid cut with the same restriction enzymes.

Second, five OCT1 isoforms representing the five common haplotypes with reduced or lost function known in Caucasians were constructed by site-directed mutagenesis. The haplotypes carried either single amino acid substitutions, i.e. Arg61Cys (rs12208357), Gly401Ser (rs34130495), and a single deletion of Met420 (rs72552763), or combination of Met420 deletion with Cys88Arg (rs55918055) or Met420 deletion with Gly465Arg (rs34130495). Briefly, the pcDNA3::hOCT1 plasmid was amplified by PCR using the KOD Hot Start DNA Polymerase Kit and site-directed mutagenesis primers (Supplementary Table 1) under the following conditions: initial denaturation for 3 min at 95 °C and 19

amplification cycles of 30 s at 95 °C, 30 s at 65 °C and 3 min 30 s at 72 °C. The non-mutated template plasmids were removed by methylation-specific digestion using *Dpn*I and the PCR products were transfected in *E. coli* TOP10 cells. The pcDNA3::OCT1 plasmids carrying the required mutations were identified by sequencing of the entire *OCT1* reading frame. The *OCT1* was then re-cloned into the pcDNA5.1 plasmid following the procedure used to re-clone the wild-type *OCT1*.

Third, the pcDNA5.1::OCT1 constructs were integrated in the genome of T-REx™ cells using the Flp-In™ system according to the manufacturer's instructions. Briefly, 400 ng of the pcDNA5::OCT1 plasmid were co-transfected with 3.6 μg DNA of the helper plasmid pOG44 in 1×10^6 T-REx™ cells, which were plated in a 6-well plate the day before. The transfection was carried out using 12 μl FuGene6 transfection reagent according to the manufacturer's instructions. After 72 h the cells were transferred to a 100 mm diameter Petri dish and grown on Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen). After overnight incubation to allow the cells to attach, hygromycine B was added to a final concentration of 300 μg/ml. The cells were incubated until single colonies appeared (approximately 10 days) and the medium was changed once at day 4. Single colonies of cells resistant to hygromycine B were selected and transferred and grown in 12-well and later in 6-well plates in DMEM supplemented with fetal calf serum, penicillin, streptomycin, and reduced concentration of 100 μg/ml hygromycine B.

The correct genomic integration of the pcDNA5.1::OCT1 constructs was verified by integration-specific PCR. For this purpose, genomic DNA was obtained from the stably transfected cells using automatic DNA extraction with the DNeasy® blood and tissue kit and the QIACube robot (Qiagen, Hilden, Germany). Two independent PCR reactions were performed to confirm correct integration. First, an integration-specific PCR was performed with the forward primer 5′-AGCTGTGGAATGTGTGTCAGTTAGG-3′ located in the SV40 promoter of the pFRT/lacZeo plasmid (which is pre-integrated in the genome of the T-REx HEK293 cells and is the target of the integration), and with the reverse primer 5′-ACGCCCTCCTACATCGAAGCTGAAA-3′ located in the hygromycin B resistance gene of the pcDNA5.1 plasmid (which should be integrated within the Flp recombination target (FRT) sites of the pFRT/lacZeo plasmid) (Supplementary Fig. 1A). The PCR was carried out using KOD Hot Start DNA Polymerase Kit under the following conditions: denaturation for 2 min at 95 °C, followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 68 °C and 1 min 30 s at 72 °C, and a final elongation of 10 min at 72 °C. A 614 bp PCR product that spans the upstream FRT site was obtained, confirming the correct chromosomal integration of the pcDNA5 constructs (Supplementary Fig. 1B). Second, the *OCT1* open reading frame was amplified using the primers 5′-CCATGGTGATGCGGTTTTGGCAGTA-3′ and 5′-CCTTCCTGTAGCCAGCTTTCATCAA-3′ and the KOD Hot Start DNA Polymerase Kit under the following conditions: denaturation for 2 min at 95 °C, followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 68 °C and 2 min at 72 °C, and a final elongation of 10 min at 72 °C. The expected 2532 bp PCR products were obtained (Supplementary Fig. 1C) and the presence of the required mutation in the *OCT1* open reading frame was verified by sequencing.

Additionally, the overexpression of OCT1 was validated by quantitative RT-PCR. For this purpose, total RNA was obtained from the stably transfected cells using automatic DNA extraction with the RNeasy® Plus Mini Kit and the QIACube robot. Reverse transcription reaction was performed using random hexanucleotides as primers and SuperScript® II reverse transcriptase according to the manufacturer's instructions. Next, OCT1 expression was quantified using ready-to-use TaqMan® gene expression assays for the OCT1 gene and the house-keeping gene TBP as an internal control. All stably transfected OCT1 cell clones showed a uniform 15-fold increase in the OCT1 mRNA expression compared to the control cells transfected with the empty pcDNA5.1 plasmid (Supplementary Fig. 2).

Finally, the OCT1 activity of the stably transfected cells was quantified by measuring the uptake of the model organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+) following the procedure described by Wilde et al. [30] with modification [28]. The cells overexpressing wild-type OCT1 showed a 9.8-fold increase in ASP^+ uptake at 1 μM ASP^+ (K_M 19.8 μM, Supplementary Fig. 3). The changes in the uptake kinetics of the mutated OCT1 isoforms (Supplementary Fig. 3) were comparable with those previously reported in the literature [31].

2.4. Inhibition of the OCT1-mediated MPP^+ uptake

OCT1-overexpressing or control HEK293 cells were plated at density of 0.6×10^6 cells/well in 24-well plates pre-coated with poly D-lysine (molecular weight of 1000–5000) and were grown for two days to reach confluence. The cells were washed with 1 ml 37 °C Hank's Buffered Salt Solution (HBSS). Uptake was measured by incubating the cells for 1 min with 400 μl 37 °C HBSS containing 5 nM ^{3}H-labeled 1-methyl-4-phenylpyridinium (MPP^+) and increasing concentration of non-radioactively-labeled debrisoquine. The uptake was stopped by adding 1 ml ice-cold HBSS. The cells were washed twice with 2 ml ice-cold HBSS and lysed with 500 μl solution of 0.1N NaOH and 0.1% (w/v) SDS. The intracellularly accumulated MPP^+ was quantified by measuring the radioactivity in 400 μl aliquots of the cell lysates using a liquid scintillation counter Beckman LS 5000 (Beckman, Krefeld, Germany). The total protein content of the lysates was measured using a bicinchoninic acid assay [32] and was used to normalize the intracellular concentration of MPP^+.

2.5. Measuring cellular uptake of debrisoquine

Cell culture Petri dishes 100 mm in diameter (BD Falcon, Heidelberg, Germany) were precoated with poly D-lysine (molecular weight of 1000–5000). Nine million HEK293 cells overexpressing wild-type or variant OCT1 were plated and incubated for 48 h to reach confluence. Prior to the uptake measurements, cells were washed with 10 ml 37 °C HBSS. The uptake was initiated by adding 5 ml 37 °C HBSS supplemented with debrisoquine. The concentrations of debrisoquine used varied between 1 and 40 μM. After 1 min the reaction was stopped by adding 20 ml ice-cold HBSS. Alternatively, if the time dependence of the debrisoquine intracellular accumulation was analysed, the reaction was stopped after 30 s, 1, 5, 15, or 30 min. The cells were washed once with 20 ml and once with 2 ml ice-cold HBSS. An aliquot of 25% of the cells was kept for protein measurements and the rest of the cells were lysed in 1 ml of a mixture of 80% acetonitrile and 20% 50 mM sodium acetate (pH 5.0). The cell debris were pelleted by centrifugation (10 min at 16 000 × *g*, 4 °C). The supernatant was transferred into 10 ml glass tubes and evaporated to dryness under a nitrogen stream at 40 °C. The dry residue was reconstituted with 200 μl 50 mM sodium acetate buffer (pH 5.0) supplemented with 5% acetonitrile. The amount of debrisoquine was quantified by HPLC and normalized to the total protein content of the sample as measured using a bicinchoninic acid assay [32].

Inhibition of OCT1-mediated debrisoquine uptake was performed using tetra-N-butylammonium (TBA^+), tropisetron, ondansetron, tramadol and paroxetine.

2.6. High-performance liquid chromatography detection of debrisoquine

The concentrations of debrisoquine from the cellular uptake and PAMPA experiments were measured by HPLC using a

modification of the method described by Pereira et al. [33]. Briefly, for the HPLC analyses a LaChrom system (Merck Hitachi, Darmstadt, Germany) was used with an interface (D-7000, Merck Hitachi), a pump (L-7100, Merck Hitachi), an autosampler (L-7200, Merck Hitachi), a fluorescence detector (L-7400, Merck Hitachi) and a degasser (L-7614, Merck Hitachi). Separation was carried out at room temperature on a LiChrospher 100 CN (5 μm, 4 × 150 mm) column with a LiChrospher100CN guard column (5 μm, both from Merck). Fluorescence detection was performed using excitation and emission wavelengths of 210 nm and 290 nm, respectively. Isocratic elution was performed with a mobile phase of 95% 50 mM sodium acetate buffer (pH 5.0) and 5% acetonitrile at a flow rate of 0.7 ml/min. Venlafaxin was used as internal standard. The peaks of debrisoquine and venlafaxin were detected with retention times of 9 min and 15 min, respectively, and were quantified using the peak areas. We used calibrator concentrations of 20, 200 and 2000 ng/ml and the limit of the quantification was 10 ng/ml.

2.7. Data analyses

Graphical representations and regression analyses were performed using Sigma Plot® version 11.0 (Systat Software Inc., Chicago, IL, USA). The half-maximal inhibitory concentration (IC_{50}) was calculated by regression analyses of the equation:

$$Y(C) = Y_{min} + (Y_{max} - Y_{min})/1 + (C/IC_{50})^{-H}$$

where C is the debrisoquine concentration, $Y(C)$ is the MPP^+ uptake at a debrisoquine concentration of C, Y_{min} and Y_{max} are the maximal and minimal MPP^+ uptakes, respectively, and H is the Hill slope. The K_M and V_{max} values were estimated by regression analyses using the Michaelis–Menten equation.

Statistical analyses were performed using SPSS® Statistics version 19 (SPSS Inc., IBM, Chicago, USA). Analyses of variance (ANOVA) were applied for multi-group comparisons. If two groups among the multiple groups were compared this was done using ANOVA followed by *post hoc* analyses applying Tukey's Honestly Significant Difference (HSD) test. P-values <0.05 were regarded as significant.

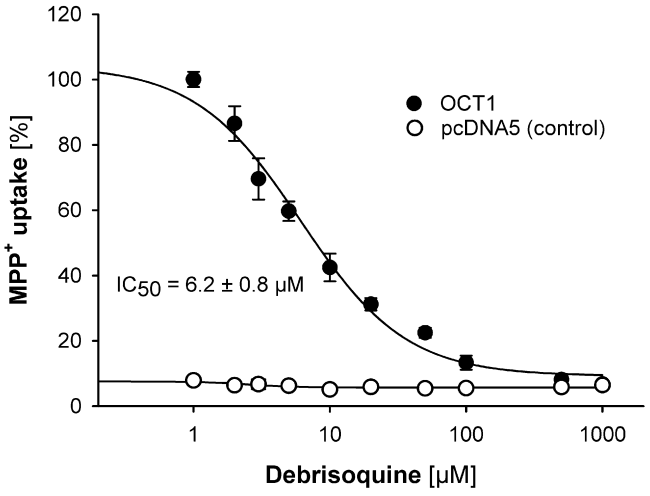

Fig. 2. Debrisoquine inhibits the uptake of the model OCT1 substrate MPP^+. The cellular accumulation of 1-methyl-4-phenylpyridinium (MPP^+) was measured after 1 min exposure to 10 nM ^3H MPP^+ in cells overexpressing OCT1 and in control cells transfected with the empty plasmid pcDNA5.1. The graph shows mean values and standard errors from three independent experiments.

3. Results

3.1. Carrier-independent membrane permeability of debrisoquine

In order to assess the ability of debrisoquine to penetrate cellular membranes in a carrier-independent manner, we

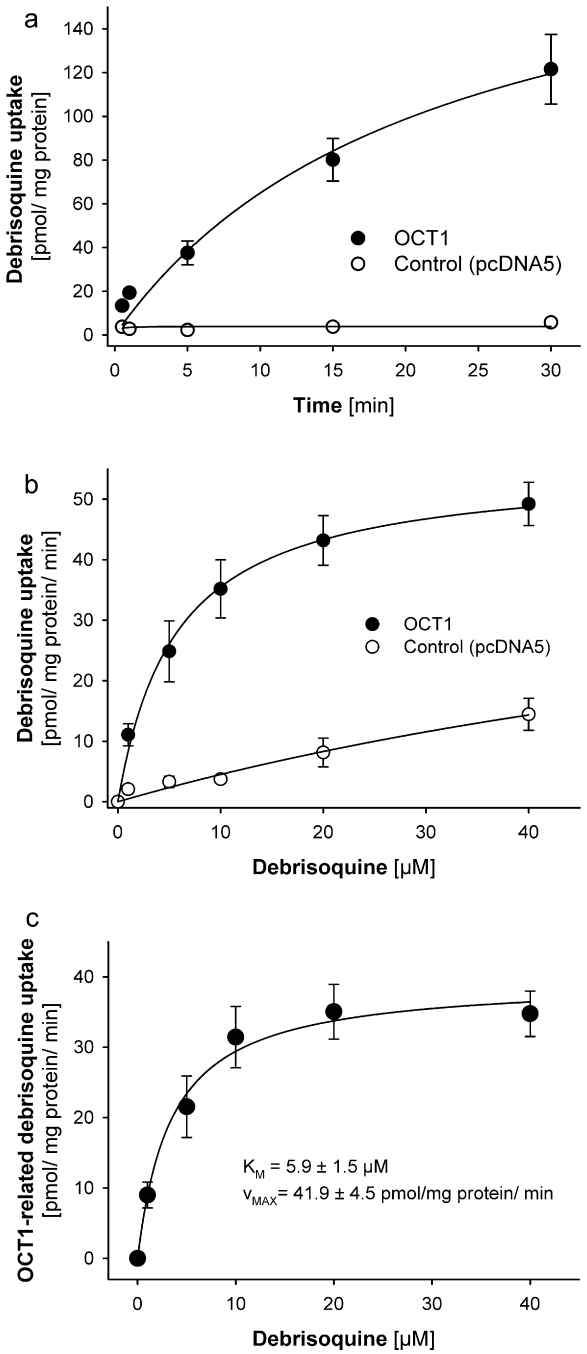

Fig. 3. Time and concentration dependence of the OCT1-caried cellular uptake of debrisoquine. (A) Time dependence of debrisoquine accumulation. OCT1-overexpressing and control cells (transfected with the empty pcDNA5.1 vector) were incubated for 1, 5, 15, and 30 min with 1 μM debrisoquine. (B) Concentration dependence of debrisoquine uptake. OCT1-overexpressing and control cells were incubated for 1 min with increasing concentrations of debrisoquine. (C) The OCT1-attributed (also called OCT1-mediated in the text) debrisoquine uptake calculated by subtracting the uptake in the control (pcDNA5.1) cells from the uptake in the OCT1-overexpressing cells using the dataset shown in (B). Intracellular debrisoquine accumulation was measured by means of high-performance liquid chromatography and normalized to the total protein amounts in the cells. The graphs show mean values and standard errors from three independent experiments.

measured debrisoquine diffusion through artificial PAMPA membranes. Debrisoquine showed very low membrane permeability (P_e 0.01 × 10^{-6} cm/s), suggesting that it will not be able to penetrate cellular membranes in a carrier-independent manner. This observation is in concordance with the low calculated lipophilicity of debrisoquine (C log $D_{7.4}$ of −1.0; calculated using ADMED Predictor™ Software Version 5.0, Simulations Plus Inc., Lancaster, CA, USA).

3.2. Debrisoquine as a substrate of OCT1

Initially we tested whether debrisoquine may inhibit OCT1-mediated uptake. For that purpose, we measured the ability of debrisoquine to inhibit the uptake of MPP^+ (1-methyl-4-phenylpyridinium), a model substrate of OCT1. We used HEK293 cells stably transfected to overexpress human OCT1, and compared them to control cells transfected with an empty pcDNA5.1 vector. Without inhibition, MPP^+ uptake was 13.6-fold higher in the OCT1 overexpressing compared to the control cells (Fig. 2). Debrisoquine strongly inhibited the MPP^+ uptake. The difference in the MPP^+ uptake between the OCT1 overexpressing and the control cells was completely reversed at 5 mM debrisoquine and half-maximal inhibitory concentration (IC_{50}) of debrisoquine was 6.2 μM (Fig. 2).

Next we tested whether debrisoquine is a substrate of OCT1. Therefore we measured the OCT1-mediated uptake of debrisoquine by comparing the intracellular debrisoquine accumulation between the OCT1-overexpressing and control cells. OCT1-overexpressing cells showed strong time- and concentration-dependent uptake of debrisoquine that was absent in the control cells (Fig. 3A and B). To evaluate the OCT1-specific debrisoquine uptake, we subtracted the uptake of the control cells from the uptake of the OCT1-overexpressing cells. The OCT1-specific debrisoquine uptake showed typical Michaelis–Menten kinetics with K_M of 5.9 ± 1.5 μM and V_{max} of 41.9 ± 4.5 pmol/min/mg of protein (Fig. 3C). From this data, we can conclude that debrisoquine is a high affinity substrate of OCT1.

3.3. Effects of drug–drug interactions on OCT1-madiated debrisoquine uptake

We analysed whether known model OCT1 substrates or clinically relevant drugs may inhibit the OCT1-mediated cellular uptake of debrisoquine (Fig. 4). We used the well known OCT1 model substrates MPP^+, ASP^+ (4-(4-(dimethylamino)styryl)-N-methylpyridinium) and the inhibitor TBA^+ (tetra-N-butylammonium) and the drugs tropisetron, ondansetron, tramadol and paroxetine (all known from earlier data as OCT1 substrates or inhibitors). All substances tested led to significant inhibition of the OCT1-mediated uptake ($P < 0.003$, ANOVA with Tukey-HSD *post hoc* test). Of all the tested substances, the least inhibition was observed with tramadol (66% reduction of the OCT1-mediated uptake, $P = 0.003$). This result was expected given that tramadol has a higher IC_{50} value for OCT1 than do tropisetron and ondansetron [28,29]. In conclusion, the involvement of OCT1 in the cellular uptake of debrisoquine was confirmed using inhibition with known OCT1 substrates. Furthermore, drug–drug interactions affecting the cellular uptake of debrisoquine were detected *in vitro*.

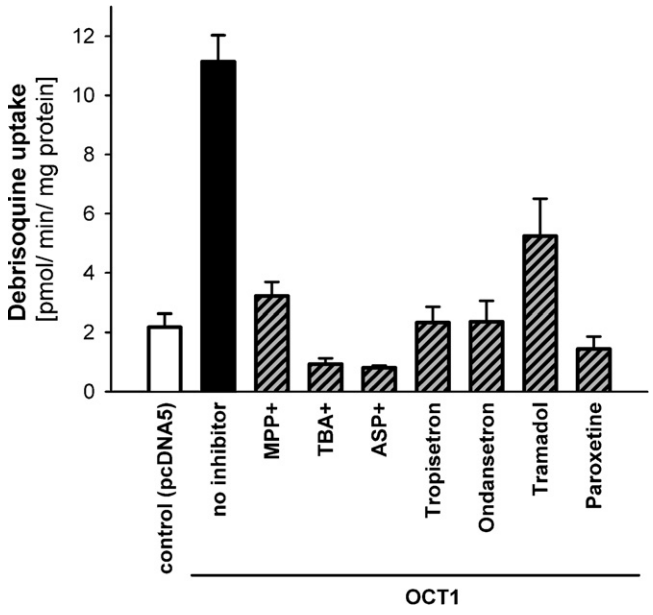

Fig. 4. Inhibition of the OCT1-mediated debrisoquine uptake. HEK293 cells overexpressing OCT1 were incubated for 1 min with 1 μM debrisoquine in the absence of any inhibitors (black bar) or in the presence of 200 μM model OCT1 substrates or drugs known to be substrates or inhibitors of OCT1 (hatched gray bars). As a control, HEK293 cells transfected with the empty pcDNA5.1 plasmid were incubated with debrisoquine in the absence of inhibitors (white bar). The cellular uptake of debrisoquine was measured by means of high-performance liquid chromatography and normalized to the total protein amounts in the cells. The graph shows mean values and standard errors from three independent experiments.

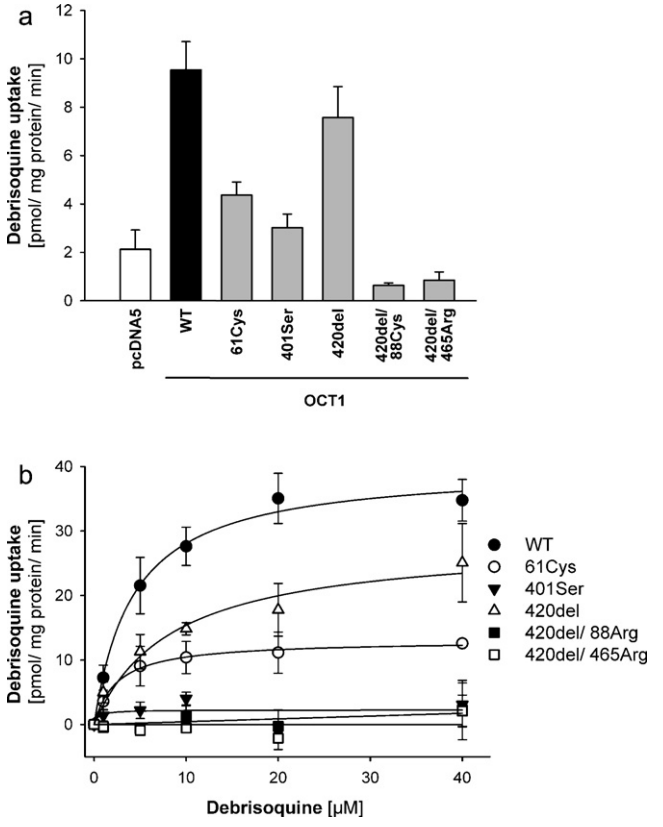

Fig. 5. Effects of common loss-of-function polymorphisms in OCT1 on the uptake of debrisoquine. (A) Differences in debrisoquine uptake between wild-type OCT1 and loss-of-function OCT1 isoforms after incubation with 1 μM debrisoquine for 1 min. (B) Differences in the concentration dependence of OCT1-mediated debrisoquine uptake between the wild-type and loss-of-function OCT1 isoforms after incubation with increasing concentrations of debrisoquine. The cellular uptake of debrisoquine was measured by means of high-performance liquid chromatography and normalized to the total protein amount of the cells. The graphs show mean values and standard errors from three independent experiments.

Table 1
Effects of the common functional amino acid substitutions in OCT1 on the kinetics of debrisoquine uptake.

SNP	V_{max}		K_M [μM]		CL_{int}
	Mean	SEM	Mean	SEM	(V_{max}/K_M)
Reference (WT)	41.9	4.5	5.9	1.5	7.1
61Cys	15.6*	0.5	6.2	3.5	2.5
401Ser	3.6*	2.1	2.6	2.5	1.4
420del	21.7*	1.1	4.8	1.5	4.5
420del/88Arg	n.a.	n.a.	n.a.	n.a.	n.a.
420del/465Arg	n.a.	n.a.	n.a.	n.a.	n.a.

n.a., not applicable.
* $P < 0.01$ compared to the WT reference in a Tukey's HSD *post hoc* analysis following one-way ANOVA ($P < 10^{-4}$).

3.4. Effects of OCT1 genetic polymorphisms on debrisoquine uptake

After having demonstrated that debrisoquine is a substrate of OCT1, we analysed how common coding polymorphisms in *OCT1* affect debrisoquine uptake. We analysed Arg61Cys, Cys88Arg, Gly401Ser, Gly465Arg and a deletion of Met420. These are all common polymorphisms in Caucasians that were previously shown to cause decreased or absent OCT1 activity in studies using substrates other than debrisoquine [22,28,29,31,34]. In the presence of 1 μM extracellular debrisoquine, all variants tested showed lower debrisoquine uptake compared to the wild-type OCT1 (Fig. 5A). However, the observed reduction in the uptake varied among the isoforms carrying different polymorphisms. We were unable to demonstrate a significant decrease in debrisoquine uptake when the Met420 deletion variant was present. In contrast, the Cys88Arg and Gly465Arg substitutions led to complete lack of OCT1-mediated uptake. Analyses of the concentration dependence of the debrisoquine uptake showed that the V_{max} of OCT1 was affected by all of the polymorphisms tested ($P < 0.003$, ANOVA with Tukey-HSD *post hoc* test; Fig. 5B and Table 1). In contrast, the K_M values were not significantly affected by any of the polymorphisms ($P = 0.73$, ANOVA).

4. Discussion

Debrisoquine metabolism is highly variable and debrisoquine has been extensively studied as a probe drug for the variable CYP2D6 oxidative phenotype in humans. Debrisoquine is more than 99.9% protonated at physiological pH. In this study we showed that debrisoquine has very low carrier-independent membrane permeability (P_e 0.01×10^{-6} cm/s) and that debrisoquine is a high affinity substrate of OCT1 (K_M 5.9 μM, Fig. 3). Our data suggests that OCT1 may mediate the cellular uptake of debrisoquine in the liver and thus may modulate the hepatic metabolism of debrisoquine.

The maximal plasma concentrations following oral administration of debrisoquine are 30–70 nM, which is 100-fold below the K_M value of OCT1. We did not measure OCT1-mediated uptake at these concentrations. However, by extrapolating our data using the Michaelis–Menten equations, one may calculate that OCT1 overexpression will increase the uptake of debrisoquine by more than 20 fold. Therefore significant involvement of OCT1 may also be expected at clinically relevant concentrations of debrisoquine.

OCT1 is genetically highly polymorphic and in this study we present *in vitro* evidence that common genetic polymorphisms and commonly used drugs affect the OCT1-mediated cellular uptake of debrisoquine. These findings have at least two direct consequences. First, *OCT1* polymorphisms and drug–drug interactions at OCT1 may contribute to the high and thus far unexplained inter-individual variability in debrisoquine hydroxylation. Second, next to CYP2D6 phenotyping, debrisoquine may have the potential to be developed as probe drug for phenotyping OCT1 activity *in vivo*.

The high inter-individual variation in debrisoquine hydroxylation capacity may represent variation not only in CYP2D6, but also in OCT1 activity. The debrisoquine hydroxylation capacity, measured as the ratio of debrisoquine over its major metabolite 4-hydroxydebrisoquine in urine, varies by more than 100-fold among individuals [2,17,35,36]. The observed bi-modal distribution could be explained by the lack of CYP2D6 activity due to genetic polymorphisms in the individuals with very low hydroxylation capacity, so-called poor metabolizers (reviewed in [37]). Still, in the rest of the population the *CYP2D6* genotypes could explain only part of the variability with strong inter-individual differences among persons with same *CYP2D6* genotypes [17,18]. Our data suggest that beyond the previously suggested effects of environmental factors [38] or downstream metabolism reactions [18], the variations in debrisoquine hydroxylation may also be due to variations OCT1-mediated hepatocellular uptake.

Correlations between debrisoquine hydroxylation rates and disease susceptibility or drug response should be re-discussed, taking into account that inter-individual variations in debrisoquine hydroxylation may be caused by genetic variants in *OCT1*. A number of clinical studies have shown a correlation of incidences of lung cancer with debrisoquine hydroxylation, but the correlation of lung cancer with CYP2D6 genotypes was found less consistently [20]. Furthermore, the data about CYP2D6 expression in the lungs are controversial, with some reports showing CYP2D6 expression in the lungs [39], but others not [40,41]. In contrast, OCT1 is expressed in bronchial epithelial cells in the lungs [42] and may be involved in the epithelial transport of carcinogenic substances in the lung epithelial cells.

Our data suggest that debrisoquine may have the potential to be used as a probe drug for *in vivo* phenotyping of OCT1 activity in humans. A number of factors beyond the known loss-of-function amino acid polymorphisms may affect the OCT1 ability to take up drugs on the liver, i.e. unknown amino acid substitutions, drug–drug interactions and suggested high variable expression of OCT1 [21]. *In vivo* phenotyping in humans using model drugs will be a powerful tool to study these effects. A number of *in vitro* model OCT1 substrates like MPP^+, TEA^+ and ASP^+ exist. However, all these model substrates are highly toxic and cannot be given directly to humans [43]. In contrast, small amounts of debrisoquine are safe to be administered to humans, and debrisoquine is a well established *in vivo* probe drug [12–16]. However, clinical studies are required to evaluate to what extent the dependence of debrisoquine cellular uptake on OCT1, which was demonstrated here *in vitro*, will manifest as inter-individual variations *in vivo*.

Several factors may mask the effects of OCT1 on debrisoquine pharmacokinetics in humans *in vivo*. First, debrisoquine pharmacokinetics highly depend on common genetic variations in *CYP2D6*. The effects of CYP2D6 may completely mask the OCT1 effects, especially in the group of poor CYP2D6 metabolizers. Second, debrisoquine elimination in urine may involve complex transport processes. Debrisoquine and its major metabolite 4-hydroxydebrisoquine have renal clearances of 282 and 371 ml/min, respectively, suggesting active tubular secretion for both substances [44]. Organic cation transporters like OCT2 may affect the tubular secretion of debrisoquine. OCT2 is strongly expressed in the proximal kidney tubules [45]. Data about the affinity of OCT2 for debrisoquine are missing, but OCT2 has been shown to have a similar or even higher affinity than OCT1 for a number of other guanidines [25]. In addition, OCT1 has been shown to be expressed on the luminal membrane of the epithelial tubular cells in the kidney and to affect the tubular reuptake of metformin [34]. Therefore, additional effects of OCT1 polymorphisms on renal excretion of debrisoquine cannot be excluded. However, the

expression levels of OCT1 in the kidney are very low [45]. Model animals like rodents could not be used to address the effects of OCT1 in the kidney, as the expression levels and subcellular localization of OCT1 in rodents differ substantially from those in humans [26,46]. All this suggest that the ratio of debrisoquine to 4-hydroxydebrisoquine eliminated over 24 h in urine, a ratio that is typically used in the debrisoquine-based phenotyping, may be not so useful for OCT1 phenotyping. Plasma debrisoquine concentration may better represent OCT1 effects. However this remains speculation that requires validation in human pharmacokinetic studies.

Debrisoquine is a much higher affinity OCT1 substrate than metformin. OCT1 mediates the uptake of debrisoquine with a K_M of 5.9 μM (Fig. 3) compared to K_M above 2 mM for metformin [21,22]. This could be explained by the presence of a highly hydrophobic aromatic tetrahydroisoquinoline ring in the debrisoquine molecule. In contrast, metformin has a hydrophilic second guanidine structure instead of this tetrahydroisoquinoline ring (Fig. 1). As all available pharmacophores for OCT1 substrates or inhibitors contain at least one hydrophobic moiety of the molecule next to a positive charge [47–51], debrisoquine fits much better in the structural requirements for OCT1 substrates. Therefore, it could be concluded that because of its structural specificities, debrisoquine is a better model substrate of OCT1 than metformin.

In conclusion, debrisoquine is a high affinity substrate of OCT1. The cellular uptake of debrisoquine depends on genetic variants in *OCT1* and drug–drug interactions at OCT1. Therefore, debrisoquine has the potential to be used as a probe drug for analyzing OCT1 activity *in vivo* in humans. Furthermore, genetic polymorphisms in *OCT1* should be analysed in relation to clinical phenotypes that have been previously related to inter-individual variations in debrisoquine hydroxylation, but could not be explained by polymorphisms in *CYP2D6*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2012.01.032.

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