The Carnitine Transporter SLC22A5 Is Not a General Drug Transporter, but It Efficiently Translocates Mildronate

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ABSTRACT:

In addition to its function as carnitine transporter, novel organic cation transporter type 2 (OCTN2; human gene symbol SLC22A5) is widely recognized as a transporter of drugs. This notion is based on several reports of direct measurement of drug accumulation. However, a rigorous, comparative, and comprehensive analysis of transport efficiency of OCTN2 has not been available so far. In the present study, OCTN2 orthologs from human, rat, and chicken were expressed in 293 cells using an inducible expression system. Uptake of trans-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (ASP\(^+\)), cephaloridine, ergothioneine, gabapentin, mildronate, pyrilamine, quinidine, spironolactone, tetraethylammonium, verapamil, and vigabatrin was determined by liquid chromatography/mass spectrometry. For reference, uptake of carnitine was measured in parallel. Our results indicate that OCTN2-mediated uptake of drugs was not significantly different from zero or, with tetraethylammonium and ergothioneine, was minute relative to carnitine. The carnitine congener mildronate, by contrast, was transported very efficiently. Thus, OCTN2 is not a general drug transporter but a highly specific carrier for carnitine and closely related molecules. Transport parameters (cellular accumulation, transporter affinity, sodium dependence) were similar for mildronate and carnitine. Efficiency of transport of mildronate was even higher than that of carnitine. Hence, our results establish that OCTN2 is a key target of the cardioprotective agent mildronate because it controls, as integral protein of the plasma membrane, cellular entry of mildronate and enables efficient access to intracellular targets. The highest levels of human OCTN2 mRNA were detected by real-time reverse transcription-polymerase chain reaction in kidney, ileum, breast, small intestine, skeletal muscle, and ovary but also in some heart and central nervous system tissues.

Novel organic cation transporter type 2 (OCTN2; human gene symbol SLC22A5) is widely recognized as a carnitine transporter because it efficiently catalyzes, as integral protein of the plasma membrane, the cotransport of Na\(^+\) and L-carnitine into cells. Carnitine can be synthesized in the human body, principally in liver and kidney, but it is largely acquired from food, in particular from red meat. Carnitine is required as a catalyst in the transport of long-chain acyl groups (fatty acids) from the cytosol into the mitochondrial matrix to fuel \(\beta\)-oxidation and hence ATP production.

The physiological importance of the carnitine transporter was realized from its role in primary carnitine deficiency (PCD; also known as systemic carnitine deficiency), an autosomal recessive hereditary disorder. Mutations in the SLC22A5 gene can cause the production of defective transporters. As a result of reduced plasma membrane transport capacity, carnitine is lost from the body, and cells are supplied insufficiently. During infancy or early childhood, the subadequate capacity for fat processing and energy production develops into characteristic symptoms that include cardiomyopathy, encephalopathy, muscle weakness, and hypoglycemia; serious complications in acute illness may involve heart and liver failure, coma, and sudden death. The consequences of defective OCTN2 were clearly shown using jvs mice, which express an inactive transporter variant (Leu352Arg) and exhibit the PCD phenotype (Lu et al., 1998; Nezu et al., 1999). Fortunately, PCD can often be treated successfully by supplementation of L-carnitine.

In addition to its undisputed function as carnitine (and acyl-carnitine) transporter, OCTN2 is regularly perceived as an important transporter of drugs (Hilgendorf et al., 2007). In contemporary drug development, early pharmacokinetic analysis is a key feature. Large efforts are made to optimize drug structures for favorable characteristics of absorption, distribution, metabolism, elimination, and toxicity. This increasingly involves direct testing of individual drug carriers expressed heterologously in cultured cell lines. For proper selection of relevant transporters in this process, it is important to understand the respective substrate selectivity. The notion that OCTN2 functions as drug transporter is based on several publications where transport of drugs was inferred from direct uptake measurements mostly with...
RESULTS. The 5'-interface between SLC22A4 (Gene Bank entry NM_003060) corresponds to GenBank entry NM_001045828 except for a single nucleotide substitution as detailed under Results. The 5'-interface between pEBTetD and cDNA is GTTAAACTCT AAGCTT GCCACC (polylinker in bold, cDNA in italics); the 3'-interface is ATGCGGGACTACGAC GCCACC (polylinker in bold, cDNA in italics). The cDNA sequence of OCTN2h corresponds to GenBank entry NM_000306. The 5'-interface is GTTTAAACTCT AAGCTT GCCACC ATCCGGAAGTCCAGC; the 3'-interface is ACGCTCTCTAA CTCGGA CGATCGC.

Fig. 1. Compound structures.

To measure transport activity reliably, our experimental approach differed from the above reports in three major aspects. 1) We have used freshly dissolved unlabeled contrast, with an inducible expression system, two states of a single substrate accumulation can be mistaken for transport by the carrier under study. By contrast, with an inducible expression system, two states of a single cell line are compared. 2) We have used highly selective detection of substrate molecules. By contrast, there is an inevitable chemical decay of radiotracers, and uptake of resulting radiolabeled decomposition fragments into cells cannot be discerned from uptake of true substrate in liquid scintillation counting. 3) All the substrates were probed with OCTN2 orthologs from human, rat, and chicken. This broad evolutionary coverage allows us to recognize conserved transporter features.

Amazingly, our results indicate that carrier-mediated uptake of drugs was mostly not significantly different from zero or, with TEA and ergothioneine, was minute relative to carnitine. Only mildronate was efficiently transported; however, this is closely related in structure to carnitine. Thus, OCTN2 is a highly specific carrier for carnitine and closely related molecules and not a general drug transporter.

Materials and Methods

Plasmid Constructs. The cDNAs of OCTN2 from chicken (OCTN2ch) and OCTN2 from human (OCTN2h) were generated by reverse transcriptase-polymerase chain reaction (RT-PCR), cloned into pUC19, fully sequenced, and finally inserted into expression vector pEBTetD. Human kidney total RNA was from Clontech (San-Germain-en-Laye, France). The cDNA sequence of OCTN2ch corresponds to GenBank entry NM_001045828 except for a single nucleotide substitution as detailed under Results. The 5'-interface between pEBTetD and cDNA is GTTAAACTCT AAGCTT GCCACC GTCCGGGGACTCCAGC (polylinker in bold, cDNA in italics); the 3'-interface is ACGCTCTCTAA CTCGGA CGATCGC. The cDNA sequence of OCTN2h corresponds to GenBank entry NM_000306. The 5'-interface is GTTTAAACTCT AAGCTT GCCACC ATCCGGAAGTCCAGC; the 3'-interface is ACGCTCTCTAA CTCGGA CGATCGC.

Cell Culture. The 293 cells [American Type Culture Collection (Manassas, VA) CRL-1573; also known as HEK 293 cells], a transformed cell line derived from HEK, were grown at 37°C in a humidified atmosphere (5% CO2) in plastic culture flasks (Falcon 3112; BD Biosciences, Heidelberg, Germany). The growth medium was Dulbecco's modified Eagle's medium (Life Technologies 31885-023; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Cölbe, Germany). Medium was changed every 2 to 3 days, and the culture was split every 5 days.

Stably transfected cell lines were generated as reported previously (Bach et al., 2007); cell culture medium always contained 5 µg/ml puromycin (PAA Laboratories GmbH) to ascertain plasmid maintenance. To turn on protein expression, cells were cultivated for at least 20 h in regular growth medium supplemented with 1 µg/ml doxycycline (MP Biomedicals, Eschwege, Germany).

Transport Assays. For measurement of solute uptake, cells were grown in surface culture on 60-mm polystyrol dishes (Nunclon 150288; NUNC A/S, Roskilde, Denmark) precoated with 0.1 g/l poly-L-ornithine in 0.15 M boric acid-NaOH, pH 8.4. Cells were used for uptake experiments at a confluence of at least 70%. Uptake was measured at 37°C. Uptake buffer contains 125 mM NaCl, 25 mM HEPES-NaOH, pH 7.4, 5.6 mM (+)-glucose, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM CaCl2, and 1.2 mM MgSO4. After preincubation for at least 20 min in 4 ml of uptake buffer, the buffer was replaced with 2 ml of unlabeled substrate or 3 ml of [3H]carnitine (at 0.1 µM) in uptake buffer. Incubation was stopped after 1 min by rinsing the cells four times each with 4 ml of ice-cold uptake buffer. Radioactivity was determined after cell lysis with 0.1% v/v Triton X-100 in 5 mM Tris-HCl, pH 7.4, by liquid scintillation counting. For LC/electrospray ionization/MS/MS analysis, the cells were solubilized with 4 mM HClO4 or methanol and stored at −20°C. After centrifugation (1 min, 16,000 g; 20°C) of the thawed lysates, 100 µl of supernatant was mixed with 10 µl of unlabeled 1-methyl-4-phenylpyridinium (MPP+) iodide (5.0 ng/µl), which served as internal standard. Of this mixture, 20-µl samples were analyzed by LC/MS/MS on a triple quadrupole mass spectrometer (TSQ Quantum; Thermo Fisher Scientific, Waltham, MA). Atmospheric pressure ionization with positive electrospray was used. The LC system consisted of Surveyor LC pump and autosampler (flow rate 250 µl/min). For spironolactone, a solvent gradient (A: 0.1% formic acid, B: 0.1% formic acid, pH 8.0). flow rate 250 µl/min. For spironolactone, a solvent gradient (A: 0.1% formic acid, B: 0.1% formic acid, pH 8.0).
methanol; 5 min 90 to 30% A, 5 min 30% A, 4 min 30 to 90% A) was used with a Waters (Milford, MA) Atlantis T3 reversed-phase column (100-mm length, 3-mm diameter, 5-μm particle size). For all the other compounds, isocratic chromatography (70% methanol and 30% 0.1% formic acid) was used with a Waters Atlantis HILIC silica column (50-mm length, 3-mm diameter, 5-μm particle size). For quantification by selected reaction monitoring (scan time 0.3 s), at first the optimal collision energy for argon-induced fragmentation in the second quadrupole was determined for each analyte. From the product ion spectra, the following fragmentations were selected for selected reaction monitoring (m/z parent, m/z fragment, collision energy): ASP$: 239, 194, 38 V; cephaloridine: 416, 337, 12 V; ergothionine: 230, 186, 16 V; gabapentin: 172, 154, 18 V; mildronate: 280, 171, 34 V; MPP*: 170, 128, 25 V; pyriformine base: 286, 121, 17 V; quinidine: 325, 307, 28 V; spironolactone: 417, 341, 20 V; TEA: 130, 86, 26 V; verapamil: 455, 165, 26 V; and vigabatrin: 130, 112, 10 V. For each analyte, the area of the intensity versus time peak was integrated and divided by the area of the MPP* peak to yield the analyte response ratio. Linear calibration curves ($R^2 > 0.99$) were constructed from at least six standards that were prepared using control cell lysates as solvent. Sample analyte content was calculated from the analyte response ratio and the slope of the calibration curve, obtained by linear regression.

Protein was measured by the bicinchoninic acid assay with bovine serum albumin as standard. The protein content of MS samples was estimated from four to six mixed cell dishes.

**Expression Profiling by Real-Time PCR.** For relative quantification of OCTN2h mRNA levels in human cells and tissues, a TaqMan real-time assay was used on a 7900 HT Sequence Detection system (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s protocols. For first-strand cDNA synthesis, 85 μg of total RNA was incubated for 1.5 h at 37°C with 2 U/μl Omniscript reverse transcriptase (QIAGEN GmbH, Hilden, Germany) in the supplied buffer plus 9.5 μM random hexamer primer, 0.5 mM dNTP, and 3000 U of RNaseOUT (Invitrogen) in a final volume of 680 μl. The resulting cDNA was diluted 1:10 with water and directly used in PCR (5 μl/reaction). A PCR reaction mix (25 μl) contained, in addition to cDNA, 0.2 μM OCTN2h amplification primer (forward, 5'-CAA AGA CCA ATG AGA TTG TT and reverse, 5'-CTG TTT CTT CTT GGA ACT TAG GTC TT), 0.2 μM 5-carboxyfluorescein-5-carboxytetramethylrhodamine-labeled OCTN2h probe (5'-CTC ACT ACT TTT GAC CCC AGT, 0.2 mM dATP, dCTP, dGTP, and dUTP, 5.5 mM MgCl2, 0.01 U/μl AmplErase uracil N-glycosylase, and 0.025 U/μl AmpliTaq Gold DNA polymerase in TaqMan buffer A. The thermal protocol was set to 2 min at 50°C, followed by 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To normalize the amount of cDNA per assay, the expression of multiple housekeeping genes (e.g., hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, and ß-actin) was measured in parallel assays. Relative expression of OCTN2h was then calculated using the normalized expression values.

**Calculations and Statistics.** The clearance is directly proportional to $K_m$ and thus a valid measure of efficiency of transport (provided that the substrate concentration is much smaller than the respective $K_m$) (Gründemann et al., 1999; Schömig et al., 2006). The clearance equals initial rate of specific uptake (uptake mediated by expressed carrier) divided by substrate concentration. Specific uptake equals total uptake minus uptake into control cells (nonspecific uptake).

Analysis of saturation curves has been reported previously (Schömig et al., 1993). $K_n$ and $V_m$ values are given as geometric mean with 95% confidence interval. In the figures, symbols and bars represent arithmetic mean ± S.E.M. ($n = 3$) if not indicated otherwise in the legend. The unpaired $t$ test was used for test for significance; $P$ values are two-tailed.

**Drugs.** The drugs and sources follow: ASP$: (336408; Sigma-Aldrich, Munich, Germany), t-carnitine (C-0283; Sigma-Aldrich), cephaloridine (Fluka 53477; Sigma-Aldrich), l-(+)-ergothioneine (F-3455; Bachem, Bubendorf, Switzerland), gabapentin (G154; Sigma-Aldrich), 1-methyl-4-phenylpyridinium iodide (D-048; Sigma-Aldrich), mildronate tablets (JSC Grindeks, Riga, Latvia), pyriformine base (P-5514; Sigma-Aldrich), quinidine (Q-0875; Sigma-Aldrich), spironolactone (S3378; Sigma-Aldrich), verapamil-HCl (Abbott Laboratories, Liestal, Switzerland), and vigabatrin (V8261; Sigma-Aldrich). L-Carnitine hydrochloride (H-5, 3.1 kBq/pmol, ART-293; ARC, St. Louis, MO) was used as radiotracer. All the other chemicals were at least of analytical grade.

**Results**

**Cloning of OCTN2ch.** To attain broad evolutionary coverage, it was our aim to analyze the substrate specificity of the OCTN2 orthologs from human, rat, and chicken. So far, no functional characterization of the OCTN2ch cDNA has been reported. OCTN2ch was cloned from chicken (Gallus gallus) kidney by RT-PCR based on GenBank entry NM_001045828. The amino acid sequence coded by our consensus cDNA is identical to the GenBank sequence except for amino acid position 165; here, all our cDNA clones (n = 8) indicate an Asp (GAC) instead of Tyr (TAC). Indeed, Asp (D) is more plausible because it is fully conserved with other orthologs at this position. Overall, the amino acid sequences of OCTN2ch and OCTN2h are markedly homologous (76% identity, 82% similarity).

The OCTN2ch cDNA was inserted into our pEBTetD expression vector and transfected into 293 cells. pEBTetD is an episomal Epstein-Barr plasmid vector for doxycycline-inducible protein expression in human cell lines based on the simple tetracycline repressor (Bach et al., 2007). Expression is turned on by addition of 1 μg/ml doxycycline to the culture medium for approximately 20 h. With, for example, the ETT from human, this system provides a high rate of carrier-mediated transport in the on-state (≈ 100%) and a low rate (4%) in the off-state (Bach et al., 2007).

Uptake of t-carnitine by OCTN2ch was saturable (Fig. 2) with a $K_m$ of 21 (95% confidence interval: 14–31) μM, which for a transporter signifies high affinity. In good agreement, $K_n$ values of 4 (Tamai et al., 1998), 4 (Ganapathy et al., 2000), 5 (Wagner et al., 2000), and 25 μM (Sekine et al., 1998) have been reported for the human and rat orthologs.

**Transport of Drugs by OCTN2 from Human, Rat, and Chicken.** A 293 cell line for the inducible expression of OCTN2 from rat was available from a previous study (Gründemann et al., 2005). The cDNA of OCTN2h was cloned from kidney total RNA by RT-PCR based on GenBank entry NM_0030960, inserted into pEBTetD, and transfected into 293 cells. The amino acid sequence coded by our cDNA is identical to the GenBank sequence.
Fig. 3. Transport of [3H]carnitine by OCTN2 from human, rat, and chicken. Initial rates of total uptake of [3H]carnitine (0.1 μM) into 293 cells with (“on”) and without (“off”) heterologous expression of OCTN2 were determined (paired assays, 37°C, 1 min). The difference of uptake rates was divided by the substrate concentration to yield the transporter-mediated clearance as shown (mean ± S.E.M.; n = 7–8).

With these three cell lines, transporter-mediated (= specific) uptake of selected drugs (Fig. 1) was determined, using paired dishes, as total uptake into induced cells minus total uptake into noninduced cells. In each experiment with each carrier, uptake of carnitine was measured in parallel to gauge active transporter. Figure 3 shows the aggregate of these reference measurements. It indicates that all the OCTN2 orthologs were very active in carnitine transport, with an average clearance (= velocity of uptake divided by substrate concentration) of at least 60 ± 7 μl min/mg protein (OCTN2 from rat). Figure 4 shows total uptake of drugs into cells with and without expression of OCTN2h. It illustrates the widely different levels of accumulation of compounds with uninduced control cells. Similar results were obtained with rat and chicken carriers (data not shown). With these data, carrier-mediated drug transport was calculated as explained above; Fig. 5 shows transport efficiency relative to carnitine (= 100%). It is apparent that only mildronate (at 160% on average) qualifies as a good substrate for OCTN2 from human, rat, and chicken. The other 10 compounds cannot be considered substrates: carrier-mediated uptake was mostly not significantly different from zero or was minute relative to carnitine (TEA and ergothioneine). All the results were consistent across species.

The high apparent uptake of some drugs evident from Fig. 4 was investigated further. Accumulation of verapamil was examined with paired dishes at 4°C (incubation on ice) and at 37°C; as control, uptake of carnitine was measured with paired dishes. OCTN2-mediated uptake of carnitine was abolished almost entirely at 4°C (Fig. 6, left); with OCTN2h-expressing cells the residual total accumulation was 2.7% only. Thus, at 37°C carnitine accumulation with OCTN2-expressing cells via binding or diffusion is negligible. By contrast, with verapamil (Fig. 6, right) there was still marked accumulation at 4°C (34% residual total uptake). Thus, we conclude that at least a major portion of verapamil accumulation is caused by diffusion or membrane insertion or binding. Note that one would not expect 100% at 4°C because these processes also depend on temperature, but to a lesser extent than protein activity.

Analysis of Mildronate Transport. Transport of mildronate was analyzed in more detail with the above-described cell lines; all the cell lysates were analyzed by LC/MS/MS. The time course of uptake (Fig. 7) reveals that mildronate accumulates to much higher levels in induced cells compared with uninduced control cells. With an extracellular mildronate concentration of 10 μM, cells with heterologous expression of OCTN2h turned on reached a steady intracellular concentration of 830 μM after approximately 30 min [calculated with an intracellular water space of 6.7 μl/mg protein (Martel et al., 1996) and a conjectured rate of carrier-expressing cells of 100%]. Thus, the carrier catalyzes intracellular accumulation of mildronate by a factor of approximately 80 (or higher because the true expression rate will be lower than 100%) over the external medium. By contrast, off-state cells reached an intracellular plateau concentration of 57 μM only; this 5.7-fold accumulation over the external medium is probably caused by leak expression of the cloned transporter (on the order of 3–5% of the on-state expression, see above) and by low endogenous expression of the transporter (see Fig. 10). Similar results were obtained with OCTN2ch (data not shown). To approximate initial rates of uptake, an uptake time of 1 min was used in subsequent experiments.

Saturation analysis (Fig. 8) of OCTN2-catalyzed mildronate uptake revealed $K_m$ values of 26 (95% confidence interval: 14–46) μM for the human variant and 29 (22–38) μM for the chicken variant. Clearances, calculated from $V_{	ext{max}}/K_m$, were high (155 μl/min/mg protein for human
and 170 μl/min/mg protein for chicken) and thus confirm mildronate as an excellent substrate. Total initial uptake into uninduced control cells, presumably via leak expression plus endogenous carrier (see Fig. 10) plus diffusion, was substantial (= 11 μl/min/mg protein on average; n = 25).

Still, at an extracellular mildronate concentration of, for example, 1 μM, the expression of OCTN2 increases initial cellular uptake by a factor of approximately 14.

Transport of carnitine or acyl-carnitines by OCTN2 is strongly stimulated by sodium ions (Wagner et al., 2000). Mildronate does not contain a hydroxyl or acyl but a hydrazinium residue (Fig. 1). To investigate whether this structural deviation affects utilization of the driving force, mildronate uptake by OCTN2 was measured as a function of the sodium ion concentration. In these experiments, Na⁺ in the uptake buffer was iso-osmotically replaced by N-methyl-D-glucamine. Our results with OCTN2 both from human and chicken indicate that carrier-mediated uptake of mildronate is strongly stimulated by sodium ions in the uptake buffer (Fig. 9, left). The sodium affinity constant was 14 (95% confidence interval: 11–18) mM for the
human and 18 (13–24) mM for the chicken variant. By contrast, with carnitine, the uptake of which by OCTN2h was analyzed in the same setup for reference (Fig. 9, right), the affinity constant was 4.5 (2.6–7.8) mM. Thus, coupling of transport to Na\(^+\) is less efficient for mildronate by a factor larger than 3. However, at high (=physiological) Na\(^+\) concentrations, the difference in transport stimulation is small (e.g., 90 versus 97% for OCTN2h at 125 mM Na\(^+\)).

Uptake of mildronate (10 \(\mu\)M, 1 min), like carnitine (Wagner et al., 2000), was only slightly affected by the uptake buffer pH; the pH was acutely altered by washing cells twice before addition of mildronate. Specific uptake decreased steadily from pH 8.5 to 6.5 for OCTN2h (pH 8.5: 0.91 \(\pm\) 0.02 nmol/min/mg protein; pH 6.5: 0.66 \(\pm\) 0.02 nmol/min/mg protein) and for OCTN2ch (pH 8.5: 1.19 \(\pm\) 0.04 nmol/min/mg protein; pH 6.5: 0.59 \(\pm\) 0.04 nmol/min/mg protein).

**Analysis of OCTN2h mRNA Levels.** The expression of OCTN2 was investigated by real-time RT-PCR in several human tissues (Fig. 10). The highest levels were found in kidney, ileum, breast, small intestine, skeletal muscle, and ovary (>20% relative to kidney). Note that some heart and central nervous system tissues contain OCTN2 mRNA at high levels, whereas little mRNA was detected in aorta, arteries, bone marrow, and peripheral leukocytes.

**Discussion**

OCTN2 has repeatedly been suggested to play an important role in the transport of drugs (Wagner et al., 2000). However, our present results show that OCTN2 cannot translocate with significant rate any of the previously suggested drugs. The only exception was mildronate, which is closely related in structure to carnitine, the physiological substrate of the carrier. Thus, in striking contrast to what can be learned from current original and review literature, OCTN2 should not be considered a general drug transporter.

To obtain meaningful comparative data on drug transport, we have strictly measured initial rates of uptake under physiological conditions with three OCTN2 orthologs, i.e., from human, rat, and chicken. Uptake of carnitine was always measured as reference in parallel. Na\(^+\)-independent transport (Ohashi et al., 2001) was not examined because in vivo we consider it irrelevant. Perfectly matching control cell lines without transporter expression were procured by using an inducible expression system. A key methodological aspect of our study was the use of LC/MS for quantification of substrate accumulation. This allowed us to analyze all the suggested drugs as unlabeled compounds, which are much easier to obtain and cheaper than radiolabeled compounds. LC/MS is not as sensitive as radiotracer detection, and thus we regularly use in our functional assays, depending on the analyte, substrate concentrations of 1 or 10 \(\mu\)M, by contrast to 0.1 \(\mu\)M in our radiotracer uptake experiments. This is no real drawback because as long as the substrate concentration is much lower than the \(K_{av}\), the clearance (= v/S) is independent of substrate concentration. However, LC/MS has the advantage of high detection specificity because molecules in MS/MS mode will be detected only if they pass two separate mass filters. By contrast, the uptake of radiolabeled fragments from the chemical decay of radiotracers may generate false uptake signals in liquid scintillation counting. Nevertheless, uptake of carnitine was determined by radiotracer assay here, which gives a much better signal-to-noise ratio because of high endogenous carnitine levels in 293 cells. Note that 293 cells, like other cell lines (Wu et al., 1998), express OCTN2 endogenously (Fig. 10) and thus have
some original capacity for carnitine and mildronate uptake. However, our functional assays indicate that the constant background uptake (which also consists of leak expression and diffusion) observed with off-state cells was much lower than uptake mediated by exogenous carrier: for example, in the experiment that shows saturation of mildronate uptake (Fig. 8), background clearance was 11 μL/min/mg protein, and expressed clearance was 155 or 170 μL/min/mg protein, respectively. Thus, in the present study, the signal-to-noise-ratio was sufficient for quantitative analysis of OCTN2 transport activity.

How good is the evidence in previous reports that supports OCTN2-mediated drug transport? With stably transfected Madin-Darby canine kidney II cells expressing OCTN2h, an increase of radiotracer accumulation versus control cells was observed with 10 nM [3H]verapamil (approximately 50% increase) and 8 nM [3H]spiromolactone (approximately +40%) (Grube et al., 2006). Likewise, with stably transfected 293 cells expressing OCTN2h, an increase of radiotracer accumulation was detected with 18 nM [3H]pyrilamine (+32%), 33 nM [3H]quinidine (+27%), and 6 nM [3H]verapamil (+22%) (Okahashi et al., 1999). Similar results were reported for [3H]pyrilamine and [3H]verapamil in another publication (Ohashi et al., 2001). With a factor of increase between 1.2 and 1.5, all these gains are small. One possible explanation would be (see Fig. 6), rather than transport, mere binding of radiolabel or fragments thereof to the carrier (Harlfinger et al., 2005; Grigat et al., 2007). Binding should not be of concern in our LC/MS assays because we have used much higher concentrations (= 10 μM) of freshly dissolved unlabeled compounds.

Accumulation of unlabeled cephaloridine by HeLa cells stably transfected to express OCTN2 from human was detected by UV absorption at 254 nm (high-performance liquid chromatography assay) (Ganapathy et al., 2000); the antibiotic was not detected in control cell lysates. However, note that no initial rates of uptake were determined in that experiment because cells were incubated very long (1 h) with a very high concentration (1 mM) of cephaloridine. Thus, the efficiency of transport relative to carnitine cannot be calculated from that report and could indeed have been very low.

Uptake of ASP+ into primary human airway epithelial cells was determined by measurement of fluorescence (Horvath et al., 2007). The transport mechanism was thoroughly characterized, and it was concluded that OCTN2 is the underlying carrier. Unfortunately, the report lacks direct demonstration of ASP+ transport by heterologously expressed OCTN2.

TEA was the first compound to be reported as substrate of OCTN2 (Wu et al., 1998). Transport has been proved by trans-stimulation experiments (Ohashi et al., 2001), and our own results confirm that OCTN2 is in fact expressed in the brain (Fig. 10). It is evident now that the functional designation OCTN2 is highly misleading. Sekine et al. (1998) have previously suggested the designation CT1, but CT1 is already in use for an unrelated creatine transporter (SLC6A8), as are CRT (another SLC6A8 alias) and CNT (concentrative nucleoside transporter, e.g., SLC28A1). For a meaningful and unambiguous functional label, we suggest CTT, from CARNITINE TRANSPORTER.

Mildronate was previously reported as competitive inhibitor of OCTN2, but transport was not examined (Grube et al., 2006). Here we provide the first direct evidence that mildronate is an excellent substrate of OCTN2; efficiency of transport was even higher than that of carnitine (Fig. 5). Cellular accumulation (Fig. 7), transporter affinity (Fig. 8), and sodium dependence (Fig. 9) indicate that mildronate very much mimics carnitine, although it lacks the hydroxyl group and contains a hydrazinium residue.

Mildronate has been shown to lower plasma carnitine levels in rats after 14 days of treatment by a factor of 3 (Liepinsh et al., 2006). Under ischemic conditions, it improves cardiac cell survival (Liepinsh et al., 2006). Hence, it is currently approved for clinical use in some countries as a cardioprotective agent (Dambrova et al., 2002). The mechanism of action of mildronate is not entirely clear. One component likely is intracellular inhibition of carnitine biosynthesis at γ-butyrobetaine (GBB) hydroxylase with subsequent accumulation of GBB, depletion of carnitine (Liepinsh et al., 2006), and interference with mitochondrial metabolism. In addition, mildronate inhibits carnitine reabsorption by the kidney (Kuwajima et al., 1999) and transport into muscle cells (Georges et al., 2000), most likely at OCTN2. Our present results establish that OCTN2 is in fact a key target of mildronate because OCTN2 in the plasma membrane ensures efficient access of mildronate to intracellular targets. Thus, the treatment of brain circulation disorders with mildronate (Sjakste et al., 2005) may rely on conspicuous expression of OCTN2 in the brain (Fig. 10). It remains to be established whether OCTN2 is in fact expressed in cells of the blood-brain barrier.

In conclusion, the present rigorous analysis has clarified that OCTN2 is not a general drug transporter but rather a highly specific transporter of carnitine and related compounds [such as glycine betaine (Wagner et al., 2000) and GBB (Sekine et al., 1998; Gründemann et al., 2005)]. It is clear that some drugs—depending on individual affinities and concentrations—will block OCTN2 simply by binding to its surface. However, it must be stressed that this will not hinder transport of drugs but merely transport of carnitine. Out of such inhibition, secondary carnitine deficiency may develop; this has been suggested, e.g., for emetine (Wagner et al., 2000). The carnitine congener mildronate, by contrast to the other drugs, is an excellent substrate of OCTN2. We conclude that OCTN2 controls cellular entry of mildronate and thus its clinical effects.
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References


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