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The carnitine transporter *SLC22A5* is not a general drug transporter, but it efficiently translocates mildronate

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Running title: Analysis of drug transport mediated by OCTN2

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Abbreviations:

ASP^+, *trans*-4-[4-(Dimethylamino)styryl]-1-methylpyridinium iodide; ETT, ergothioneine transporter; GBB, gamma-butyrobetaine; K_D, dissociation constant; LC, liquid chromatography; MPP^+, 1-methyl-4-phenylpyridinium; MS, mass spectrometry; OCTN2, novel organic cation transporter type 2; RT-PCR, reverse transcriptase polymerase chain reaction; TEA, tetraethylammonium.

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Abstract

In addition to its function as carnitine transporter, 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 orthologues from human, rat, and chicken were expressed in 293 cells using an inducible expression system. Uptake of 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 efficently. 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, since 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 RT-PCR in kidney, ileum, breast, small intestine, skeletal muscle and ovary, but also in some heart and CNS tissues.

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Introduction

OCTN2 (human gene symbol *SLC22A5*) is widely recognized as a carnitine transporter, since 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 in order to fuel β-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 sub-adequate capacity for fat processing and energy production develops into characteristic symptoms which 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 demonstrated 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 ("organic cation transporter, novel") 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 favourable 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

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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 radiolabeled substrates. These drugs are tetraethylammonium (TEA) (Ohashi et al., 2001), verapamil (Ohashi et al., 1999; Ohashi et al., 2001; Grube et al., 2006), pyrilamine (Ohashi et al., 1999; Ohashi et al., 2001), quinidine (Ohashi et al., 1999), cephaloridine (Ganapathy et al., 2000), spironolactone (Grube et al., 2006), and mildronate (Grube et al., 2006). However, a rigorous, comparative, and comprehensive analysis of transport efficiency of OCTN2 has not been available so far.

The purpose of the present study was therefore to scrutinize transport of the above drugs by OCTN2. We also examined the proposed substrate ASP^+, the uptake of which was previously measured by fluorescence (Horvath et al., 2007), gabapentin and vigabatrin, which are related in structure to carnitine, and ergothioneine, the physiological substrate of the related ergothioneine transporter (ETT, gene symbol *SLC22A4*). All compound structures are shown in Fig. 1.

In order to measure transport activity reliably, our experimental approach differed from the above reports in 3 major aspects: i) We have used an inducible expression system. The straightforward way to determine carrier-mediated transport is to compare substrate accumulation between cells with and without heterologous transporter expression. With 2 separately selected cell lines as in the above reports, there is a risk of non-identical background of substrate accumulation which 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. ii) We have used freshly dissolved unlabeled compounds in our uptake experiments and have quantitated these by LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry). This ensures intactness and highly-selective detection of substrate molecules. By contrast, there is an inevitable chemical decay of radiotracers and uptake of resulting

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radiolabeled decomposition fragments into cells can not be discerned from uptake of true substrate in liquid scintillation counting. iii) All substrates were probed with OCTN2 orthologues 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; this, however, 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.

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Methods

Plasmid constructs

The cDNAs of OCTN2 from chicken (OCTN2ch) and OCTN2 from human (OCTN2h) were generated by RT-PCR, cloned into pUC19, fully sequenced, and finally inserted into expression vector pEBTetD. Human kidney total RNA was from Clontech, Saint-Germain-en-Laye, France. The cDNA sequence of OCTN2ch corresponds to GenBank entry NM_001045828 except for a single nucleotide substitution as detailed in Results. The 5'-interface between pEBTetD and cDNA is **GTTTAAACTT AAGCTT** GCCACC ATGCGGGACTACGAG (polylinker in bold, cDNA underlined); the 3'-interface is ACAGCTTTTTAA **CTCGAG CGATCGC**. The cDNA sequence of OCTN2h corresponds to GenBank entry NM_003060. The 5'-interface is **GTTTAAACTT AAGCTT** GCCACC ATGCGGGACTACGAC; the 3'-interface is ACAGCCTTCTAA **CTCGAG CGATCGC**.

Cell culture

293 cells (ATCC CRL-1573; also known as HEK-293 cells), a transformed cell line derived from human embryonic kidney, were grown at 37 °C in a humidified atmosphere (5% CO_2) in plastic culture flasks (Falcon 3112, Becton Dickinson, Heidelberg, Germany). The growth medium was Dulbecco's Modified Eagle Medium (Life Technologies 31885-023, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Cölbe, Germany). Medium was changed every 2-3 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 3 µg/ml puromycin (PAA Laboratories) to ascertain plasmid maintenance. To turn on protein expression, cells were cultivated for at least 20 h in regular

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growth medium supplemented with 1 µg/ml doxycycline (195044, 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, 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 mmol/l NaCl, 25 mmol/l HEPES-NaOH pH 7.4, 5.6 mmol/l (+)glucose, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $CaCl_2$, and 1.2 mmol/l $MgSO_4$. After preincubation for at least 20 minutes in 4 ml of uptake buffer, the buffer was replaced with 2 ml of unlabeled substrate or 3 ml of ^{3}H-carnitine (at 0.1 µmol/l) in uptake buffer. Incubation was stopped after 1 min by rinsing the cells four times each with 4 ml ice-cold uptake buffer. Radioactivity was determined, after cell lysis with 0.1% v/v Triton X-100 in 5 mmol/l TRIS-HCl pH 7.4, by liquid scintillation counting. For LC-electrospray ionization-MS/MS analysis, the cells were solubilized with 4 mmol/l $HClO_4$ or methanol and stored at -20 °C. After centrifugation (1 min, 16000 x g, 20 °C) of the thawed lysates, 100 µl of the supernatant was mixed with 10 µl unlabeled 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 Electron, Dreieich, Germany). 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: methanol; 5 min 90% to 30% A, 5 min 30% A, 4 min 30% to 90% A) was used with a Waters Atlantis T3 reversed phase column (length 100 mm, diameter 3 mm, particle size 5 µm). For all other compounds, isocratic chromatography (70% methanol and 30% 0.1% formic acid) was employed with a Waters Atlantis HILIC silica column (length 50

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mm, diameter 3 mm, particle size 5 µm). For quantification by SRM (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 SRM (m/z parent, m/z fragment, collision energy): ASP^+: 239, 194, 38 V; cephaloridine: 416, 337, 12 V; ergothioneine: 230, 186, 16 V; gabapentin: 172, 154, 18 V; mildronate: 147, 58, 34 V; MPP^+: 170, 128, 25 V; pyrilamine: 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 vs. 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 which 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 BCA assay with bovine serum albumin as standard. The protein content of MS samples was estimated from 4-6 matched cell dishes.

Expression profiling by real-time PCR

For relative quantitation of OCTN2h mRNA levels in human cells and tissues, a TaqMan™ real-time PCR assay was employed 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, Hilden, Germany) in the supplied buffer plus 9.5 µM random hexamer primer, 0.5 mM per dNTP, and 3000 U 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 per reaction). A

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PCR reaction mix (25 µl) contained, in addition to cDNA, 0.2 µM per OCTN2h amplification primer (forward: 5'-CCA AAG CCA ATG GGA TTG TT; reverse: 5'-CTG TTG CTT CTT GGA ACT TAG GTC TT) , 0.2 µM FAM™/TAMRA™-labeled OCTN2h probe (5'-CCT TCC ACT ATC TTT GAC CCG AGT), 0.2 mM of dATP, dCTP, dGTP, and dUTP, 5.5 mM MgCl2, 0.01 U/µl AmpErase® 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 β-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_{cat}/K_m (k_{cat}: turnover number) 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 (= non-specific uptake).

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

Drugs

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ASP^+ (336408, Sigma-Aldrich, Munich, Germany), L-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, Latvia), pyrilamine (P-5514, Sigma-Aldrich), quinidine (Q-0875, Sigma-Aldrich), spironolactone (S3378, Sigma-Aldrich), verapamil-HCl (Knoll AG (Abbott Laboratories), Liestal, Switzerland), vigabatrin (V8261, Sigma-Aldrich). L-carnitine hydrochloride (H-3, 3.1 kBq/pmol, ART-293, ARC, St. Louis, MO, USA) was used as radiotracer. All other chemicals were at least of analytical grade.

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Results

Cloning of OCTN2 from chicken

To attain broad evolutionary coverage, it was our aim to analyze the substrate specificity of the OCTN2 orthologues from human, rat, and chicken. So far, no functional characterization of the OCTN2ch (ch = chicken) 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 of our cDNA clones (n = 8) indicate an Asp (GAC) instead of Tyr (TAC). Indeed, Asp (D) is more plausible, since it is fully conserved with other orthologues at this position. Overall, the amino acid sequences of OCTN2ch and OCTN2h (h = human) 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 about 20 h. With *e.g.* the ergothioneine transporter from human (ETTh), 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 L-carnitine by OCTN2ch was saturable (Fig. 2) with a K_m of 21 (95% confidence interval, 14 - 31) µmol/l which for a transporter signifies high affinity. In good agreement, K_m-values of 4 (Tamai et al., 1998), 4 (Ganapathy et al., 2000), 5 (Wagner et al., 2000), and 25 µmol/l (Sekine et al., 1998) have been reported for the human and rat orthologues.

Transport of drugs by OCTN2 from human, rat, and chicken

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A 293 cell line for the inducible expression of OCTN2 from rat (OCTN2r) was available from a previous study (Gründemann et al., 2005). The cDNA of OCTN2 from human was cloned from kidney total RNA by RT-PCR based on GenBank entry NM_003060, inserted into pEBTetD, and transfected into 293 cells. The amino acid sequence coded by our cDNA is identical to the GenBank sequence.

With these 3 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 non-induced cells. In each experiment with each carrier, uptake of carnitine was measured in parallel in order to gauge active transporter. Figure 3 shows the aggregate of these reference measurements. It indicates that all OCTN2 orthologues were very active in carnitine transport, with an average clearance (= velocity of uptake divided by substrate concentration) of at least 60 ± 7 µl min^{-1} mg $protein^{-1}$ (OCTN2 from rat). Figure 4 shows total uptake of drugs into cells with and without expression of OCTN2 from human. It illustrates the widely different levels of accumulation of compounds with uninduced control cells. Similar results were obtained with rat and chicken carriers (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 can not be considered substrates: carrier-mediated uptake was mostly not significantly different from zero or was minute relative to carnitine (TEA and ergothioneine). All 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

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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). We thus conclude that at least a major portion of verapamil accumulation is due to diffusion or membrane insertion or binding. Note that one would not expect 100% at 4 °C, since 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 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 to uninduced control cells. With an extracellular mildronate concentration of 10 µmol/l, cells with heterologous expression of OCTN2h turned on reached a steady intracellular concentration of 830 µmol/l after about 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 about 80 (or higher, since 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 µmol/l 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 OCTN2 from chicken (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) µmol/l for the human variant, and 29 (22 - 38) µmol/l for the chicken variant. Clearances, calculated from V_{max}/K_m, were high (155 µl min^{-1} mg $protein^{-1}$ for human, and 170 µl min^{-1} mg $protein^{-1}$ for chicken) and thus confirm mildronate as an excellent

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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^{-1} mg $protein^{-1}$ on average; n = 25). Still, at an extracellular mildronate concentration of e.g. 1 µmol/l, the expression of OCTN2 increases initial cellular uptake by a factor of about 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 isoosmotically 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) mmol/l for the human, and 18 (13 - 24) mmol/l for the chicken variant. By contrast, with carnitine, the uptake of which by OCTN2h was analyzed in the same set-up for reference (Fig. 9 right), the affinity constant was 4.5 (2.6 - 7.8) mmol/l. 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% *vs.* 97% for OCTN2h at 125 mmol/l Na^+).

Uptake of mildronate (10 µmol/l, 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 ± 0.02 nmol min^{-1} mg $protein^{-1}$; pH 6.5: 0.66 ± 0.02 nmol min^{-1} mg $protein^{-1}$) and for OCTN2ch (pH 8.5: 1.19 ± 0.04 nmol min^{-1} mg $protein^{-1}$; pH 6.5: 0.59 ± 0.04 nmol min^{-1} mg $protein^{-1}$).

Analysis of OCTN2h mRNA levels

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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 CNS tissues contain OCTN2 mRNA at high levels, whereas little mRNA was detected in aorta, arteries, bone marrow, and peripheral leukocytes.

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Discussion

OCTN2 has repeatedly been suggested to play an important role in the transport of drugs (Wagner et al., 2000). However, our present results demonstrate that OCTN2 can not 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.

In order to obtain meaningful comparative data on drug transport, we have strictly measured initial rates of uptake under physiological conditions with 3 OCTN2 orthologues, *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, since *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 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 µmol/l, by contrast to 0.1 µmol/l in our radiotracer uptake experiments. This is no real drawback, since as long as the substrate concentration is much lower than the K_m, the clearance (= v/S) is independent of substrate concentration. However, LC-MS has the advantage of high detection specificity, since molecules in MS/MS mode will be detected only if they pass 2 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

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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 demonstrates saturation of mildronate uptake (Fig. 8), background clearance was 11 µl min^{-1} mg $protein^{-1}$, and expressed clearance was 155 or 170 µl min^{-1} mg $protein^{-1}$, 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 MDCKII cells expressing OCTN2 from human, an increase of radiotracer accumulation *vs.* control cells was observed with 10 nmol/l ^{3}H-verapamil (ca. 50% increase) and 8 nmol/l ^{3}H-spironolactone (+ ca. 40%) (Grube et al., 2006). Similarly, with stably transfected 293 cells expressing OCTN2 from human, an increase of radiotracer accumulation was detected with 18 nmol/l ^{3}H-pyrilamine (+ 32%), 33 nmol/l ^{3}H-quinidine (+ 27%), and 6 nmol/l ^{3}H-verapamil (+ 22%) (Ohashi et al., 1999). Similar results were reported for ^{3}H-pyrilamine and ^{3}H-verapamil in another publication (Ohashi et al., 2001). With a factor of increase between 1.2 and 1.5, all of these gains are small. One possible explanation would be (*cf.* 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 since we have used much higher concentrations (= 10 µmol/l) 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 (HPLC assay) (Ganapathy et al., 2000); the antibiotic was not detected in control cell lysates. Note however that no inital rates of uptake were determined in that experiment, since cells were incubated very long (1 h) with a very high

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concentration (1 mmol/l) of cephaloridine. Thus, the efficiency of transport relative to carnitine can not 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 TEA is indeed transported (Gründemann et al., 2005). However, efficiency of transport relative to carnitine is very low (Fig. 5), *i.e.* (as percentage) 0.2 ± 0.6 (human), 0.5 ± 0.1 (rat), and 0.8 ± 0.2 (chicken). Similarly, in a previous report with 293 cells expressing OCTN2 from human (Ohashi et al., 1999), the rate of TEA transport was only 0.2% relative to carnitine (see Table 2 in the cited work). No significant TEA transport was noted by another group (Tamai et al., 1998). By contrast, in another publication (Ohashi et al., 2001), uptake of TEA and carnitine into 293 cells stably transfected to express OCTN2 from mouse would seem to be of similar magnitude (see Fig. 2A in the cited work). However, if different uptake times are taken into account and if initial rates of uptake are assumed (which was not documented), then the TEA transport rate was only 3% relative to carnitine (see Table 3 in the cited work). Thus, evidence from other groups also suggests that TEA is only a poor substrate of OCTN2.

Taken together, our literature review suggests that previous evidence in support of OCTN2-mediated drug transport is weak. It is thus not implausible that our present results based on refined methodology have failed to demonstrate drug transport. Differences in the accumulation of radiolabel between cells with and without heterologous expression of transporter must be interpreted cautiously. In case of doubt only trans-stimulation experiments can provide definitive

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proof of translocation of substrate (Stein, 1986). Such proof is available solely for TEA (see above).

Gabapentin has been reported as substrate of the related ergothioneine transporter ETT (Urban et al., 2008). Our results establish that neither gabapentin nor vigabatrin are translocated by OCTN2. We speculate that this may be due to the lack of a tetraalkylammonium structure. The lack of transport of ergothioneine underscores the exquisite selectivity of OCTN2.

It is evident now that the functional designation OCTN2 is highly misleading. Sekine *et al.* have previously suggested the designation CT1 (Sekine et al., 1998), 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 $C_{ARNI}T_{INE}$ $T_{RANSPORTER}$.

Mildronate was previously reported as competitive inhibitor of OCNT2, 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). It is hence 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 gamma-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

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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, since 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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Footnotes

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Figure legends

Fig. 1. Compound structures.

Fig. 2. Saturation of uptake of ^{3}H-carnitine mediated by OCTN2 from chicken. An uptake period of 1 min was chosen to approximate initial rates of transport. Carrier-mediated uptake was calculated as the difference of total uptake between cells with and without heterologous carrier expression. Uptake into control cells (= OCTN2 expression off) could be described as a linear function of carnitine concentration; the slope was 4.1 µl min^{-1} mg protein^{-1}. Inset: Eadie-Scatchard transformation.

Fig. 3. Transport of ^{3}H-carnitine by OCTN2 from human, rat, and chicken. Initial rates of total uptake of ^{3}H-carnitine (0.1 µmol/l) 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 ± SEM; n = 7-8).

Fig. 4. Total accumulation of drugs in 293 cells with or without heterologous expression of OCTN2 from human. Cells grown in dishes (paired assays) were incubated for 1 min at 37 °C with 1.0 (quinidine and verapamil) or 10 µmol/l drug in uptake buffer, washed, and lysed. The drug content of cell lysates was determined by LC-MS/MS and expressed as clearance. A correction for endogenous ergothioneine (Grigat et al., 2007) was not necessary here, since OCTN2 is virtually inactive for this compound. Uptake of carnitine was measured by radiotracer assay as explained for Fig. 3.

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Fig. 5. Efficiency of transport of drugs by OCTN2 from human, rat, and chicken. For each compound, the transporter-mediated clearance (*cf.* Fig. 4) was divided by the corresponding carnitine clearance (*cf.* Fig. 3). Statistical significance of increased compound accumulation in cells with heterologous transporter expression *vs.* without is denoted as follows: *: $0.05 \geq P > 0.01$; **: $0.01 \geq P > 0.001$; ***: $P \leq 0.001$.

Fig. 6. Accumulation at 37 °C or at 4 °C of verapamil or carnitine in 293 cells with or without heterologous expression of OCTN2 from human. Cells grown in dishes (paired assays) were incubated for 1 min with 1.0 µmol/l verapamil or 0.1 µmol/l carnitine in uptake buffer at the indicated temperature, washed, and lysed. Verapamil and carnitine were measured as explained (Figs. 3, 4).

Fig. 7. Time course of mildronate uptake into cells with and without heterologous expression of OCTN2h. Cells grown in dishes were incubated at 37 °C with 10 µmol/l mildronate for the indicated time. Exponential functions were fitted to the experimental data (Schömig et al., 1992).

Fig. 8. Saturation of specific uptake of mildronate mediated by OCTN2 from human and chicken. An uptake period of 1 min was used to approximate initial rates of transport. Specific uptake was calculated as the difference of total uptake between cells with and without heterologous carrier expression. Uptake into control cells (= OCTN2 expression off) could be described as a linear function of mildronate concentration; the slope was 12.6 and 10.0 µl min^{-1} mg $protein^{-1}$ for OCTN2h and OCTN2ch cells, respectively.

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Fig. 9. Sodium dependence of uptake of mildronate and carnitine mediated by OCTN2. Cells were rapidly washed twice with modified uptake buffers in which N-methyl-D-glucamine isoosmotically substituted Na^+ as indicated and then assayed for solute uptake in the same buffer with an uptake period of 1 min. The modified Hill function $v = V_0 + V_{lim}/(1 + K_D/c_{Na^+})$ was fitted to the data, with v = uptake velocity, V_0 = basal uptake velocity, V_{lim} = maximum Na^+-stimulated uptake velocity, K_D = Na^+ affinity constant, and c_{Na^+} = sodium ion concentration. This function, which adequately described experimental data, implies a Hill coefficient of 1 as reported previously for carnitine (Wagner et al., 2000). With the V_0 and V_{lim} thus obtained, data was normalized and plotted as shown.

Fig. 10. Tissue distribution of OCTN2 from human analyzed by real-time RT-PCR. Results are given relative to the mRNA level of kidney. NS: no signal. Note that real-time PCR data inherently have limited accuracy; the data should only be used to identify possible sites of expression.

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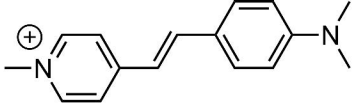
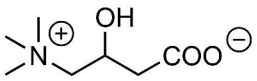
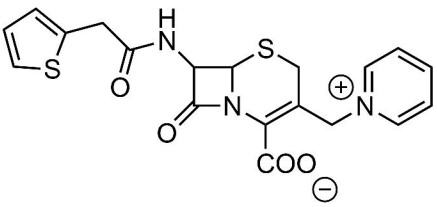


◂ ASP+

FIG. 1

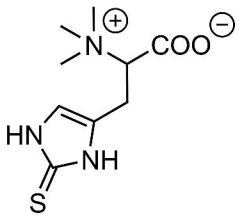
Carnitine ▸

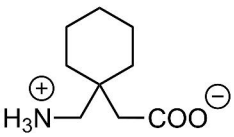




◂ Cephaloridine

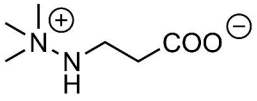
Ergothioneine ▸

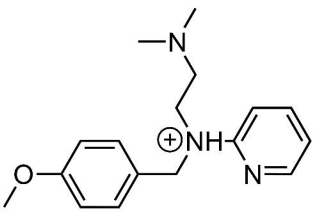




◂ Gabapentin

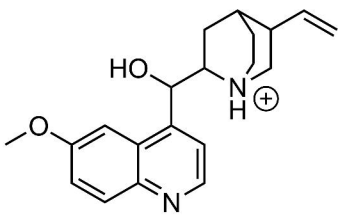
Mildronate ▸

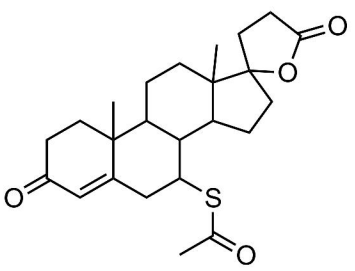




◂ Pyrilamine

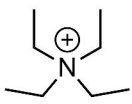
Quinidine ▸

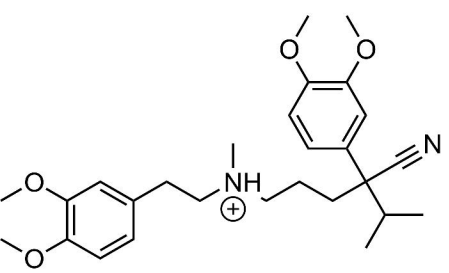




◂ Spironolactone

TEA ▸





◂ Verapamil

Vigabatrin ▸

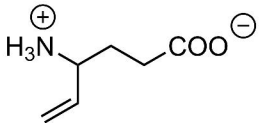


FIG. 2

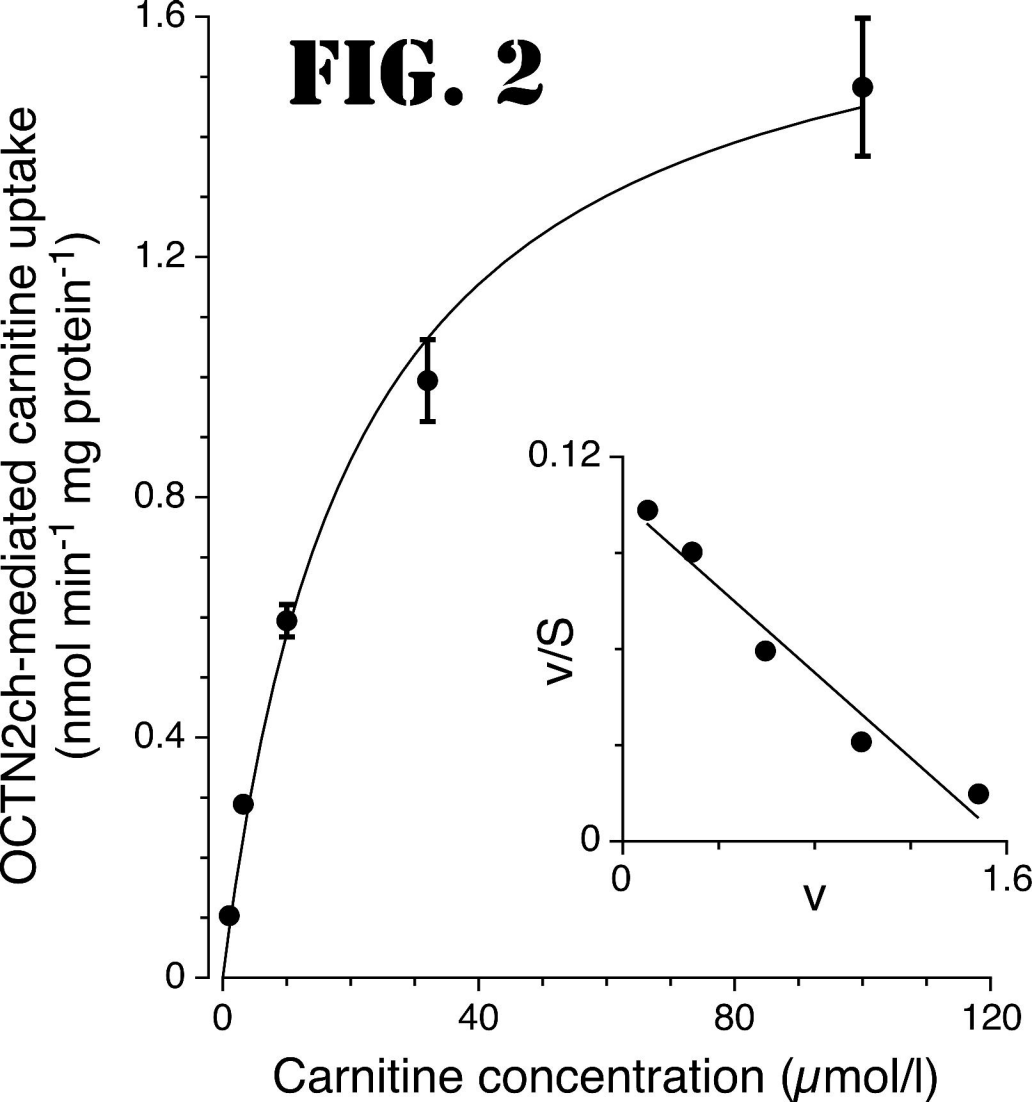
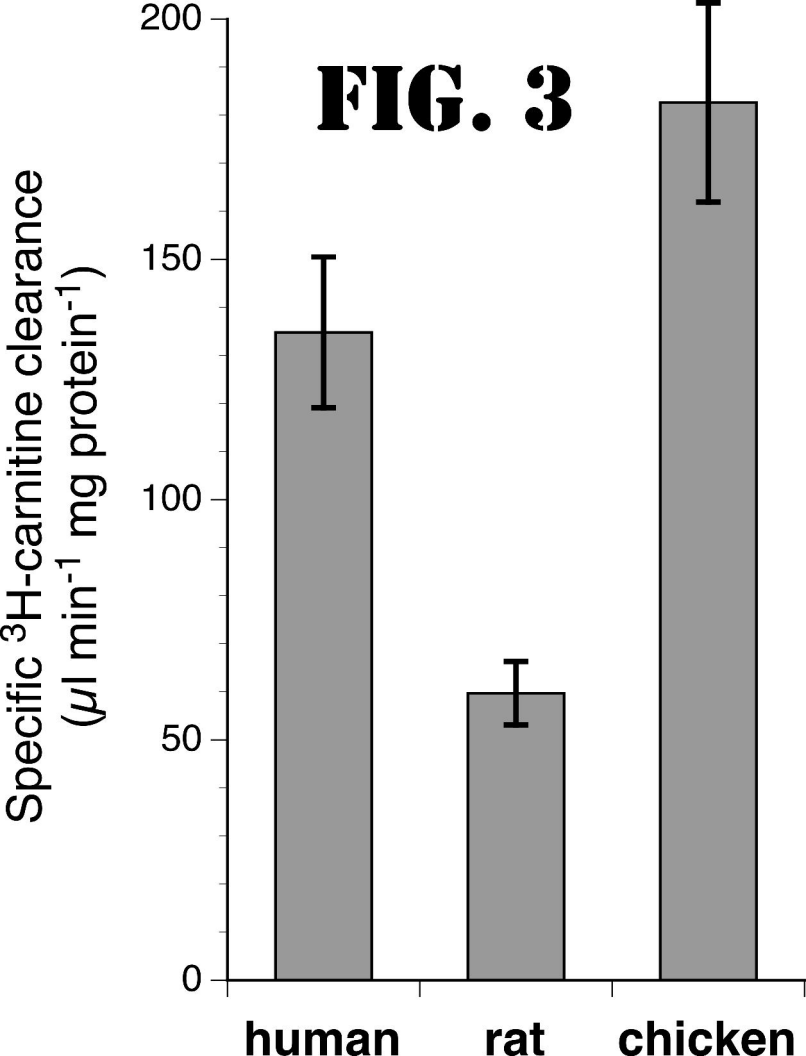

OCTN2ch-mediated carnitine uptake
(nmol min-1 mg protein-1)
1.6
1.2
0.8
0.4
0
0
40
80
120
Carnitine concentration (μmol/l)
0.12
v/S
0
0
v
1.6

FIG. 3


Specific ^{3}H-carnitine clearance
(μl min^{-1} mg protein^{-1})
200
150
100
50
0
human
rat
chicken

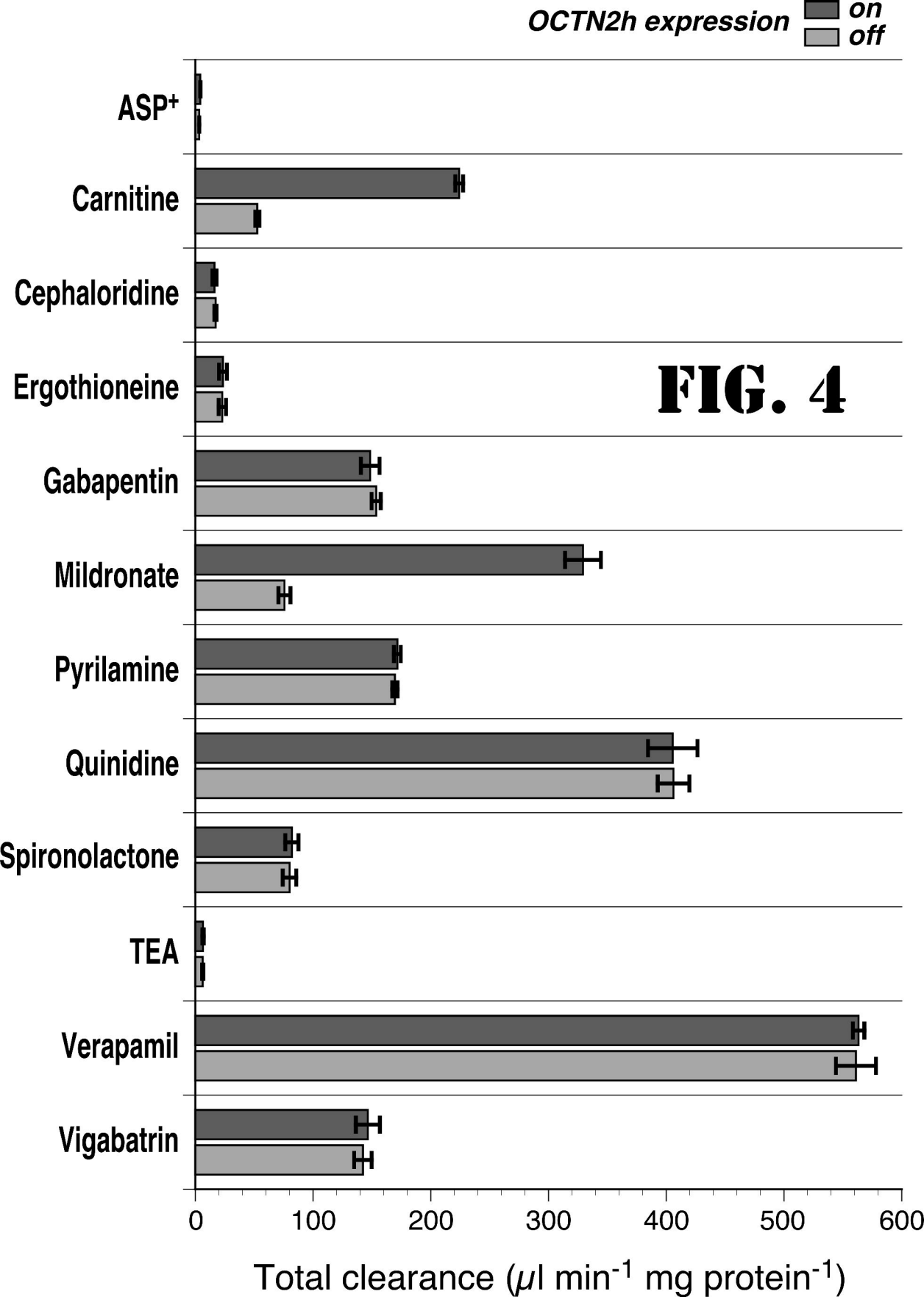

OCTN2h expression
on
off
ASP+
Carnitine
Cephaloridine
Ergothioneine
Gabapentin
Mildronate
Pyrilamine
Quinidine
Spironolactone
TEA
Verapamil
Vigabatrin
0
100
200
300
400
500
600
Total clearance (μl min-1 mg protein-1)

FIG. 4

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FIG. 5

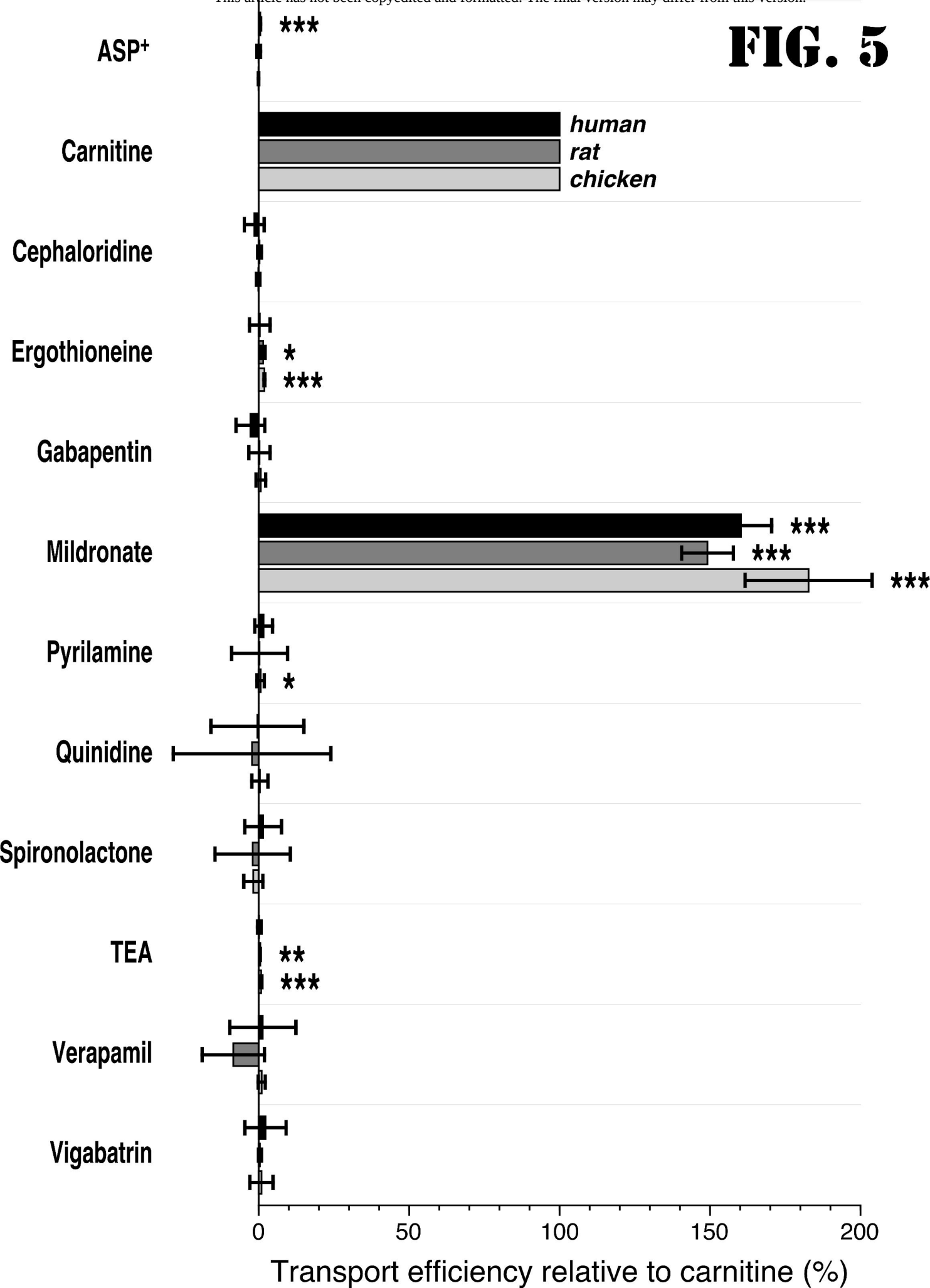
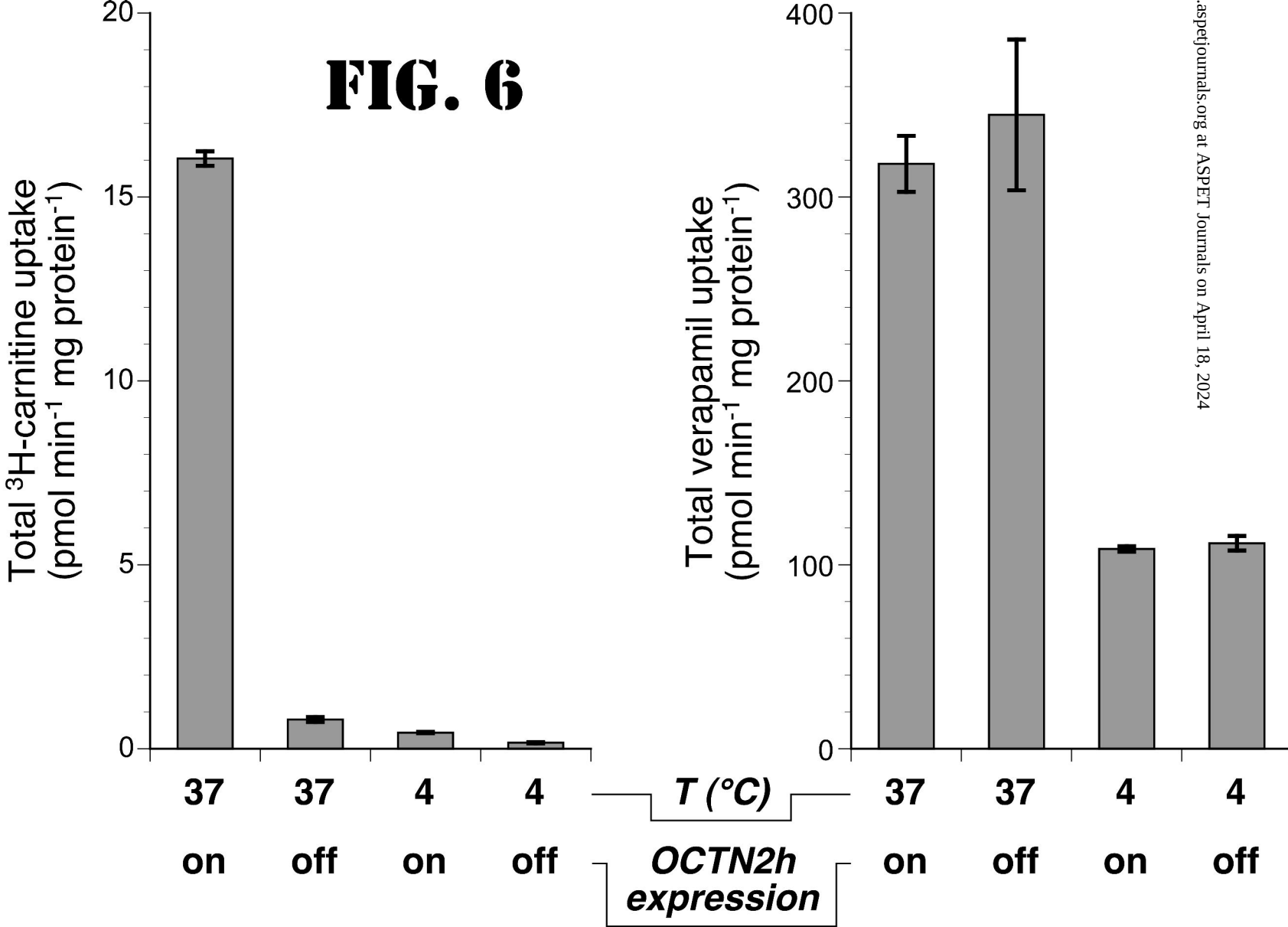


FIG. 6



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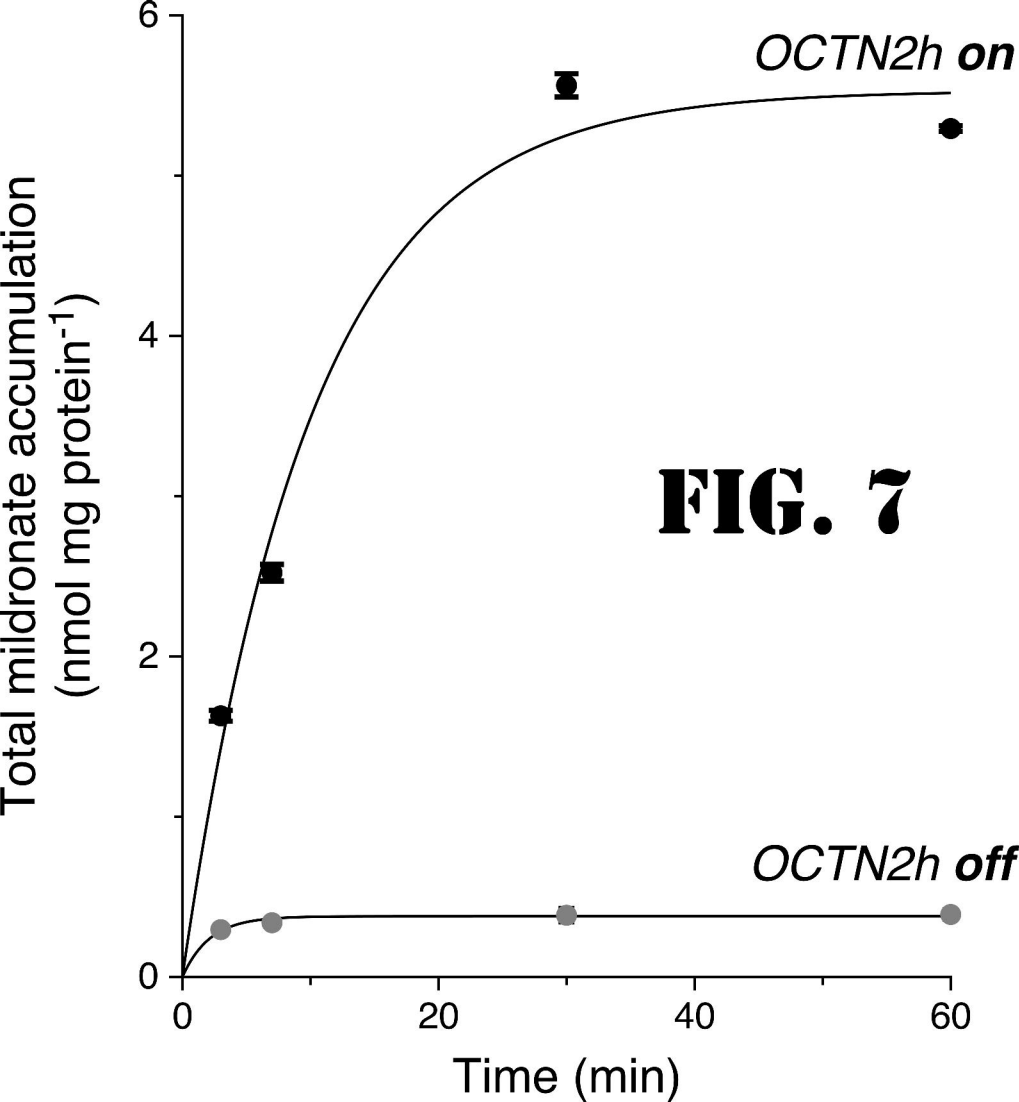

OCTN2h on
OCTN2h off
Total mildronate accumulation (nmol mg protein-1)
Time (min)
0
2
4
6
0
20
40
60

FIG. 7

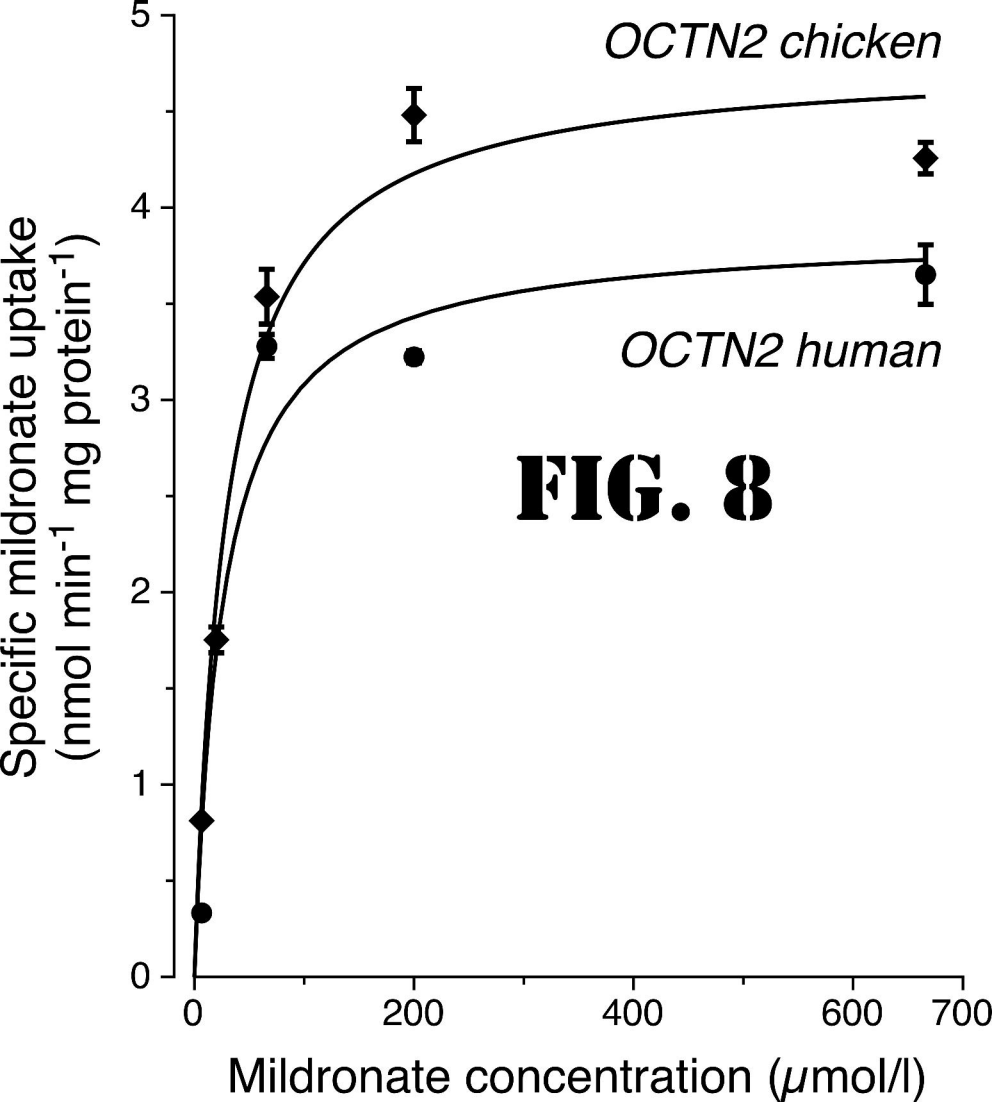
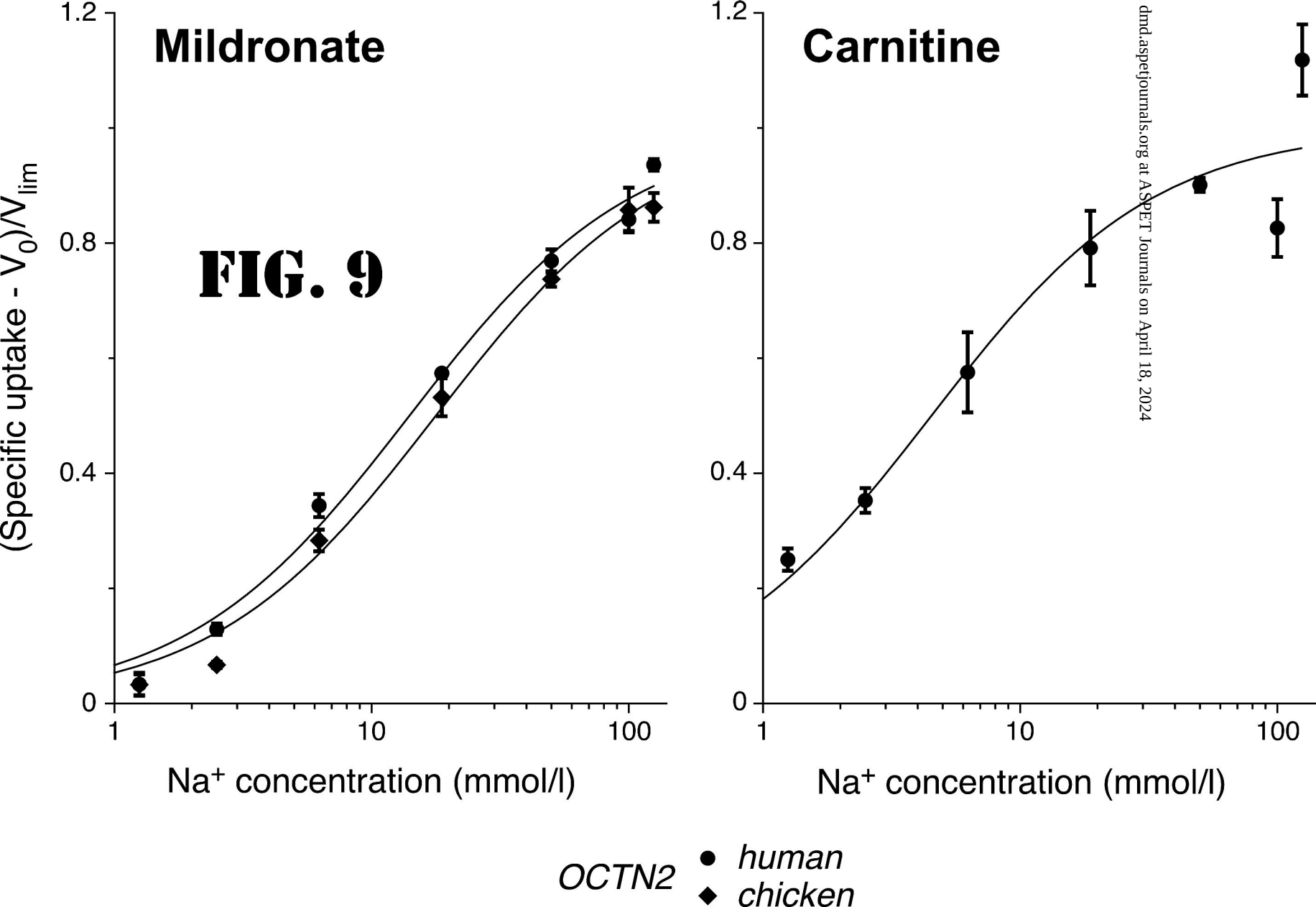

OCTN2 chicken
OCTN2 human
Specific mildronate uptake
(nmol min-1 mg protein-1)
Mildronate concentration (µmol/l)
0
1
2
3
4
5
0
200
400
600
700

FIG. 8



dmd.aspetjournals.org at ASPET Journals on April 18, 2024

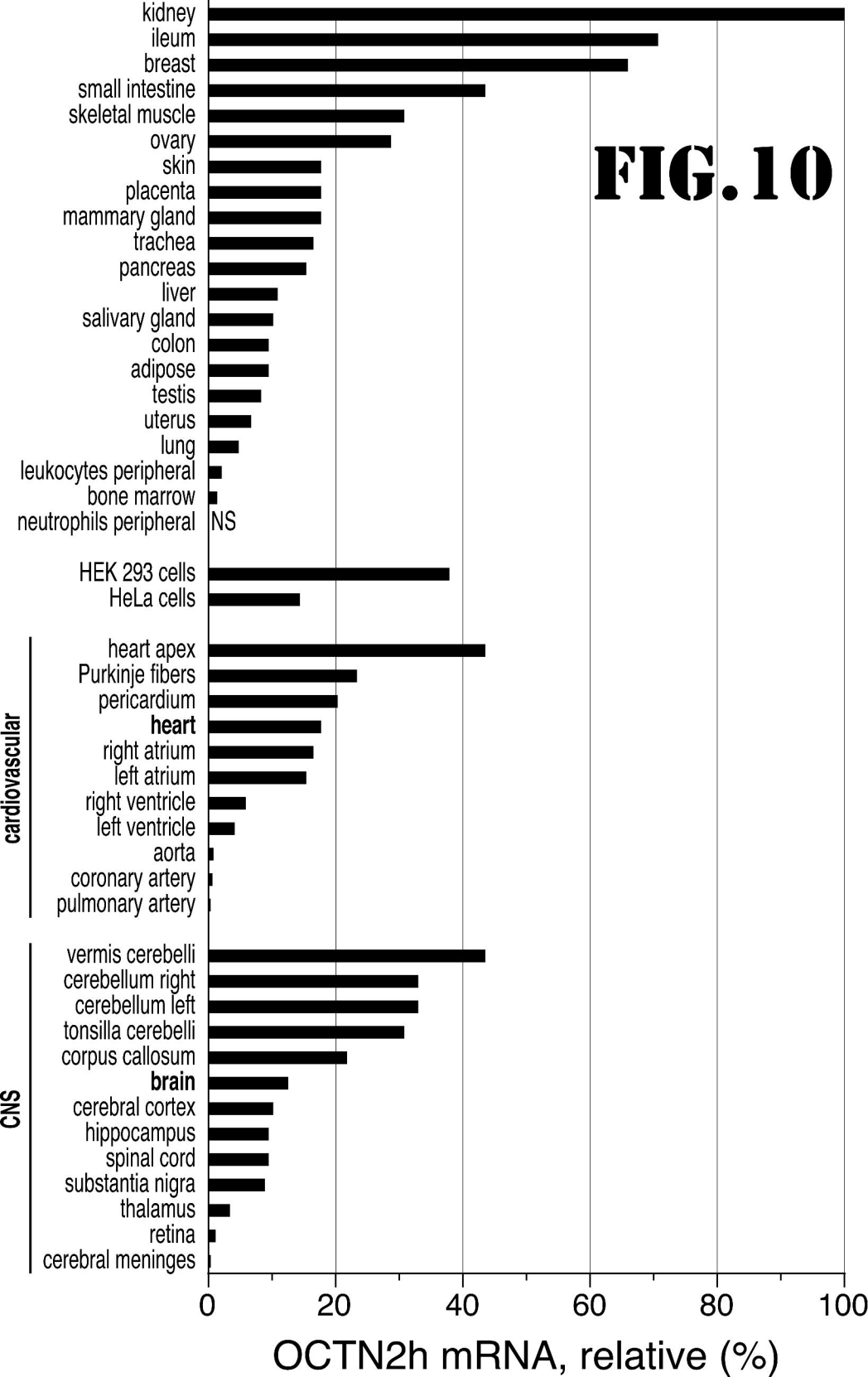

kidney
ileum
breast
small intestine
skeletal muscle
ovary
skin
placenta
mammary gland
trachea
pancreas
liver
salivary gland
colon
adipose
testis
uterus
lung
leukocytes peripheral
bone marrow
neutrophils peripheral
NS
HEK 293 cells
HeLa cells
cardiovascular
heart apex
Purkinje fibers
pericardium
heart
right atrium
left atrium
right ventricle
left ventricle
aorta
coronary artery
pulmonary artery
CNS
vermis cerebelli
cerebellum right
cerebellum left
tonsilla cerebelli
corpus callosum
brain
cerebral cortex
hippocampus
spinal cord
substantia nigra
thalamus
retina
cerebral meninges
0
20
40
60
80
100
OCTN2h mRNA, relative (%)

FIG. 10