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Functional analysis of organic cation transporter 3 (OCT3) expressed in human placenta

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Functional analysis of OCT3 in human placenta

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Abbreviations:

ALP : alkaline phosphatase

ANOVA : analysis of variance

BBMVs : human placental microvillous membrane vesicles

BLMVs : human placental basolateral membrane vesicles

MPP^+ : 1-methyl-4-phenylpyridinium

NET : norepinephrine transporter

OCT : organic cation transporter

PAH : *p*-aminohippuric acid

SERT : serotonin transporter

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Abstract

The aim of this study is to investigate the placental transport mechanism of cationic compounds by comparison of the uptake of an organic cation into human placental basal membrane vesicles (BLMVs) with that into organic cation transporter 3 (OCT3)-expressing cells. RT-PCR analysis demonstrated that OCT3 is the only OCT isoform expressed in the human placenta. The function of OCT3 was investigated by measuring the uptake of 1-methyl-4-phenylpyridinium (MPP^+) into HEK293 cells stably expressing OCT3 (HEK/OCT3 cells). The OCT3-mediated uptake of MPP^+ was sodium- and chloride-independent and saturable, with a Michaelis constant (Km) of 70 μM. The OCT3-mediated uptake was inhibited by various cationic drugs in a concentration-dependent manner, but not by anionic compounds, such as *p*-aminohippuric acid and captopril, or a zwitterion, carnitine. Western blotting analysis of membrane

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vesicles prepared from human term placenta revealed that OCT3 is expressed only in BLMVs, but not in microvillous membrane vesicles (BBMVs). The uptake of MPP^+ into BLMVs was membrane potential-dependent and saturable, with a Km value of 39 μM, which is similar to that in HEK293/OCT3 cells. The inhibitory spectrum of various compounds on MPP^+ uptake by BLMVs was also similar to that in HEK293/OCT3 cells. These results suggest that OCT3 is expressed on the basal membrane of human trophoblast cells and plays an important role in the placental transport of cationic compounds.

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Introduction

Throughout gestation, the placenta plays an important role in regulating the supply of nutrients to the fetus, excretion of metabolic waste products from the fetus, and so on. In the placenta, trophoblast cells, which face the maternal blood, are considered to be the functional entity of the blood-placental barrier. Various transporters have been identified on both microvillous membrane and basal membrane, which face the maternal and fetal side, respectively, of trophoblast cells and are considered to regulate the exchange of various materials between mother and fetus.

Monoamines, including serotonin and norepinephrine, and cationic drugs, such as cimetidine and procainamide, are transported by organic cation transport systems in the kidney and liver (Dresser *et al.*, 2000; Koepsell *et al.*, 1998; Hohage *et al.*, 2000;

Kamisako *et al*., 1999; Suzuki *et al*., 2000). Rat organic cation transporter 1 (rOCT1) was first cloned from rat kidney as a component of the organic cation transport system (Gründemann *et al*., 1994). While mouse and rat OCT1 are expressed in both liver and kidney (Schweifer *et al*., 1996; Gründemann *et al*., 1994), hOCT1 is mainly expressed in the liver (Gorboulev *et al*., 1997; Zhang *et al*., 1999). It has also been shown by immunohistochemical study that rOCT1 is expressed on the sinusoidal membrane of hepatocytes (Meyer-Wentrup *et al*., 1998) and the basolateral membrane of renal tubular epithelium (Karbach *et al*., 2000; Sugawara *et al*., 2000).

rOCT2 was cloned as a homologue of rOCT1 from rat kidney (Okuda *et al*., 1996). Unlike hOCT1, OCT2 is expressed predominantly in the kidney (Gorboulev *et al*., 1997; Okuda *et al*., 1996) and is localized on the basolateral membrane of renal proximal

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tubules (Karbach *et al*., 2000; Sugawara *et al*., 2000).

Organic cation transporter 3, OCT3, was first cloned from rat placenta (Kekuda *et al*., 1998), and its orthologues were also cloned from humans and mice (Gründemann *et al*., 1998; Verhaagh *et al*., 1999). Since hOCT3 has high affinity for monoamines, such as histamine, it is also designated as extraneuronal monoamine transporter (EMT) (Gründemann *et al*., 1998). In contrast to OCT1 and 2, OCT3 is widely expressed (Kekuda *et al*., 1998; Verhaagh *et al*., 1999), though its expression is particularly high in the placenta (Verhaagh *et al*., 1999). Recently, it has been reported that pregnant OCT3-knockout mice exhibit reduced accumulation of MPP^+ in the embryo compared with pregnant control mice, although the MPP^+ concentration in placenta and amniotic fluid was not affected, suggesting that OCT3 mediates the transport of MPP^+ from the placenta to the

fetus, but not from the maternal circulation (Zwart *et al*., 2001). These findings emphasize the importance of OCT3 in the placental transfer of cationic compounds, although its subcellular localization in the placenta and other tissues still remains unknown.

Besides OCTs, various transporters of organic cations have been identified in the human placenta. The expression of OCTN1, a member of a new subfamily of OCTs, has been reported in the human placenta, but its subcellular localization remains to be identified (Tamai *et al*., 1997). Another OCTN, OCTN2, has been demonstrated to be expressed on the maternal side (microvillous membrane) of trophoblast cells by Western blotting (Lahjouji *et al*., 2004). An organic cation/proton antiporter, norepinephrine transporter (NET), serotonin transporter (SERT) and P-glycoprotein (P-gp) have been

found on the microvillous membrane of trophoblast cells (Ganapathy *et al*., 2000; Ushigome *et al*., 2003), but the identity of the transporter of organic cations on the basolateral membrane remains unknown.

The aim of this study is to investigate the placental transport mechanism of cationic compounds by comparison of the uptake of a model organic cation into human placental BLMVs with that into OCT3-expressing cells.

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Methods

Materials and reagents

[^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) (85.0 Ci/mmol), [^{3}H]quinine (14.5 Ci/mmol) and [^{3}H]dihydroalprenolol (60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). [^{14}C]Tetraethylammonium (2.4 Ci/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, MA, USA). [^{3}H]Theophylline (18.5 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Anti-OCT3 goat polyclonal antibody OCT3 (C-14) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HRP-labeled anti-goat IgG antibody was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA).

All other chemicals used in this study were commercial products of reagent grade.

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Functional expression of OCT3 in mammalian cells

Human OCT3 cDNA was obtained from human kidney total RNA by RT-PCR using KOD-plus-polymerase (Toyobo, Osaka, Japan). Sequence analysis revealed that the obtained OCT3 cDNA was 300 bp shorter than the OCT3 previously cloned (Gene Bank accession No. AJ001417; Gründemann *et al.*, 1998). The missing part of the OCT3 cDNA was obtained by PCR using an Advantage-GC Genomic PCR Kit (BD Biosciences Clontech, Palo Alto, CA, USA) and human liver cDNA (BD Biosciences Clontech) under the following conditions: (1) 94˚C × 3 min (2) 94˚C × 30 sec, 68˚C × 3 min (35 cycles) (3) 68˚C × 3min. The full-length OCT3 cDNA was generated using the restriction enzyme *Bgl* II and subcloned into pIRESneo vector.

Sequencing of the cDNA was carried out with an ABI PRISM™ BigDye® Terminator Cycle Sequencing Kit and 373 DNA sequencer (PE Applied Biosystems, Foster

City, CA, USA) and the sequence was analyzed by Sequencing Analysis™ 3.0 (PE Applied Biosystems, Foster City, CA, USA). Sequence analysis showed that there was a G1260A mutation compared with the sequence already published by Gründemann *et al.* (1998), but it was a silent mutation.

An aliquot of 1 μg of OCT3/pIRESneo vector was transfected into HEK293 cells with Lipofect AMINE^{TM} 2000 Reagent (GIBCO BRL Life Technologies, Tokyo, Japan). After incubation for 24 hours, cells were released by trypsin treatment and cultured in MEM medium containing 500 μg/mL geneticin.

The cells were grown for 3 to 4 weeks and then used as HEK293 cells stably expressing OCT3 (HEK/OCT3 cells).

Preparation of human placental basal membrane vesicles (BLMVs) and microvillous

membrane vesicles (BBMVs)

Human placental basal membrane vesicles (BLMVs) were prepared by the method of Inuyama *et al*. (2002) with minor modifications. Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or cesarean delivery and placed in 0.9% NaCl. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in phosphate-buffered saline (PBS) (-). Tissue was stirred in PBS (-) for 30 min and collected on a nylon mesh. The filtrate was washed three times with ice-cold 50 mM Tris-HCl (pH 7.4), collected on a 250 μm pore size nylon mesh and divided into several equal portions. Each portion was sonicated in 100 ml of the same Tris buffer using a 3/4-inch high-gain probe for 10 sec at 240 W (Vibra-cell, Sonics and Materials, CT, USA). The suspensions were kept on ice. The sonication procedure selectively removes any remaining microvillous membrane.

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Sonicated tissue was collected on the mesh, washed three times with 5 mM Tris-HCl (pH 7.4) and then stirred gently for 60 min in the same buffer. Tissue was then collected on the nylon mesh and washed again in the same buffer. This procedure disrupts and removes the intracellular components, thus exposing the basolateral membranes. Tissue portions of 25-30 g were resuspended in about 100 mL of 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 250 mM sucrose and incubated for 30 min with occasional stirring. The portions were then sonicated twice for 20 sec at 250 W to release the basolateral membranes. The suspensions were strained through nylon mesh and the supernatant was centrifuged at 3,430 $\times$ *g* for 10 min to remove debris. The supernatant from this spin was re-centrifuged at 80,000 $\times$ *g* for 40 min to yield the basolateral membrane pellet, which was resuspended, using a Dounce homogenizer, in 25 mM HEPES-Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. This fraction was

further purified by centrifugation on a discontinuous gradient of 10% (w/v) Ficoll (Pharmacia, Peapack, NJ, USA) in the resuspension buffer overlaid with 4% Ficoll (as described by Kelley et al., 1983) prepared in 25 mM HEPES-Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. Ficoll gradient tubes were spun at 90,000 $\times$ *g* for 6-8 h. The material at the density gradient interfaces was collected, washed and resuspended in 25 mM HEPES-Tris (pH 7.4) containing 275 mM sucrose. The suspension from this run was resuspended in 25 mM Hepes-Tris buffer containing 150 mM KCl (pH 7.4; E buffer) with a 25 gauge syringe needle. All the operations were carried out at 4˚C.

BBMVs were prepared according to the method described by Nakamura *et al.* (2002) with minor modifications. Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or cesarean delivery and placed in 0.9% NaCl. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the

maternal side and washed in 250 mM mannitol, 10 mM HEPES-Tris at pH 7.4 (MHT buffer). The mince was stirred for 1 hr to loosen the microvilli and filtered through two layers of woven cotton gauze. A sample of this starting mince was taken for enzyme analysis. The filtrate was centrifuged at 800 × *g* for 10 min. The pellet was discarded and $MgCl_2$ was added to the supernatant to a final concentration of 10 mM. After 10 min, with occasional stirring, the supernatant was centrifuged at 10,500 × *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet from this run was suspended in E buffer with a 25-gauge syringe needle. All the subsequent procedures were performed at 4˚C.

BLMVs and BBMVs were quickly frozen and stored at -80˚C, and used within a month.

Tissue homogenate was prepared by the method previously described by Kelley *et*

al. (1983). Approximately 3 g of whole villous tissue was homogenized in 10 mL of buffer E using a Waring blender (PHYSCOTRON, Micro Teq. Nichion Co., Chiba, Japan) for 2.25 min and further with a homogenizer for 8 strokes. The material was filtered through six layers of gauze.

Binding activity of [^{3}H]dihydroalprenolol as a marker of the basal membrane and alkaline phosphatase (ALP) activity as a marker of the microvillous membrane were assayed as reported by Kelley *et al*. (1983) and Bessey *et al*. (1946), respectively. The dihydroalprenolol binding of BLMVs was 25.2-fold higher than that of the homogenate, while the ALP activity was only 3.07-fold higher. On the other hand, the dihydroalprenolol binding of BBMVs was only 1.07-fold higher than that of the homogenate, while the ALP activity was 18.3-fold higher. The amount of protein in the sample was measured by the method of Lowry *et al*. (1951).

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RT-PCR analysis of OCTs mRNA in human placenta and BeWo cells

The total RNAs of BeWo cells and human placenta were extracted with an RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). Human liver total RNA was purchased from Cell Applications, Inc. (San Diego, CA, USA), and human kidney total RNA was purchased form Stratagene (La Jolla, CA, USA). First-strand cDNA was synthesized from total RNA (50 μg), using random primer and SuperScript^{TM}II Reverse Transcriptase (Invitrogen Corp, Carlsbad, CA, USA). PCR was performed with a T-Gradient Thermoblock (Biometra, Gottingen, Germany) using KOD-plus-polymerase (Toyobo, Osaka, Japan) and primers specific for each family member. Table 1 shows the primer sets used in RT-PCR and the accession number of each transporter.

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Western blotting

HEK293/OCT3 cells, BLMVs and BBMVs were collected and suspended in lysis buffer containing 100 mM Tris HCl (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/mL leupeptin, 0.01 mg/mL aprotinin and 1 mM sodium vanadate, and incubated for 30-45 min at 4˚C. After incubation, the suspension was centrifuged at 15,000 $\times$ *g* for 15 min at 4˚C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli *et al*. (1970). The proteins were transferred electrophoretically onto a 0.2 μm pore size Clear Blot Membrane-P (Atto Corporation, Tokyo, Japan). Blots were blocked overnight at 4˚C with 5% nonfat powdered milk in PBS (-). OCT3 (C-14) was used as the primary antibody for OCT3, and horseradish peroxidase anti-goat IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA) was used as the secondary antibody.

Detection was done with ECL reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the instructions of the manufacturer.

Uptake experiment into HEK/OCT3 cells

HEK/OCT3 cells were seeded at 5×10^4 cells/well on 96-well plates (Nunc, Denmark) and grown for 2 days until used for the uptake study.

After reaching confluence, cells were washed twice with uptake buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ 25 mM HEPES, pH 7.4) before the study. Uptake was initiated by adding 100 µL of uptake buffer containing unlabelled MPP^+ and 10 nM [^{3}H]MPP^+. For the Na^+-dependency study, NaCl was replaced with LiCl, mannitol or sodium gluconate. After incubation at 37˚C for an appropriate time, uptake was terminated by aspiration of the buffer, followed by two

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washes with 1 mL of ice-cold uptake buffer. Cells were then dissolved in 150 µL of 1 N NaOH and the solution was neutralized with 150 µL of 1 N HCl. Scintillation cocktail (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added, and the radioactivity of [^{3}H]MPP$^+$ was determined with a liquid scintillation counter (LS6500, Beckman Instruments, Inc., Fullerton, CA, USA). The amount of protein in the cells was measured by the method of Lowry *et al*. (1951).

Measurement of uptake into BLMVs and BBMVs

The uptake of [^{3}H]MPP$^+$ into membrane vesicles was measured by using a rapid filtration technique (Russel *et al*., 1988). Uptake was initiated by the addition of 90 µL of incubation buffer to 10 µL of BLMV suspension containing 40-50 µg protein. The incubation buffer consisted, in general, of unlabelled MPP$^+$, 25 mM Hepes-Tris (pH 7.4),

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150 mM NaCl, and [^{3}H]MPP^+ (0.08 μCi/point).

At the designated time, uptake was terminated by adding 1 mL of ice-cold stop buffer, followed immediately by filtration (HAWP 0.45 μm; Millipore Intertech, MA, USA). The filter was washed twice with 4 mL of ice-cold stop buffer. Stop buffer contained, in general, 10 mM sucrose in incubation buffer. Non-specific binding was determined by adding 1 mL of ice-cold stop solution and 90 μL of ice-cold incubation buffer to the ice-cold BLMV suspension, followed by the same treatment as in the uptake experiments.

To assay radiolabeled compounds, filters were placed in counting vials, and mixed with 4 mL of scintillation fluid, Clear-sol I. Radioactivity was measured with a liquid scintillation counter (LS6500).

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Data analysis

The [^{3}H]MPP$^+$ uptake into HEK/OCT3 cells is expressed as the cell/medium ratio calculated from the intracellular uptake per mg protein (dpm/mg protein) of the cells relative to the initial drug concentration (dpm/μL).

As for the uptake into membrane vesicles, obtained radioactivity was normalized with respect to the protein amount of vesicles. Values were determined by subtracting non-specific binding from total uptake (in the investigation of osmolarity effects as well), and data are presented as the vesicle/medium ratio (μL/mg protein).

To determine the kinetic parameters, K_t, J_{max} and k_d, the following Michaelis-Menten equation was fitted to the data using the nonlinear least-squares regression analysis program MULTI (Yamaoka *et al*., 1981):

$$J = Jmax \times S/(Kt + S) + kd \times S \quad (1)$$

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, where J and S represent the transport rate and concentration of substrate, respectively. J_{max} (nmol/mg protein/30 sec), K_t (mM) and k_d (μL/mg protein/30 sec) represent the maximum uptake rate for a carrier-mediated process, the Michaelis constant and the rate constant for the nonsaturable component.

Comparisons between two and among more than three groups were performed with the unpaired Student's *t*-test and with non-repeated analysis of variance (ANOVA) followed by Dunnett's test, respectively. The Spearman rank correlation test was used to determine the degree of association between the inhibitory effects on MPP^+ uptake into HEK/OCT3 cells and BLMVs. A *P* value of less than 0.05 was considered statistically significant.

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Results

Expression of OCT mRNAs in human placenta and BeWo cells

The expression of OCTs in human placenta and BeWo cells was examined by RT-PCR analysis. Total RNAs from human liver and kidney were used as positive controls for OCT1 and OCT2 and 3, respectively. In placenta, only OCT3 and the positive control were detected (Figure 1). The PCR product was confirmed to be in accord with the sequence of human OCT3. With regard to BeWo cells, no band was detected, suggesting that OCTs are not expressed in these cells.

Expression of OCT3 protein in HEK/OCT3 cells and human placenta

The expression of OCT3 was examined by Western blotting analysis of HEK/OCT3 cells and mock cells (HEK293 cells transfected with vector alone). As

expected, a band of 70 kDa was detected only in HEK/OCT3 cells (Figure 2A).

The expression of OCT3 on human placental trophoblast membrane was also examined by Western blotting analysis using BLMVs and BBMVs prepared from human placenta. The band corresponding to OCT3 was detected in BLMVs at about 70 kDa, as well as in HEK/OCT3 cells, but not in BBMVs (Figure 2B).

Time-course of the uptake of MPP^+

The uptake of 1 μM MPP^+ into HEK293/OCT3 cells was significantly higher than that into mock cells (Figure 3). This uptake was linear for at least 2 minutes and was attenuated at 4˚C. In the following experiments, the initial uptake rate was determined at 30 seconds.

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The effects of extracellular ion composition on the uptake of MPP^+

The effects of Na^+ and Cl^- on the uptake of MPP^+ were examined by isoosmotic replacement of NaCl (Na^+ : +, Cl^- : +) with mannitol (Na^+ : -, Cl^- : -), sodium gluconate (Na^+ : +, Cl^- : -) and LiCl (Na^+ : -, Cl^- : +) in the uptake buffer. As shown in Figure 4, the uptake of MPP^+ was not affected by any replacement of NaCl, suggesting that OCT3-mediated uptake of MPP^+ is independent of both Na^+ and Cl^-.

Concentration-dependent uptake of MPP^+

The OCT3-mediated uptake was saturable with a Michaelis constant (Kt) of 82.5 [59.7 – 113.8] µM (mean [mean-SD – mean+SD]) and a maximal uptake velocity (Jmax) of 2,538 ± 567.4 pmol/mg protein/30 sec (mean ± SD) (Figure 5), obtained from three separate experiments.

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Inhibitory effects of various cationic compounds on the OCT3-mediated uptake of MPP^+

The OCT3-mediated uptake of 10 μM MPP^+ was significantly inhibited by various cationic drugs, such as cimetidine, ranitidine, verapamil, quinine, quinidine, imipramine, trimethoprim and procainamide, in a concentration-dependent manner (Figures 6, 7).

On the other hand, anionic compounds (*p*-aminohippuric acid (PAH), captopril and phenobarbital), a zwitterion (carnitine), and a cationic compound (lamivudine), showed little or no inhibitory effect.

Uptake of various cationic compounds

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To search for novel substrates of OCT3, we examined the uptake of 10 μM tetraethylammonium, quinine, theophylline and ramosetron into HEK/OCT3 cells. No OCT3-mediated uptake of these compounds was observed (data not shown).

Uptake of MPP^+ into BLMVs

The vesicle-to-medium ratio of MPP^+ in BLMVs was linear for at least 30 seconds (Figure 8). The vesicle-to-medium ratio at 10 minutes was reduced with increasing extracellular osmolarity, suggesting that MPP^+ was not only bound to the vesicles, but also was taken up into the vesicles.

This uptake was significantly attenuated by replacing extravesicular NaCl with KCl, and was potentiated by the addition of valinomycin, a potassium ionophore. The uptake of MPP^+ was not affected by the replacement of NaCl with LiCl (Figure 9). These

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results suggested that the uptake of MPP^+ into BLMVs is dependent on membrane potential and independent of Na^+.

Concentration-dependent uptake of MPP^+ into BLMVs

The uptake of MPP^+ into BLMVs was saturable with a Michaelis constant (Kt) of 51.8 [34.9 – 113.8] μM (mean [mean-SD – mean+SD]) and a maximal uptake velocity (Jmax) of 332 ± 30.8 pmol/mg protein/30 sec (mean ± SD) (Figure 10), obtained from three separate experiments.

Effects of various compounds on the uptake of MPP^+ into BLMVs

We examined the effects of various compounds of the uptake of MPP^+ into BLMVs. We selected non-inhibitors (PAH, captopril and lamivudin), modest inhibitors

(carnitine and procainamide) and strong inhibitors (cimetidine, imipramine, quinidine, verapamil and quinine) of OCT3-mediated uptake. As shown in Figure 11, PAH and procainamide showed little or no inhibitory effect, while quinine, quinidine, imipramine and verapamil inhibited the uptake of MPP^+ into BLMVs. The rank order of the inhibitory effects of MPP^+ uptake into HEK/OCT3 cells was highly correlated with that in BLMVs ($r^2 = 0.688$, $P < 0.05$; Figure 12).

Discussion

To investigate the placental transport mechanism of cationic compounds, we focused on OCTs in this study. Since the only OCT detected in human placenta by RT-PCR analysis was OCT3, this molecule is expected to play an important role in the transport of cationic compounds across the human placenta.

hOCT1 and hOCT2 are predominantly expressed in the liver and kidney, respectively (Gorboulev *et al*., 1997). Unlike OCT1 and OCT2, OCT3 is widely expressed (Kekuda *et al*., 1998; Verhaagh *et al*., 1999). The OCT3 mRNA level in the placenta is particularly high, being 2.5 and 5 times those in the liver and kidney, respectively. OCT3 is considered to be one of the most abundantly expressed transporters in the placenta (Leazer *et al*., 2003). Our results are consistent with that finding, and underscore the key role of OCT3 in the transport of cationic compounds in the placenta.

We also investigated OCTs in a human choriocarcinoma trophoblast cell line, BeWo cells, and found no expression of OCTs. This finding is in accordance with the results of Wu *et al.* (Wu *et al*., 2000). Although the BeWo cell line originated from placenta, it has the characteristics of cytotrophoblast cells and is functionally and morphologically distinct from normal syncytiotrophoblast cells (Bernhard *et al.*, 1999). Thus, it is not surprising that OCT3 is not expressed in BeWo cells.

Western blotting demonstrated predominant expression of OCT3 in HEK/OCT3 cells. It was detected as a band of 70 kDa, which is larger than expected from the amino acid sequence (62 kDa) (Figure 2). Since both mouse and rat OCT3 have 4 to 5 N-glycosylation sites (Burckhardt *et al*., 2000), and hOCT3 shows 90% homology to them, the difference in size is likely to be attributable to glycosylation. Indeed, the size of the bands of OCT3 from both BLMVs and HEK/OCT3 cells was 70 kDa. The established

HEK/OCT3 cells transported MPP^+ with a Kt value of 69 μM (Figure 5), which is in good agreement with the values reported for HRPE cells (47 μM; Wu *et al*., 2000) and HEK293 (104 μM; Martel *et al*., 2001).

The uptake of MPP^+, a typical substrate of OCT3, into HEK/OCT3 cells was inhibited by cationic compounds, such as cimetidine, verapamil, quinine, quinidine, ramosetron and trimethoprim, in a concentration-dependent manner. On the other hand, anionic compounds including PAH, captopril and phenobarbital, and a zwitterion, carnitine, showed little or no inhibitory effect (Figures 6, 7). In addition to well-known inhibitors of OCT3, such as cimetidine, imipramine and quinine (Gründemann *et al*., 1998; Wu *et al*., 2000), we have identified a variety of cationic drugs that inhibit OCT3. Interestingly, a cationic compound, lamivudine, did not inhibit OCT3 function, suggesting that cationic character alone is not sufficient for recognition by OCT3. Among therapeutic drugs, only

cimetidine is known to be a substrate of OCT3 (Gründemann *et al*., 1999). As various cationic drugs were found to inhibit OCT3, some of them might be substrates of OCT3.

To search for novel substrates of OCT3, we also examined the uptake of tetraethylammonium, quinine, theophylline and ramosetron into HEK/OCT3 cells. Although tetraethylammonium is a typical substrate of OCT1 and 2 as well as MPP^+, none of the above drugs was a substrate for OCT3. Our finding in the case of tetraethylammonium is consistent with previous reports that the affinity of tetraethylammonium for mouse and rat OCT3 is about 10-fold weaker than that for OCT1 and 2 (Wu *et al*., 2000; Kekuda *et al*., 1998), and that hOCT3 does not interact with tetraethylammonium (Gründemann *et al*., 1999). On the other hand, OCT3 has high affinity for monoamines (Gründemann *et al*., 1998). Overall, OCT3 appears to have a substrate specificity distinct from those of OCT1 and 2.

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Western blot analysis of BLMVs and BBMVs showed that OCT3 is predominantly expressed on the basolateral membrane of trophoblast cells (Figure 2). Recently, it has been reported that accumulation of MPP^+ in the embryo was reduced in OCT3-knockout pregnant mice, although MPP^+ concentrations in the placenta and amniotic fluid were comparable (Zwart *et al*. 2001), suggesting that OCT3 mediates the transport of MPP^+ from the placenta to the fetus, but not from the maternal circulation to the placenta. This finding is consistent with our results that OCT3 is expressed on the basolateral membrane of trophoblast cells. Though various transporters, such as organic cation/proton antiporter, NET and SERT, were shown to be localized at the microvillous membrane of placental trophoblast cells (Ganapathy *et al*., 2000), nothing was known about the expression of transporters for cationic compounds on the basolateral membrane of trophoblast cells. We have here demonstrated for the first time the expression and

function of OCT3 on the basal membrane, where it presumably plays a role in the excretion of metabolic waste products or xenobiotics from the fetus. With regard to the transport direction, OCT3 has been considered to transport cationic compounds bidirectionally in a concentration-dependent manner (Kekuda *et al.*, 1998). OCT3 may transport the maternally administered cationic compounds from the placenta to the fetal side and transport oppositely the cationic compounds whose level is higher in the fetal blood.

We compared quantitatively the transport properties of cationic compounds in human placental membrane vesicles with those of HEK/OCT3 cells. The uptake of MPP^+ into BLMVs was membrane potential-sensitive and Na^+-independent (Figure 9). The Km values of MPP^+ uptake into BLMVs and HEK/OCT3 cells were similar (39 μM and 70 μM, respectively). Moreover, the inhibitory effects of various compounds on the uptake

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of MPP^+ into BLMVs were highly correlated with those on uptake into HEK/OCT3 cells (Figure 12). Since we did not examine detailed mechanism for the inhibition by drugs, we cannot exclude the possibility that they inhibited OCT3 in a non-competitive manner by a non-specific mechanism such as membrane depolarizaion and so on. Although the inhibitory mechanism by these compounds remains to be further investigated, the inhibitory nature is unlikely to affect significantly the conclusion that OCT3 plays an important role in the placental transport of cationic compounds.

In summary, we have identified the expression of OCT3 on the basal membranes of trophoblast cells. Since the properties of MPP^+ uptake into BLMVs are similar to those in HEK/OCT3 cells, OCT3 is likely to be predominantly responsible for the transport of cationic compounds across the basolateral membranes of trophoblast cells.

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Footnotes

(Footnote to title)

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Legends for Figures

Figure 1 RT-PCR analysis of the distribution of OCT mRNAs in the human placenta and BeWo cells. Total RNAs from the placenta, liver, kidney and BeWo cells were reverse-transcribed and used for PCR amplification with oligonucleotide primers specific for OCT1, 2 and 3.

Figure 2 Western blotting analysis of OCT3 in HEK/OCT3 cells and human placenta. A) 10 μg each of HEK/OCT3 cells and mock, and B) 50 μg each of BLMVs and BBMVs were resolved by SDS-PAGE with a 10% polyacrylamide gel and transfered onto Clear Blot Membrane-P. Immunoblots were performed with OCT3-C14 and anti-β-actin mouse monoclonal antibody, and developed with the ECL detection reagent. OCT3 and ß-actin

were detected at 70 kDa and 40 kDa, respectively.

Figure 3 Time course of OCT3-mediated uptake of 1 μM MPP^+. HEK/OCT3 cells were incubated at 37˚C (closed circle) and 4˚C (closed triangle). HEK293 cells transfected with vector alone (open circle) were incubated at 37˚C. Each point represents the mean ± S.E.M. of four determinations.

* ; $P < 0.05$ vs control (Student's *t*-test)

Figure 4 OCT3-mediated uptake of MPP^+ in the presence or absence of Na^+ or Cl^-. HEK/OCT3 cells were incubated at 37˚C for 30 seconds with 10 μM MPP^+. Uptake buffers of different ionic composition were used : NaCl (Na^+ : +, Cl^- : +), mannitol (Na^+ : -, Cl^- : -), sodium gluconate (Na^+ : +, Cl^- : -), LiCl (Na^+ : -, Cl^- : +).

Each point represents the mean ± S.E.M. of four determinations.

Figure 5 Concentration-dependent uptake of MPP^+ into HEK/OCT3 cells. Uptake of MPP^+ into HEK/OCT3 cells and mock was measured at concentrations between 1 μM and 3 mM. Data are presented as the OCT3-specific uptake calculated by subtracting the uptake obtained with mock from that obtained with HEK/OCT3.

Each point represents the mean ± S.E.M. obtained from three different experiments.

Figure 6 Inhibitory effects of several drugs on the uptake of MPP^+ into HEK/OCT3 cells. HEK/OCT3 cells were incubated at 37˚C for 30 seconds with 10 μM MPP^+, in the absence or presence of 1 mM various compounds. Results are given as percent of control uptake measured in the absence of inhibitors. The control and control (+0.5% DMSO) values were

27.38 µL/mg protein /30 sec and 25.04 µL/mg protein/30 sec.

Each point represents the mean ± S.E.M. of four determinations.

* ; P<0.05 vs respective control (ANOVA; Dunnett's test)

Figure 7 Inhibitory effects of several drugs on the uptake of MPP^+ into HEK/OCT3 cells. HEK/OCT3 cells were incubated at 37˚C for 30 seconds with 10 µM MPP^+, in the absence or presence of 10, 100 µM or 1 mM inhibitors. Results are given as percent of control uptake measured in the absence of inhibitors. The control and control (+0.5% DMSO) values were 29.97 µL/mg protein /30 sec and 28.54 µL/mg protein/30 sec.

Each point represents the mean ± S.E.M. of four determinations.

* ; P<0.05 vs respective control (ANOVA; Dunnett's test)

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Figure 8 Influence of Na^+ on the uptake of MPP^+ into BLMVs. Uptake was initiated by the addition of 10 μM MPP^+ in the presence of Na^+ 100 mM / K^+ 3 mM (closed circle) or Na^+ 3 mM / K^+ 100 mM (open circle).

Each point represents the mean ± S.E.M. of three determinations.

* ; P< 0.05 vs control (Student's *t*-test)

Figure 9 Influence of membrane potential on the uptake of MPP^+ into BLMVs. Uptake was initiated by the addition of 10 μM MPP^+. Ionic composition of uptake buffers is shown below the figure.

Each point represents the mean ± S.E.M. of three determinations.

* ; P< 0.05 vs control (ANOVA; Dunnett's test)

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Figure 10 Concentration-dependent uptake of MPP^+ into BLMVs. Uptake of MPP^+ into BLMVs was measured at concentrations between 1 µM and 1 mM. Uptake was initiated by the addition of 10 µM MPP^+ in the presence of Na^+ 100 mM / K^+ 3 mM.

Each point represents the mean ± S.E.M. obtained from three different experiments.

Figure 11 Inhibitory effects of several drugs on the uptake of 10 µM MPP^+ into BLMVs. BLMVs were incubated at 37˚C for 30 seconds with 10 µM MPP^+, in the absence or presence of 1 mM various compounds. Results are given as percent of control uptake measured in the absence of inhibitors. The control values were 4.02 µL/mg protein/30 sec.

Each point represents the mean ± S.E.M. of three determinations.

* ; $P < 0.05$ vs control (ANOVA; Dunnett's test)

Figure 12 Comparison of inhibitory effects on MPP^+ uptake between HEK/OCT3 cells and BLMVs. The solid line shows the linear regression and the dashed line shows the 1 : 1 line. The concentration of each inhibitor was 1 mM.

Each point represents the mean ± S.E.M. of three (BLMVs) or four (HEK/OCT3 cells) determinations.

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Tables

Table 1 Oligonucleotides used for RT-PCR. Open reading frame (ORF) positions of OCT1, 2 and 3 are 1 to 1665, 1 to 1668 and 1 to 1671, respectively.

Family member		Sequence	Position	Accession No.
OCT1	Forward	5'-ACTCCGCTCTGGTCGAAATC-3'	1142→ 1161	X98332
	Reverse	5'-CGACATCGCCGCAAAACATC-3'	1670← 1689	
OCT2	Forward	5'-ACTCTGCCCTGGTTGAATTC-3'	1145→ 1164	X98333
	Reverse	5'-GCAACGGTCTCTCTTCTTAG-3'	1665← 1684	
OCT3	Forward	5'-CAGAGATCACTGTTACAGAT-3'	971→ 990	AJ001417
	Reverse	5'-GATAGCTCCTTCTTTCTGTC-3'	1685← 1704	

Figures

Figure 1

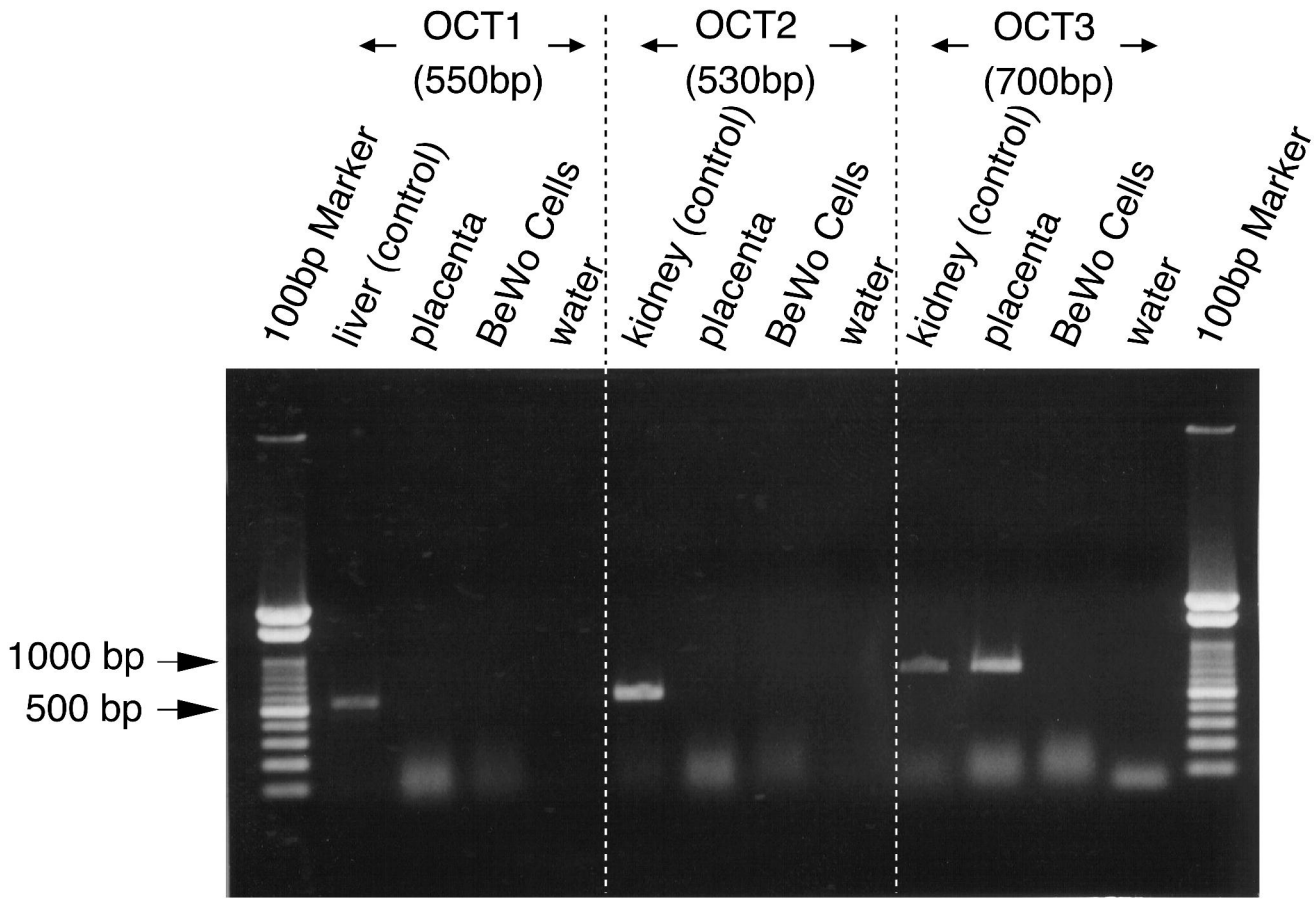


Figure 2

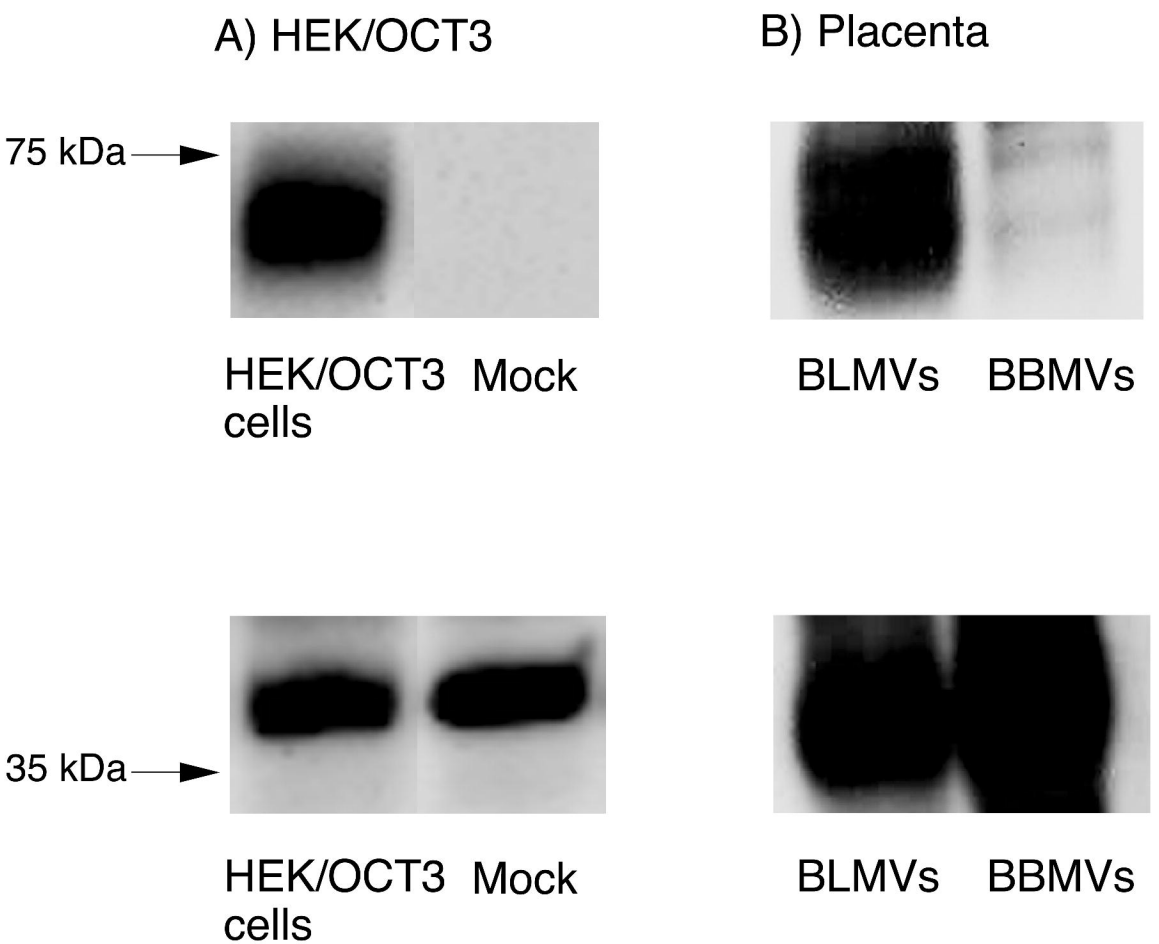


Figure 3

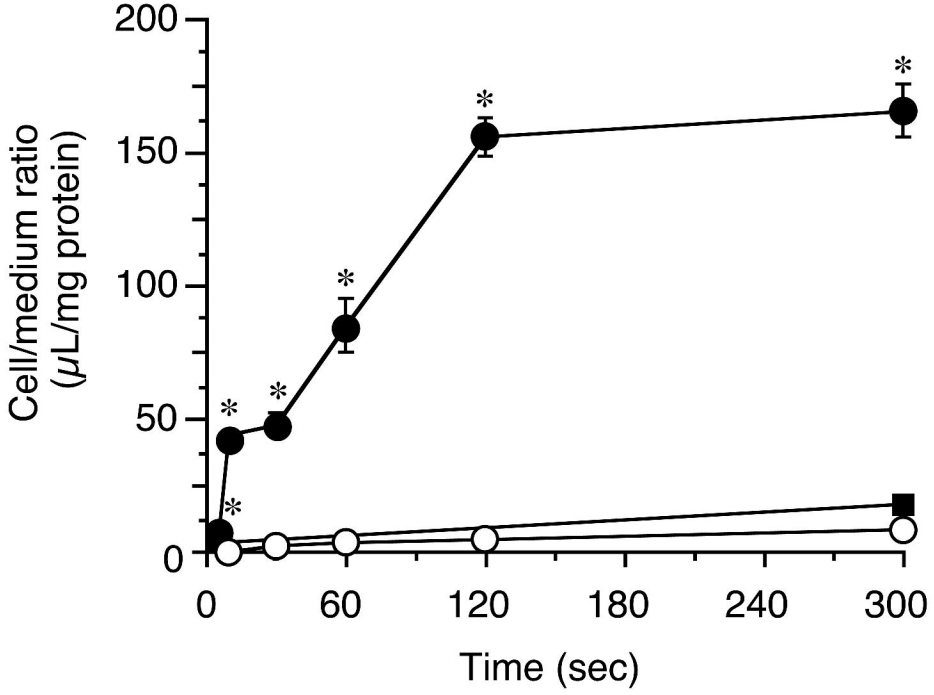


Figure 4

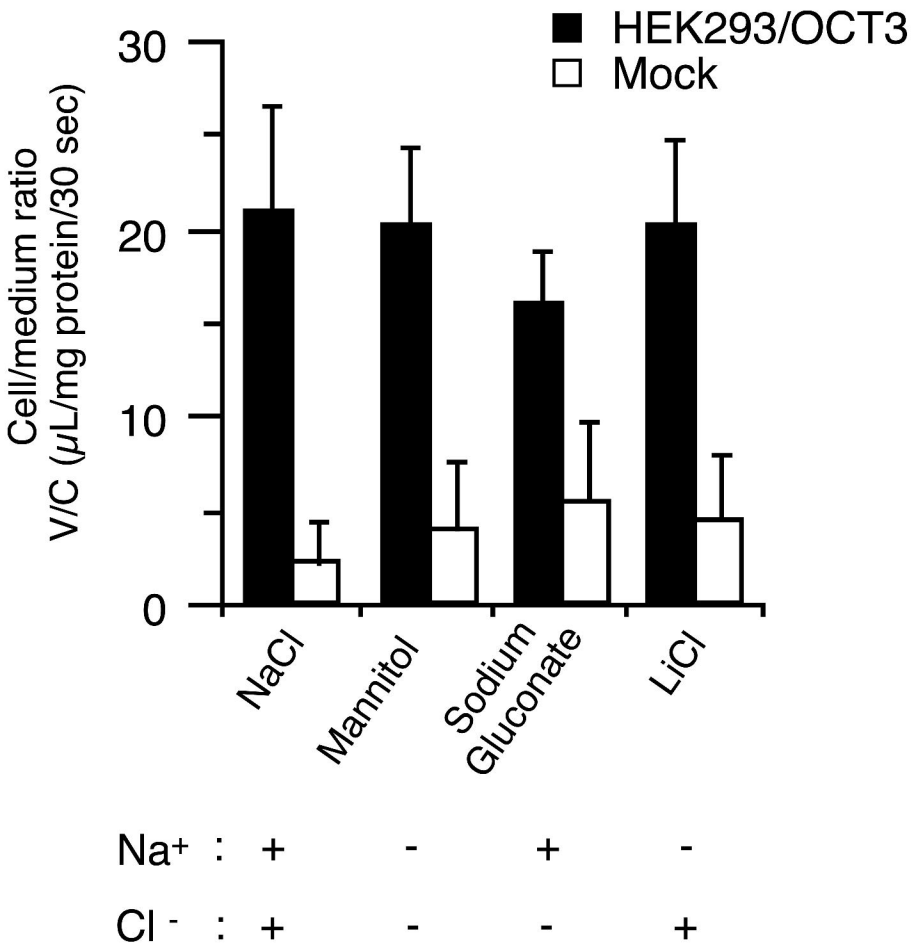


Figure 5

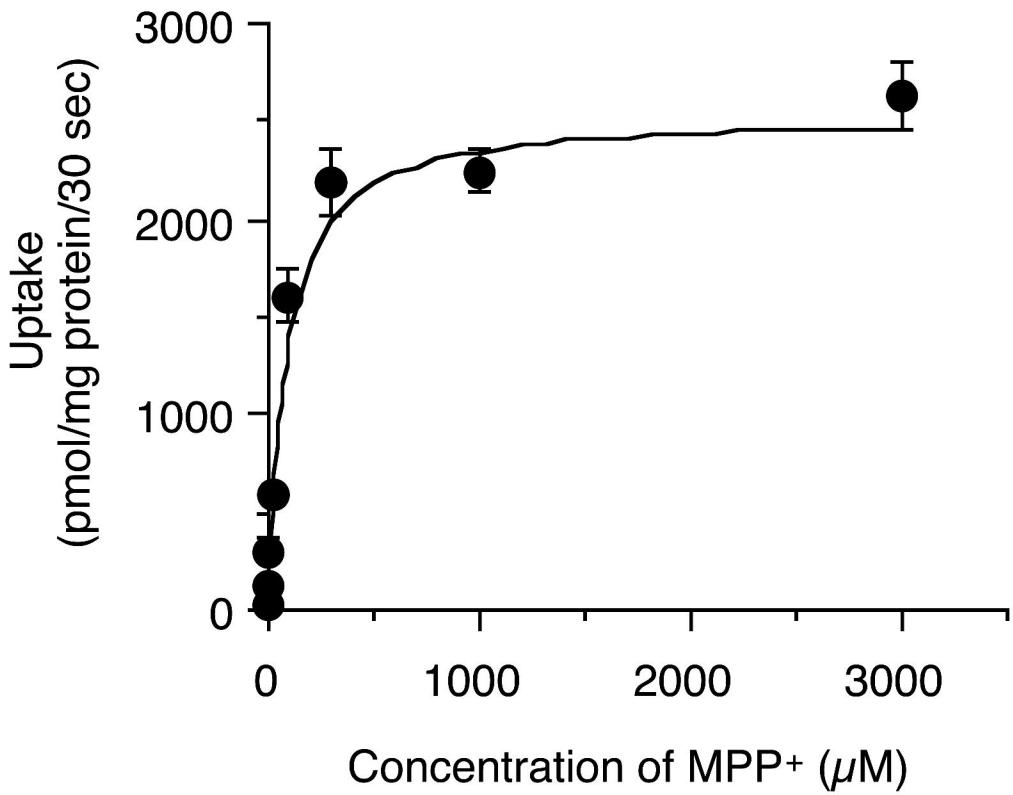


Figure 6

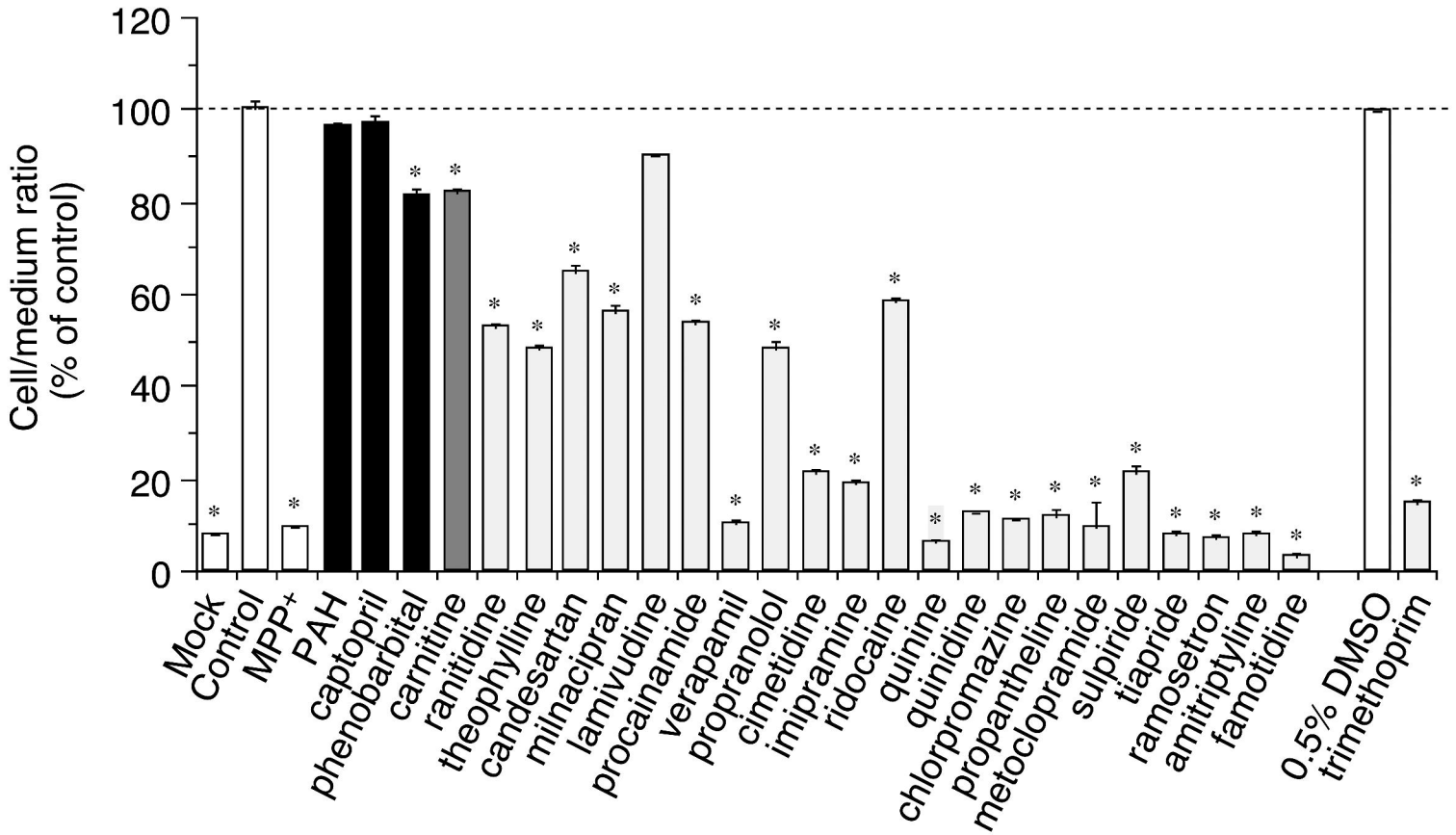


Figure 7

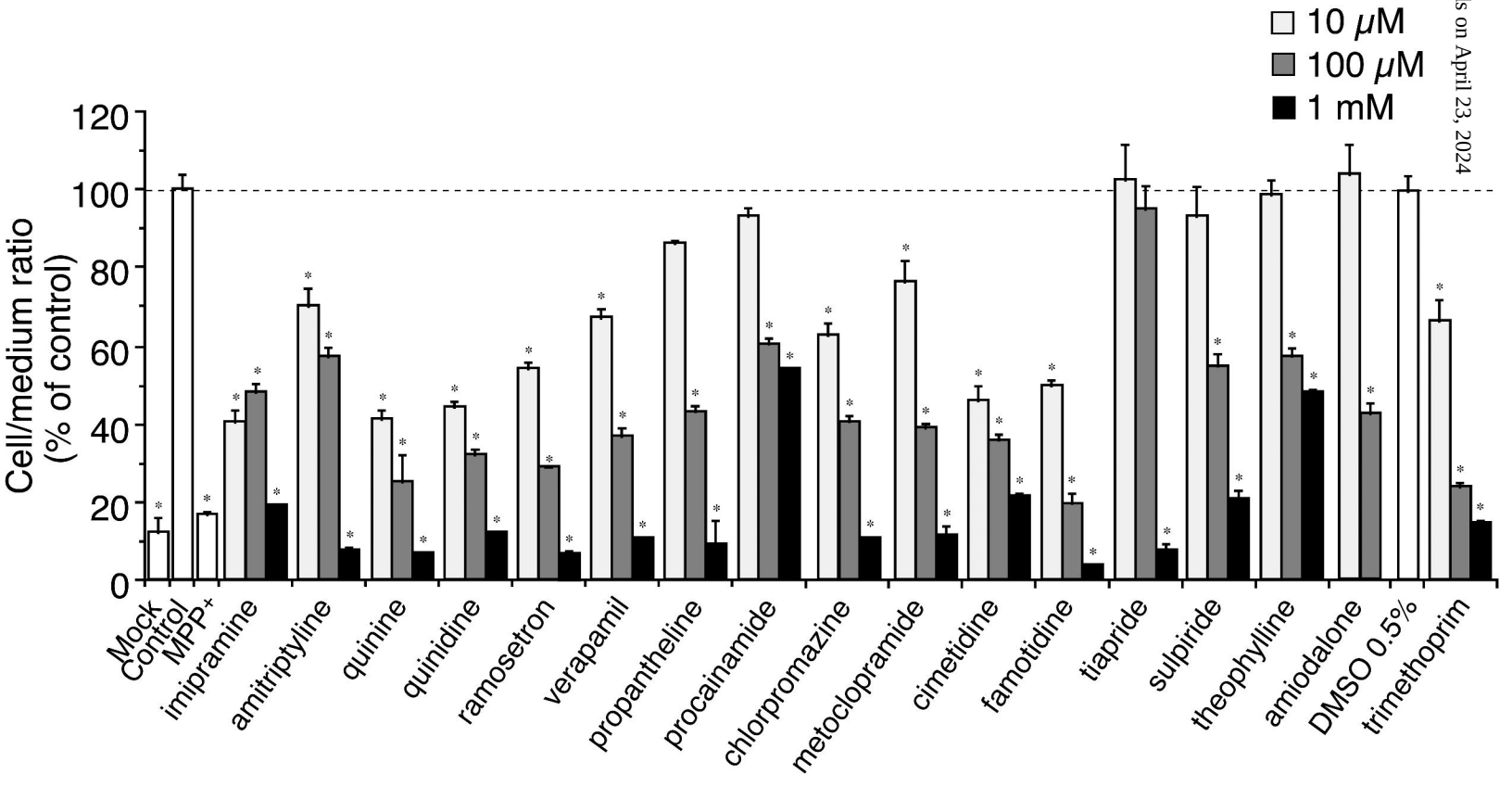


Figure 8

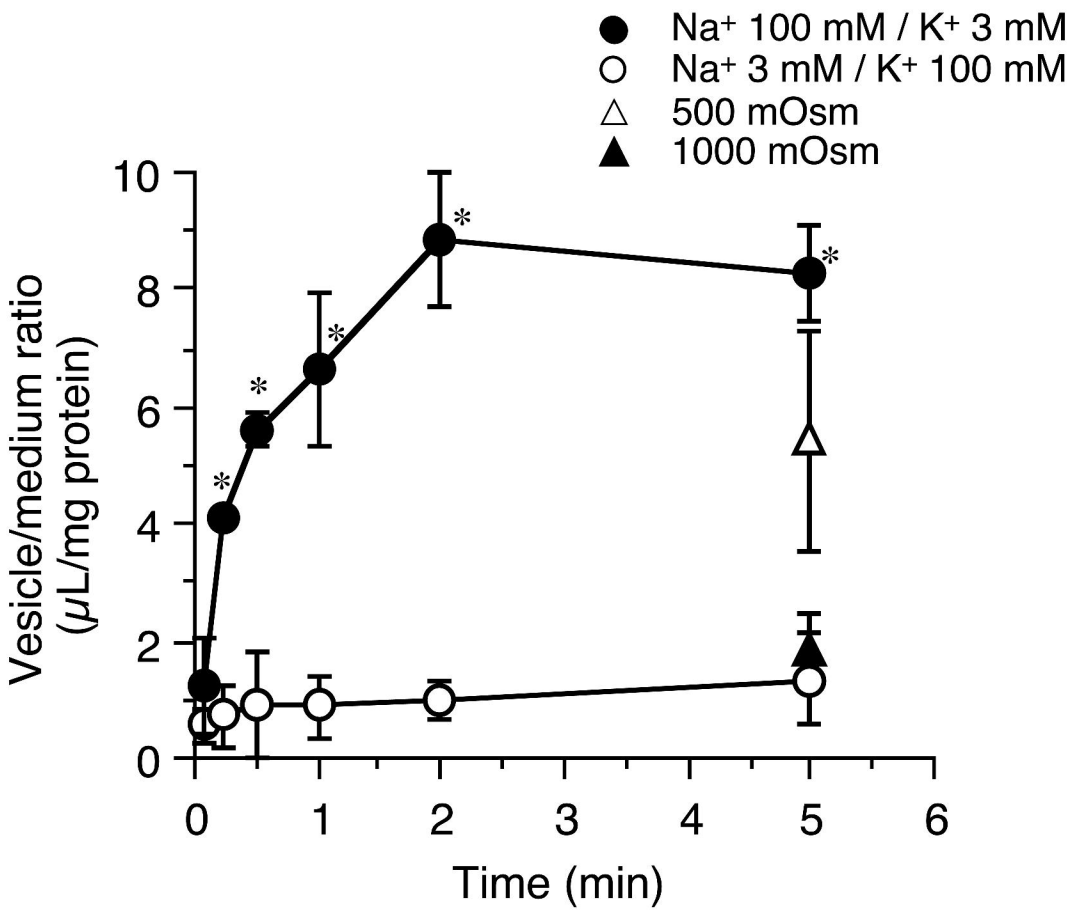


Figure 9

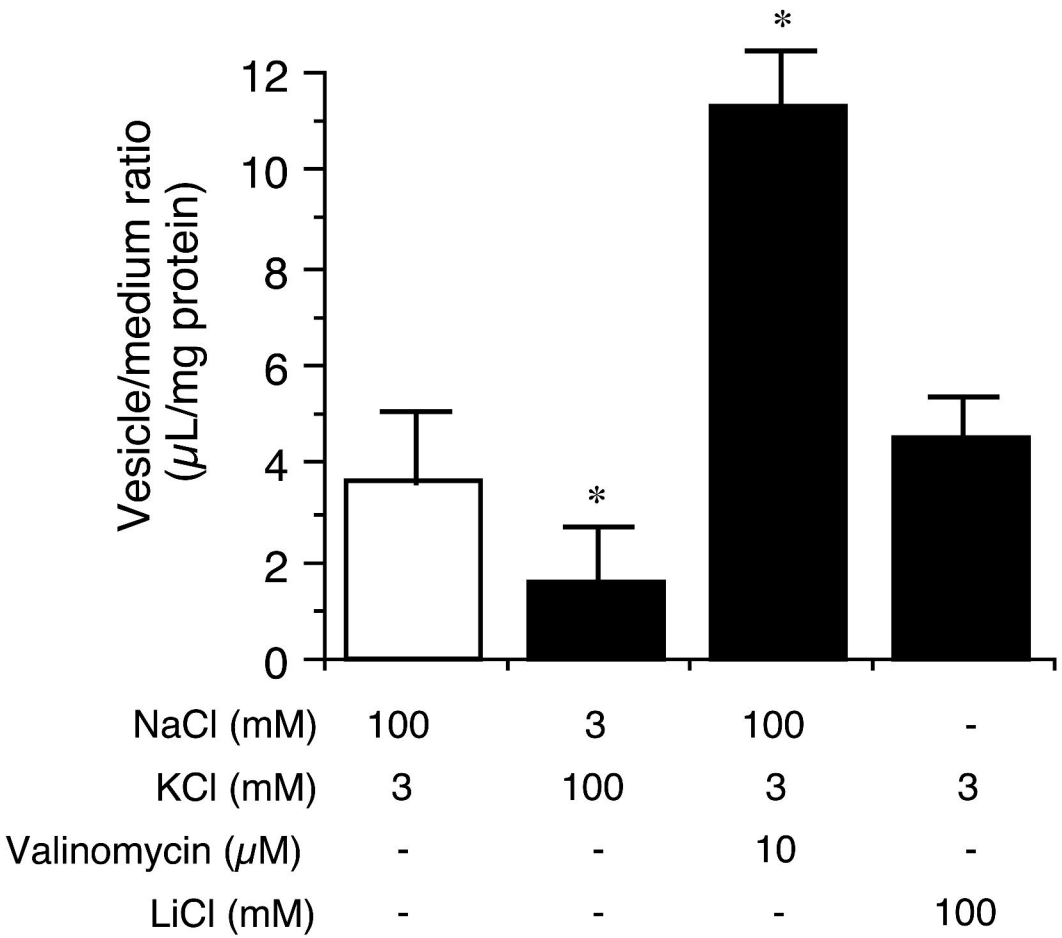


Figure 10

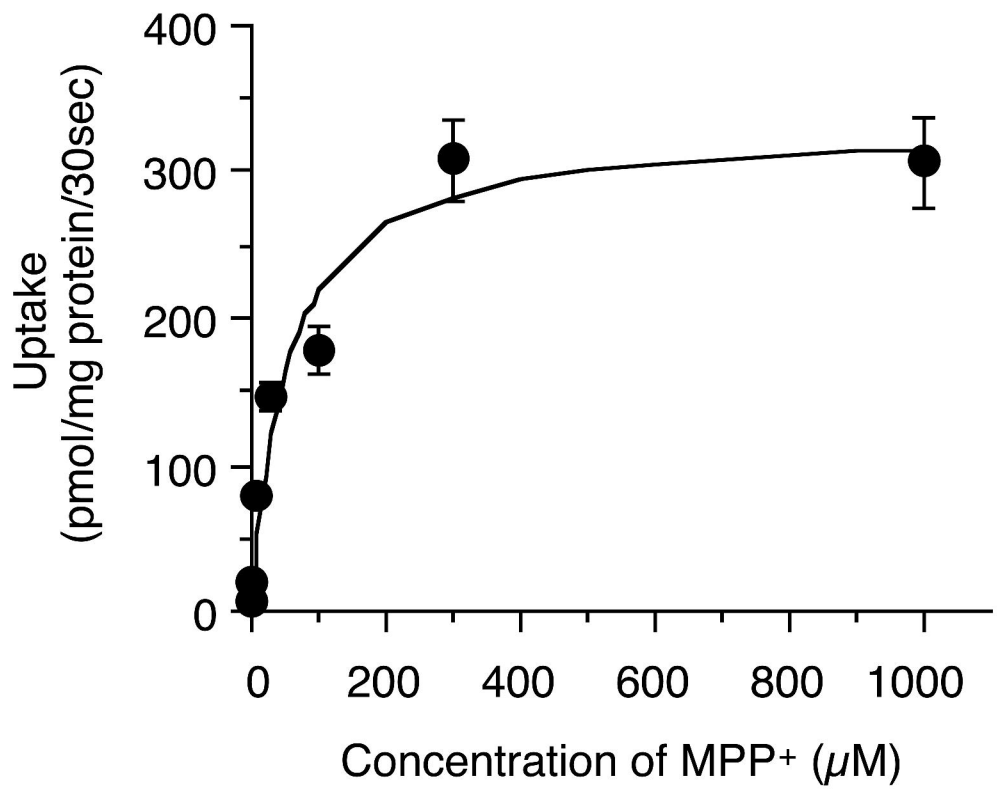

Uptake
(pmol/mg protein/30sec)
400
300
200
100
0
0
200
400
600
800
1000
Concentration of MPP+ (μM)

Figure 11

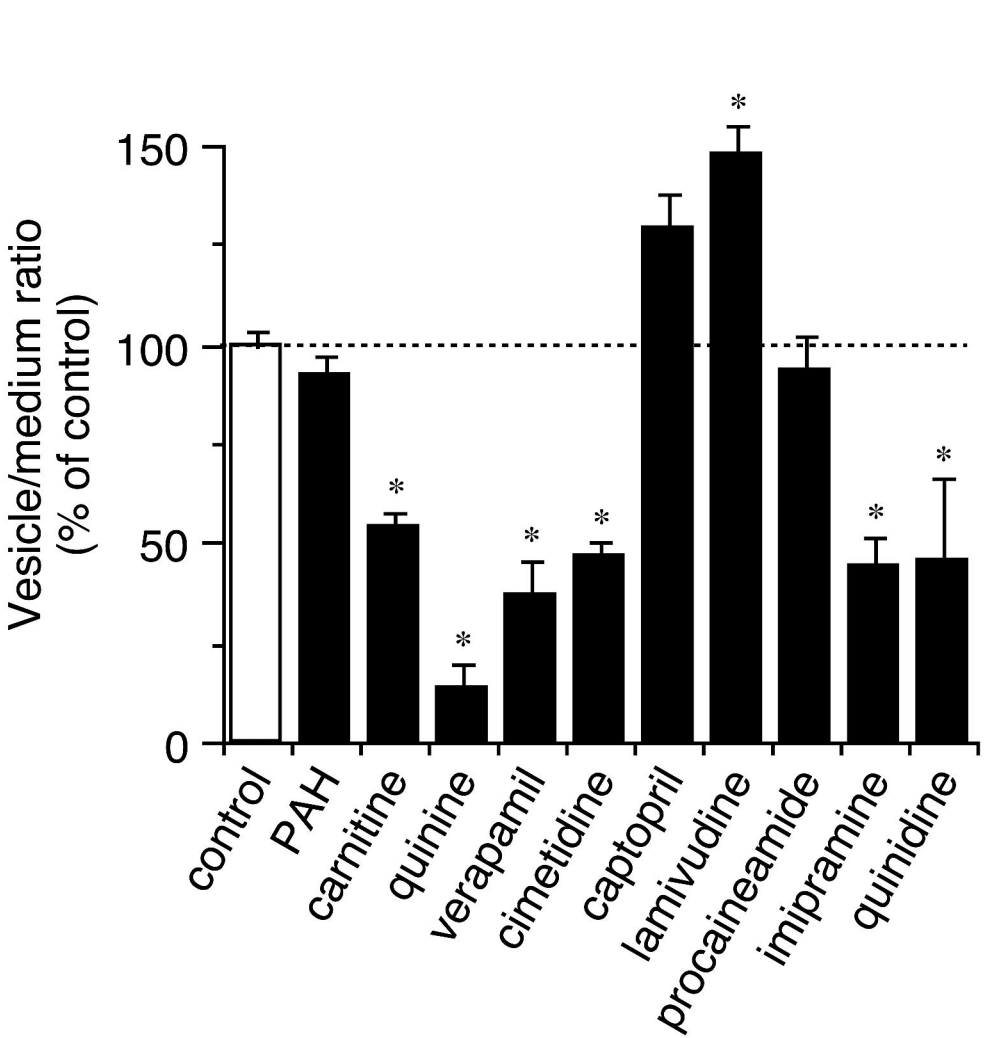
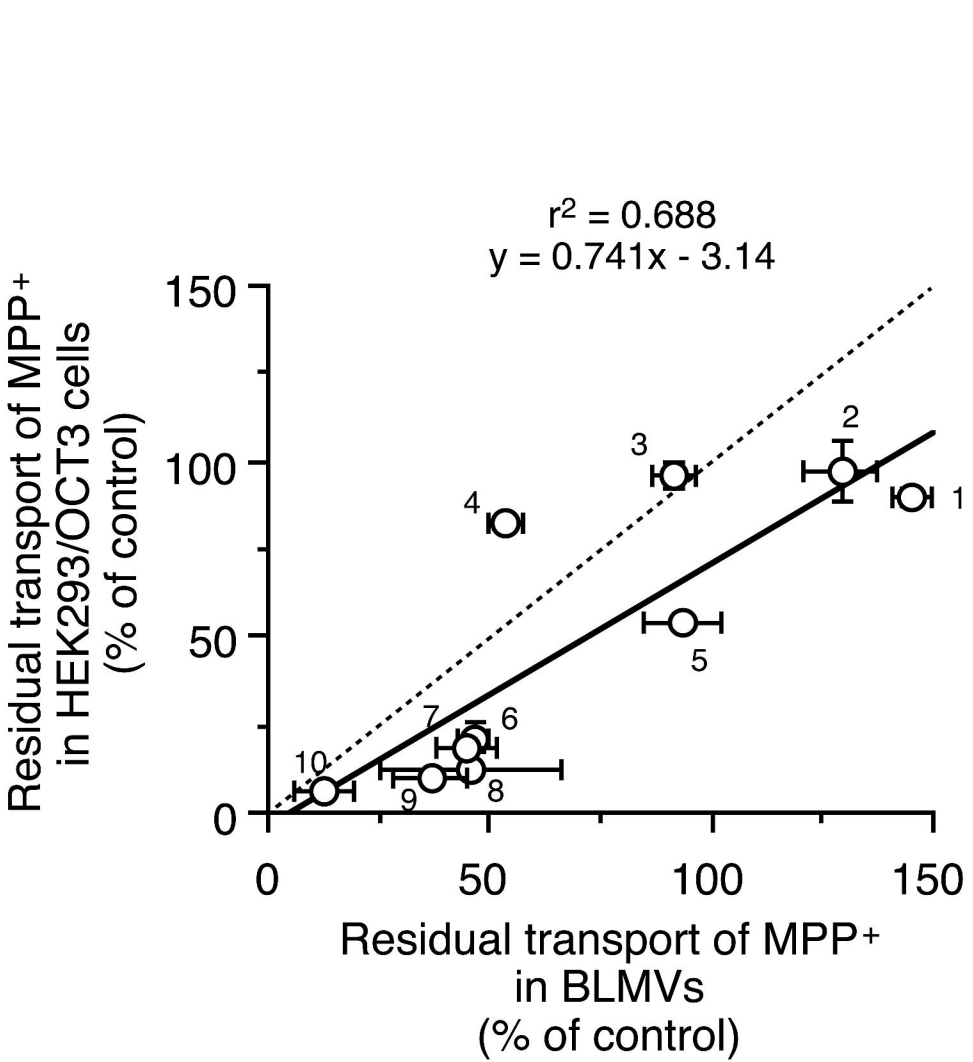


Figure 12



Inhibitors

1. lamivudine
2. captopril
3. PAH
4. carnitine
5. procainamide
6. cimetidine
7. imipramine
8. quinidine
9. verapamil
10. quinine