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The Role of Monocarboxylate Transporter 2 and 4 in the Transport of Gamma-hydroxybutyric Acid in Mammalian Cells

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Abstract

Monocarboxylate transporter 1 (MCT1) is an important determinant of the renal transport of the drug of abuse, γ-hydroxybutyric acid (GHB). The objective of this study was to investigate the role of MCT2 and MCT4, present in tissues including intestine, kidney, skeletal muscle and brain, in the membrane transport of GHB and the MCT substrate L-lactate. mRNA and protein of MCT2 and MCT4 were expressed in MDA-MB231 cells, as detected by RT-PCR and Western blot analysis; MCT1 and MCT3 were not detected. The uptake of GHB or L-lactate by MDA-MB231 cells was pH-dependent but not sodium-dependent. The concentration-dependent uptake of GHB was best-fitted to a single-transporter model with a diffusional clearance component (K_m of 17.6±1.5 mM, V_{max} of 50.6±9.0 nmol•mg^{-1}min^{-1} and diffusional clearance of 0.20±0.07 μL•mg^{-1}min^{-1}). On the other hand, the concentration-dependent uptake of L-lactate was best-fitted to a two-transporter model (K_m of 21±2.5 and 3.0±1.5 mM, and V_{max} of 268±72 and 62.9±42.2 nmol•mg^{-1}min^{-1}, respectively). The uptake of GHB and L-lactate was inhibited by MCT inhibitors α-cyano-4-hydroxycinnamate (CHC), phloretin and p-chloromercuribenzoic acid; CHC inhibited GHB and L-lactate uptake with IC_{50} values of 1.71±0.39 and 0.71±0.11 mM, respectively. siRNA treatment to silence MCT2 or MCT4 significantly decreased their protein expression and the uptake of L-lactate and GHB; however, the decrease in GHB uptake with MCT2 inhibition was smaller than for MCT4. This investigation demonstrated that GHB is a substrate for both MCT2 and MCT4; these transporters may be important in the nonlinear disposition of GHB, as well as influencing its tissue distribution.

Introduction

γ-hydroxybutyric acid (GHB) is present in mammalian brain as a metabolite of γ–aminobutyric acid (Bessman and Fishbein, 1963); studies have revealed that GHB is a neurotransmitter or neuromodulator with its own specific receptors (Snead, 2000). GHB has been detected in various tissues other than brain, including heart, kidney, liver, lung, muscle and gastrointestinal tract (Nelson et al., 1981; Tedeschi et al., 2003). GHB is approved in the USA for treatment of the sleep disorder narcolepsy (Mamelak et al., 1986), and is used in the treatment of alcohol dependence in Europe (Gallimberti et al., 2000). The abuse of GHB by body builders as a popular steroid alternative, due to its growth hormone-releasing effects (Okun et al., 2001), and by drug-abusers, as a recreational drug at nightclubs and rave parties for its euphoric effects has resulted in serious adverse effects, including coma, seizure and death (Mason and Kerns, 2002).

The pharmacokinetics of GHB has been reported to be nonlinear in rats (Lettieri and Fung, 1979) and humans (Palatini et al., 1993; Scharf et al., 1998). The nonlinear pharmacokinetics of GHB is due to capacity-limited metabolism (Lettieri and Fung, 1979; Palatini et al., 1993), absorption (Arena and Fung, 1980) and renal elimination (Morris et al., 2005). The distribution of GHB to various tissues, including the brain, is also dependent on specific transporters, since at physiologic pH, more than 99% of GHB is ionized and can not readily diffuse across cellular membranes.

Our recent studies have demonstrated that the monocarboxylate transporter 1 (MCT1) is important in the membrane transport of GHB (Morris et al., 2005; Wang et al., 2006a). Monocarboxylate transporters belong to SLC16A gene family, and consist of 14 members which differ in terms of substrate specificities and affinities (Halestrap and

Meredith, 2004). MCT 1-4 have been demonstrated to be proton-coupled transporters (Halestrap and Meredith, 2004), with similar, but not identical substrates, including the endogenous compounds, lactate and pyruvate (Garcia et al., 1994; Lin et al., 1998) and the exogenous compounds, foscarnet, nateglinide, simvastatin acid, lovastatin acid, and fluorescein (Tsuji A, 1993; Nagasawa et al., 2002; Okamura et al., 2002). However, the tissue distribution of MCT isoforms is quite different. MCT1 is ubiquitously distributed, being present in the intestine, colon, muscle, heart, brain, kidney and red blood cells (Garcia et al., 1994; Lin et al., 1998; Price et al., 1998). The tissue distribution of MCT2 is more restricted with MCT2 found mainly in testis, brain, skeletal muscle, heart and kidney (Lin et al., 1998). MCT4 is present in testis, small intestine, lung, brain, heart, kidney and spleen (Dimmer et al., 2000). Our previous studies with rat kidney cortex membrane vesicles suggested that MCT1 mediated, at least in part, the transport of GHB in mammalian kidney (Wang et al., 2006a); however, it is likely that MCT2 and MCT4, which have been shown to transport L-lactate, may also play a role in the membrane transport and disposition of GHB.

The objectives of this study were 1) to characterize the mRNA and protein expression of MCT1, MCT2, MCT3 and MCT4 in MDA-MB231 cells; 2) to investigate the transport driving forces and transport kinetics of GHB and the MCT substrate L-lactate; 3) to study the effect of MCT inhibitors on the uptake of GHB and 4) to investigate the role of MCT2 and MCT4 in GHB transport through the use of RNA interference studies.

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Materials and Methods

L-lactate, α-cyano-4-hydroxycinnamate (CHC), p-chloro-mercuribenzoic acid (pCMB), phloretin, and tetraethylammonium chloride (TEA) were purchased from Sigma (St Louis, MO). [2, 3-^{3}H]- γ-Hydroxybutyric acid (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). L-[^{14}C (U)]-lactate (56 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). MDA-MB231 cells were purchased from the American Type Culture Collection (Manassas, VA).

Cell Culture

MDA-MB231 cells were maintained in RPMI1640 (Invitrogen Corp. Carlsbad, CA) with 5% fetal bovine serum (FBS), 100 unit penicillin and 100μg/ml streptomycin, at 37 °C in 5% CO2 / 95% air environment. Cells reached confluence after 5-7 days in culture. Cells were subcultured at a ratio of 1:4 using 0.05% trypsin and 0.53 mM EDTA, and seeded in 35mm (diameter) plastic culture dishes two days before uptake studies.

RT-PCR

The primers specific to human MCT1, MCT2, MCT3, and MCT4 have been used in other studies to detect mRNA in our laboratory (Wang et al., 2006c). Briefly, first strand cDNA was synthesized from the total RNA isolated from MDA-MB231 cells. The PCR reactions were performed with 200 μM of each dNTP, 0.2 μM of each forward and reverse primer and 0.5U/reaction TAQ DNA polymerase in 1X PCR buffer through 40 cycles of 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute. The PCR products were separated by electrophoresis on 2% agarose gel and then stained by

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ethidium bromide and visualized under UV light. The PCR products were also purified using Qiaquick PCR clean-up kit (Qiagen Inc. Valencia, CA) and then sequenced.

Western blot analysis

The expression of MCT1, MCT2 and MCT4 in MDA-MB231 cells was examined by Western blot. MDA-MB231 cells were harvested and lysed with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X100, 10 mM Tris-Cl pH 7.4, 5 mM DTT, 100 μM phenylmethanesulfonyl fluoride (PMSF) in isopropanol, 5 mM ε-aminocaproic acid) on ice for 30 minutes and then centrifuged at 16,000g for 18 minutes at 4 ºC. The supernatants were collected and separated on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and then transferred to nitrocellulose membrane. After blocking with 5% (w/v) milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.05% (v/v) Tween 20 at 20 °C for one hour, the membranes were incubated with primary antibodies for MCT1 (1 μg/ml), MCT2 (1 μg/ml) and MCT4 (1 μg/ml) (US Biological, Swampscott, MA) overnight at 4°C. After incubation with specific horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA) for 1 hour at room temperature, the membranes were developed with the enhanced chemiluminescence system (ECL) (Amersham International, UK).

Uptake Studies

Growth medium was removed from the cell monolayers and cells were washed three times with uptake buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10mM HEPES, pH7.5). Uptake was initiated by adding one milliliter of buffer containing 0.05 μCi of [^{14}C]-L-lactate or 0.5 μCi [^{3}H]-GHB to the dishes. To investigate the effect of pH on uptake, the cells were incubated with uptake buffer with different pH (pH 6.0 and 7.5).

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To investigate the effect of Na^+ on uptake, the cells were incubated with uptake buffer in the presence of Na^+ (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10mM HEPES, pH 7.5 or 10 mM MES, pH 6.0) or in the absence of Na^+ (150 mM N-methyl-D-glucamine , 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ 10mM HEPES, pH 7.5, or 10 mM MES, pH6.0). To investigate the time-dependence of the uptake, cells were incubated at room temperature for 0.5, 1, 5, 10, 30, 60 and 90 minutes (if incubated longer than 90 minutes, the cells began to detach from the dishes). From these studies a time-point of 10 min was chosen to represent the linear uptake of GHB and 1 minute to represent the linear uptake of L-lactate. A short uptake period for L-lactate was chosen to minimize the cellular metabolism of L-lactate; no metabolism of GHB occurs over this 10-minute period as determined in a preliminary study. Several inhibitors were used in cellular uptake studies including CHC, pCMB, TEA, and phloretin. The uptake was stopped by aspirating the buffer, and the cells were immediately washed three times with ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5). The cells were lysed with 1ml lysis buffer containing 0.3 N NaOH and 1% SDS, and the radioactivity was determined using a liquid scintillation counter (1900 CA, Tri-carb liquid scintillation analyzer, Packard Instrument Co. Downers Grove, IL). Protein concentrations were determined using the Bicinchoninic Acid protein assay kit (BCA, Pierce Chemicals, Rockford, IL) with bovine serum albumin as standard. The results were normalized for the protein content of the cells in each dish and accumulation was expressed as pmol or nmol /mg protein/minute.

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RNA interference

Two siRNA constructs targeting MCT1, two siRNA constructs targeting MCT2 (exon4 and exon5, respectively) and two siRNA constructs targeting MCT4 (exon5), were designed using the manufacturers' supplied tools and purchased from Ambion Inc. (Houston, TX). The sequences of siRNA for MCT1 are: (sense/antisense, 5'---3') #1 GCAGUAUCCUGGUGAAUAAtt, UUAUUCACCAGGAUACUGCtg. The sequences of siRNA for MCT2 are: #5 CCCUUGAGCAAAUCUAAACtt, GUUUAGAUUUGCUCAAGGtt and #6 CCCGCCUUAACCAUAAUUGtt, CAAUUAUGGUUAAGGCGGGtt. The sequences of siRNA for MCT4 are: #3 CGUCUACAUGUACGUGUUCtt, GAACACGUACAUGUAGACGtg and #4 CCCACGUCUACAUGUACGUtt, ACGUACAUGUAGACGUGGGtc. The nonspecific scrambled control RNA was also purchased from Ambion Inc. and was used as negative control. For the transfection experiments, cells were seeded in 6-well plates at 20-40% confluence one day before transfection. Cells were transfected with siRNA at a final concentration of 20nM using Lipofectamine 2000 (Invitrogen Corp. Carlsbad, CA), according to the manufacturers' instructions. The cells were then incubated at 37ºC in 5% CO_2 for 48-96 hours before the western blot analysis and uptake experiments.

Data Analysis

All the uptake data are presented as mean ± SD. One-way analysis of variance (ANOVA) followed by a Dunnett's test was used to detect statistical significance among means of more than two groups. The differences with a p value of 0.05 or less were considered as statistically significant. Data analysis was performed using GraphPad

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Prism (GraphPad Inc., San Diego CA). The transport kinetic parameters: Michaelis – Menten constant K_m and maximum uptake rate V_{max} were determined by fitting the data using weighted nonlinear regression analysis (Adapt II, BMSR University of South California, Los Angels, CA) using the following equations:

$$v = \frac{V_{\max} * C}{K_m + C} \qquad \text{(Equation 1)}$$

$$v = \frac{V_{\max} * C}{K_m + C} + P * C \qquad \text{(Equation 2)}$$

$$v = \frac{V_{\max\_low} * C}{K_{m\_low} + C} + \frac{V_{\max\_high} * C}{K_{m\_high} + C} \qquad \text{(Equation 3)}$$

$$v = \frac{V_{\max} * C}{K_m * (1 + K_i) + C} \qquad \text{(Equation 4)}$$

$$v = \frac{V_{\max} * C}{K_m * (1 + I / K_i) + C} + P * C \qquad \text{(Equation 5)}$$

$$v = \frac{V_{\max\_low} * C}{K_{m\_low} * (1 + I / K_{i,\,low}) + C} + \frac{V_{\max\_high} * C}{K_{m\_high} * (1 + I / K_{i,\,high}) + C} \qquad \text{(Equation 6)}$$

Here *v* is the uptake rate of L-lactate or GHB and C is the concentration of L-lactate or GHB. P is the non-saturable uptake clearance. The symbol *I* represents the inhibitor concentration. K_i is the inhibition constant: K_{i_low} is the inhibition constant for low affinity transporter, and K_{i_high} is the inhibition constant for high affinity transporter. The goodness of fit was determined by the sum of the squared derivatives, the residual plot and the Akaike Information Criterion (AIC) (Kamikubo et al., 1986). The equation that provided the smallest CV% and AIC for the data, was used in obtaining K_m and V_{max} parameters for the uptake data.

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Results

RT-PCR and Western blot results

Using RT-PCR, we demonstrated that mRNA expression for MCT2 and MCT4 was present in MDA-MB231 cells (the PCR products were confirmed by sequencing) (Fig 1A), while MCT1 and MCT3 were not detected. MCT2 and MCT4 protein were also present in MDA-MB231 cells (Fig 1B).

Uptake studies

Sodium and pH effects on L-lactate uptake. A 1-minute uptake time was used to study the L-lactate uptake by MDA-MB231 cells to minimize the influence of L-lactate metabolism. Uptake of L-lactate by MDA-MB231 cells was pH-dependent, with a significantly higher uptake rate at pH 6.0 than that at pH 7.5 (Fig 2A). At pH 6.0, the uptake of L-lactate was not affected by the presence or absence of sodium, while at pH 7.5, a significant reduction was observed in the absence of sodium (Fig 2A). The uptake of L-lactate could be inhibited to the same level when using the classical MCT inhibitor CHC at the two pH values (Fig 2A).

Kinetics of L-lactate uptake Concentration-dependence of L-lactate uptake at pH 6.0 was demonstrated in this study, and the uptake rate was fitted by equations 1, 2 and 3. An Eadie-Hofstee plot indicated that more than one transporter might be involved in the uptake of L-lactate (Fig 2C). A two-transporter model described the data well with fitted K_{m_low} and K_{m_high} for low and high affinity transporters of 21.3 mM and 3.0 mM, and V_{max_low} and V_{max_high} for low and high affinity transporters of 268 and 62.9 $nmol \bullet mg^{-1} min^{-1}$, respectively (Table 1 and Fig 2B).

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Effects of MCT inhibitors on L-lactate uptake. The uptake of L-lactate by MDA-MB231 cells was significantly inhibited by the MCT inhibitors, phloretin (0.1 mM), pCMB (0.1 mM), and CHC (2mM) to about 20% of control (Fig 3A). A concentration-dependent inhibition of L-lactate uptake by CHC was demonstrated (Fig 3B), and the IC_{50} value was determined to be 0.71 mM (Table 1). CHC can competitively inhibit L-lactate uptake in human kidney HK-2 cells that express predominantly MCT1 (Wang et al., 2006b), and has been reported to be a competitive inhibitor for MCTs (Halestrap and Price, 1999). Therefore, competitive inhibition models were used to obtain the inhibition constants with the two-transporter model. The inhibition constants were determined to be 2.1 mM, and 0.03 mM for high and low K_m transporters, respectively.

Sodium and pH effects on GHB uptake. Uptake of GHB by MDA-MB231 cells was pH-dependent, with a significantly higher uptake rate at pH 6.0 than that at pH 7.5 (Fig 4A). At pH 6.0, the uptake of GHB was not affected by the presence or absence of sodium, while at pH 7.5, a significant reduction was observed in the absence of sodium ($P<0.05$) (Fig 4A). The uptake of GHB was inhibited to similar levels (no statistical difference) when using the classical MCT inhibitor CHC at the two pH values of 6.0 and 7.4 (Fig 4A).

Kinetics of GHB uptake. The concentration-dependent uptake of GHB at pH 6.0 was demonstrated in this study (Fig 4B). An Eadie-Hofstee plot suggested that more than one transporter or a transporter plus non-transporter mediated process might be involved in the uptake of L-lactate (Fig 4C). A model with one-transporter plus a diffusional component described the data well with fitted K_m and V_{max} of 17.6 mM and 50.6

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nmol•mg^{-1}min^{-1}, respectively, and a diffusional clearance of 0.20 μL•mg^{-1}min^{-1} (Table 1 and Fig 4B).

Effects of MCT inhibitors on GHB uptake. The uptake of GHB by MDA-MB231 cells was significantly inhibited by the MCT inhibitors, phloretin (0.1 mM), pCMB (0.1 mM), and CHC (2mM) to about 60% of control, but not by an organic cation transporter inhibitor TEA (Fig 5A). A concentration-dependent inhibition of GHB uptake by CHC was demonstrated (Fig 5B), and the IC_{50} value was determined to be 1.71 mM (Table 1). The uptake of GHB in the presence of 10 mM of CHC was about 30% of control, which also suggested a non-transporter mediated uptake of GHB by MDA-MB231 cells. Since previous studies have indicated that CHC is a competitive inhibitor for MCT-mediated GHB transport (Wang et al., 2006b), we used equation 5 to obtain an estimate of the inhibition constant. For these inhibition data, we also examined other kinetic models as described by equations 4 and 6; however, evaluation of goodness of fit indicated that equation 5 provided the best fitting of the data. The fitted K_i value was 1.8 mM, which is close to the K_{i_low} (2.1 mM) for the CHC inhibition of L-lactate.

Effects of siRNA treatment in MDA-MB231 cells

Treatment with siRNAs for MCT4. Transfection of MDA-MB231 cells with either of the two siRNAs for MCT4 significantly decreased the protein expression of MCT4 in MDA-MB231 cells as determined by Western blot analysis (Fig. 6A). These two siRNAs for MCT4 had no effect on the protein expression of MCT2 (data not shown). The siRNA for MCT1 had no effect on the protein expression of MCT4 or MCT2, and the uptake of GHB or L-lactate by MDA-MB231 cells was not affected by the siRNA treatment for

MCT1 (data not shown). Using ^{14}C-L-lactate as the substrate, the uptake was significantly decreased following siRNA treatment for MCT4, with #4 siRNA being the most effective (Fig 6B). The uptake of GHB was significantly decreased to 60% of the negative control in cells following siRNA treatment for MCT4 (Fig 6C).

Treatment with siRNAs for MCT2. Transfection of MDA-MB231 cells with either of the two siRNAs for MCT2 significantly decreased the protein expression of MCT2 (Fig 7A). These two siRNAs had no effect on the protein expression of MCT4 as examined by Western blot analysis (data not shown). The uptake of L-lactate was significantly inhibited by one of the siRNA treatments (#6), although the inhibition was moderate, with only a 17% decrease in uptake (Fig 7B). The uptake of GHB following siRNA treatment (with #6) was significantly lower than that of the negative control (Fig 7C); as observed with L-lactate, the inhibition was modest, with only 15% inhibition (Fig 7C).

Discussion

GHB is a monocarboxylic acid with a pKa value of 4.7, which is more than 99% ionized at physiological pH. The transport of GHB across various biological barriers and cellular membranes, such as blood-brain barrier, brain cells and proximal tubule cells requires specific transporters (Benavides et al., 1982; Bhattacharya and Boje, 2004; Wang et al., 2006a). The renal transport of GHB has been studied previously, and the monocarboxylate transporter-1 (MCT1) represents an important transporter for GHB transport across the proximal tubule cells (Wang et al., 2006a). Pyruvate uptake by MCT2 transfected oocytes can be inhibited by GHB, suggesting that GHB may be a substrate of MCT2 (Lin et al., 1998).

L-lactate, a known substrate of MCT2 and MCT4 (Halestrap and Meredith, 2004), exhibited decreased uptake in this investigation in MDA-MB231 cells following siRNA treatment for either MCT2 or MCT4. Kinetic studies of L-lactate demonstrated a high affinity transporter with a K_m of 3 mM and a low affinity transporter with a K_m of 21 mM; these values are similar to the previous reported values for MCT2- (Garcia et al., 1995; Lin et al., 1998) and MCT4-mediated (Dimmer et al., 2000; Manning Fox et al., 2000) transport of L-lactate, respectively. The K_i values for CHC for the low affinity and high affinity transporters were also similar to previous reported K_i values for MCT2 and MCT4, respectively (Juel, 1997; Broer et al., 1999). The transport of GHB by MCT2 or MCT4 has not been previously studied. Kinetic data of GHB transport in MDA-MB231 cells supported a model with a single transporter plus a diffusional component; however, RNA interference studies for both MCT2 and MCT4 demonstrated significant reductions in the uptake of GHB. The activity of the high-affinity transporter, possibly MCT2, could

not be identified using model fitting techniques, even in the presence of a transport inhibitor. The K_i value (1.8 mM) determined for GHB inhibition may well represent the K_i for the low-affinity transporter, which is very similar to that for the low-affinity L-lactate transporter (2.1 mM). Overall our data suggested that both MCT2 and MCT4 represent transporters of GHB, but the contribution of MCT2 to GHB uptake in MDA-MB231 cells was small. Indeed, the inhibition of GHB uptake after MCT4 siRNA treatment was able to decrease the uptake to a similar extent as the chemical inhibitors CHC and phoretin; on the other hand, the inhibition of GHB uptake after MCT2 siRNA treatment only explained 15% of the total GHB uptake, indicating that it is relatively minor component of the overall uptake. The transport of GHB by MDA-MB231 cells with a K_m value of 17.6 mM was much lower than a previous estimation using an indirect fluorescence indicator pHi method (> 500 mM) (Manning Fox et al., 2000). This difference in K_m value may be explained partly by the experimental differences in the studies, including the buffer pH used. It has been demonstrated previously that the affinity of substrates to MCT increases as pH decreases (Halestrap and Price, 1999).

The transport of L-lactate or GHB at pH 7.5 was significantly affected by the presence or absence of sodium, which suggested that a sodium-dependent transporter(s) might be involved in L-lactate or GHB transport. This sodium-effect was not observed at pH 6.0, suggesting that with a low pH as the driving force, MCT transporters represent the predominant transporters for L-lactate and GHB.

GHB has been found in several extraneuronal tissues, including gastrointestinal tract, kidney, muscle, heart, liver, lung, brain fat and blood (Nelson et al., 1981; Tedeschi et al., 2003). The endogenous concentration of GHB in the kidney, gastrointestinal tract, heart

and muscle are much higher than that in brain and blood; however, the origin and function of GHB in extraneuronal tissues are not known. MCT1, MCT2 and MCT4 have been found to be present in brain, muscles, kidney, heart, gastrointestinal tract and blood cells (Pellerin et al., 1998; Eladari et al., 1999; Bergersen et al., 2002; Merezhinskaya et al., 2004; Gill et al., 2005). The presence of these MCT transporters in these cellular or subcellular membranes enables the transport of GHB among various tissue types; therefore, monocarboxylate transporters are important in the physiological/pharmacological action of GHB, as well as in the absorption and disposition of GHB in the body.

It has been the reported that absorption of GHB from the gastrointestinal tract was capacity-limited (Arena and Fung, 1980); however, the identity of possible transporters was not investigated. MCT1, MCT2 and MCT4 have been reported to be present in the gastrointestinal tract, with MCT1 exhibiting an apical membrane localization and MCT4 localized to the basolateral membrane (Gill et al., 2005). The renal reabsorption of GHB is also capacity limited and represents a major mechanism of elimination following GHB overdoses (Morris et al., 2005). We have demonstrated previously that MCT1 mediated the transport of GHB in rat kidney cortex; however, whether MCT2 also represents a GHB transporter was unclear (Wang et al., 2006a). In this study, we demonstrate that MCT2 is indeed a GHB transporter. MCT2 has been demonstrated to be present in the kidney in the medullary thick limbs of Henle where MCT1 is not present (Eladari et al., 1999), so it may represent an important GHB transporter at the medullary region of kidney. The transport of GHB across the blood-brain barrier (BBB) may also be transporter-mediated, and an MCT isoform was proposed as an uptake transporter

(Bhattacharya and Boje, 2004). MCT1 has been shown to be present on both the abluminal and luminal membranes of BBB endothelial cells (Gerhart et al., 1997), suggesting that MCT1 may represent a major transporter for GHB at the BBB. MCT4 has been reported to be strongly expressed in astrocytes (Bergersen et al., 2002; Pellerin et al., 2005), suggesting the MCT4-mediated transport of GHB from BBB to neurons. MCT2 is a major MCT transporter in neurons, and is present in the postsynaptic membrane of parallel fiber-Purkinje cell synapses (Bergersen et al., 2002; Bergersen et al., 2005), suggesting its role in the uptake of GHB into neurons. More studies are needed in order to investigate the role of MCT isoforms in GHB transport in the central nervous system.

In summary, this investigation using MDA-MB231 cells that express MCT2 and MCT4 has demonstrated that the transport of the monocarboxylates L-lactate and GHB are mediated by both MCT2 and MCT4. MCT4 represents the major transporter for GHB in these cells, with MCT2 responsible for a minor component of the uptake of GHB. These transporters may be important in both the bioavailability and renal clearance of GHB, as well as influencing its pharmacological activity by influencing its brain uptake and distribution.

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Figure Legends

Figure 1. Expression of MCT isoforms in MDA-MB231 cells. A, the RT-PCR product of MCT2 (251 bp) and MCT4 (200 bp). B, Protein expression of MCT2 (36 KD) and MCT4 (50 KD). The Western blots were treated with MCT2 or MCT4 antibodies first, and then treated with the β-actin antibody. Upper lanes are for MCT isoforms protein, lower lanes are β-actin as the loading control.

Figure 2. Uptake of L-lactate in MDA-MB231 cells: Driving forces and concentration-dependence. A, pH and sodium effects on the uptake of L-lactate (0.5 μM). The sodium buffer contained 150 mM sodium, while the non-sodium buffer substituted N-methyl-D-glucamine for the sodium. All the uptake data were normalized to the uptake at pH 7.5 in the presence of sodium and compared to this uptake.. B, concentration-dependent uptake at pH 6.0, with the line representing the model-fitting of the data; C, Eadie-Hofstee plot of the concentration-dependent data. Data presented as mean ± SD, n =3-4 independent experiments with triplicates determinations. One-way ANOVA followed by a Dunnett's test was used to detect statistical significance.* $P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 3. Uptake of L-lactate in MDA-MB231 cells: Effects of inhibitors. A, effects of selected compounds on the uptake of L-lactate. The concentration of L-lactate was 0.5 mM, and those for CHC, phloretin, pCMB (p-chloro-mercuribenzoic acid) and TEA

(tetraethyl-ammonium chloride) were 2, 0.1, 0.1 and 5 mM, respectively; B, concentration-dependent inhibition of L-lactate (0.5 mM) uptake by CHC, with the line representing the model-fitting of the data to a competitive inhibition model. Data presented as mean ± SD, n =3-4 independent experiments with triplicates determinations for each experiment. One-way ANOVA followed by a Dunnett's test were used to detect statistical difference. **P<0.01.

Figure 4. Uptake of GHB in MDA-MB231 cells: Driving forces and concentration-dependence. A, pH and sodium effects on the uptake of GHB (5.7 nM). The sodium buffer contained 150 mM sodium, while the non-sodium buffer substituted N-methyl-D-glucamine for the sodium. All the uptake data were normalized to the uptake at pH 7.5 in the presence of sodium. B, concentration-dependent uptake at pH 6.0, with the lines representing the model-fitting of the data; C, Eadie-Hofstee plot the of concentration-dependent data. Data presented as mean ± SD, n = 3 to 4 independent experiments with triplicate determinations for each experiment. * P<0.05, **P<0.01, ***P<0.001.

Figure 5. Uptake of GHB in MDA-MB231 cells: Effects of inhibitors. A, effects of selected inhibitors on the uptake of GHB in MDA-MB231 cells; GHB concentration was 0.1 mM, and the concentrations of CHC, phloretin, pCMB and TEA were 2, 0.1, 0.1 and 5mM, respectively. B, concentration-dependent uptake of GHB in MDA-MB231 cells at pH 6.0, the line represents the model-fitting of the data to a competitive inhibition model;

Data presented as mean ± SD, n =3-4 independent experiments with triplicate determinations for each experiment. * P<0.05, **P<0.01, ***P<0.001.

Figure 6. Effects of treatment with MCT4 siRNA in MDA-MB231 cells. A, MCT4 protein expression after treatment of MDA-MB231 cells with two siRNAs for MCT4, with β-actin used as the loading control; B, uptake of L-lactate (0.5 μM) in siRNA-treated MDA-MB231 cells; C, uptake of GHB (0.1 mM) in siRNA-treated MDA-MB231 cells. #3 and #4 are the two siRNAs for MCT4 used in the study (see Methods for details). Data presented as mean ± SD, n =6-9. **P<0.01.

Figure 7. Effects of treatment with MCT2 siRNA in MDA-MB231 cells. A, MCT2 protein expression after treatment of MDA-MB231 cells with two siRNAs for MCT2, with β-actin used as the loading control; B, uptake of L-lactate (0.5 μM) in siRNA-treated MDA-MB231 cells; C, uptake of GHB (0.1 mM) in siRNA-treated MDA-MB231 cells. #5 and #6 are the two siRNAs for MCT2 used in the study (see Methods for details). Data presented as mean ± SD, n =6-9. * P<0.05, **P<0.01, ***P<0.001.

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Table 1. Kinetic parameters for the uptake of L-lactate and GHB.

The parameters are presented as mean ± SD, n = 3-4 independent experiments with triplicate determinations.

Parameters	L-lactate	GHB
V_{max_low} (nmol•mg^{-1}min^{-1})	268 ± 72	50.6 ±9.0
K_{m_low} (mM)	21.3 ± 12.5	17.6 ± 1.5
V_{max_high} (nmol•mg^{-1}min^{-1})	62.9 ± 42.2	
K_{m_high} (mM)	3.0 ± 1.5	
P (µL•mg^{-1}min^{-1})		0.20 ± 0.07
IC_{50} of CHC (mM)	0.71 ± 0.11	1.71 ±0.39
K_{i_low} (mM)	2.10 ± 0.32	1.82 ± 0.39
K_{i_high} (mM)	0.03 ± 0.01	

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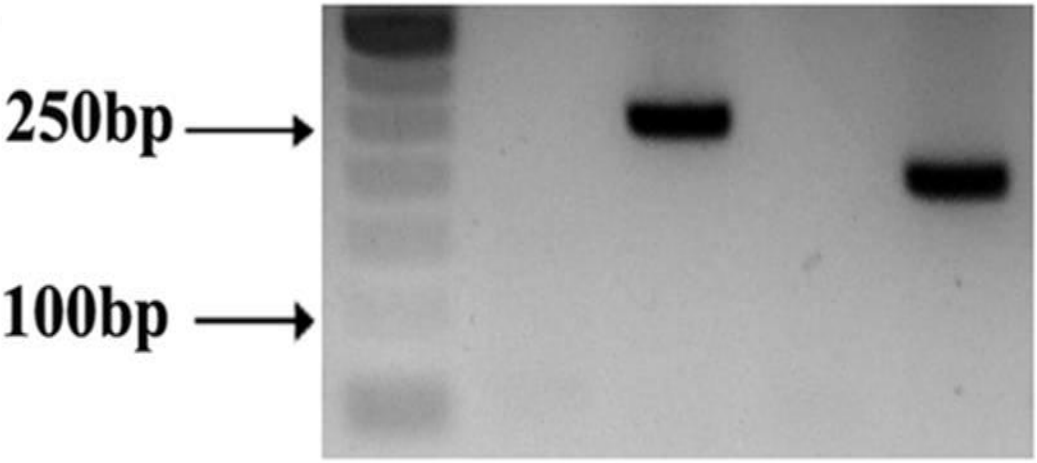
Figure 1

A



B

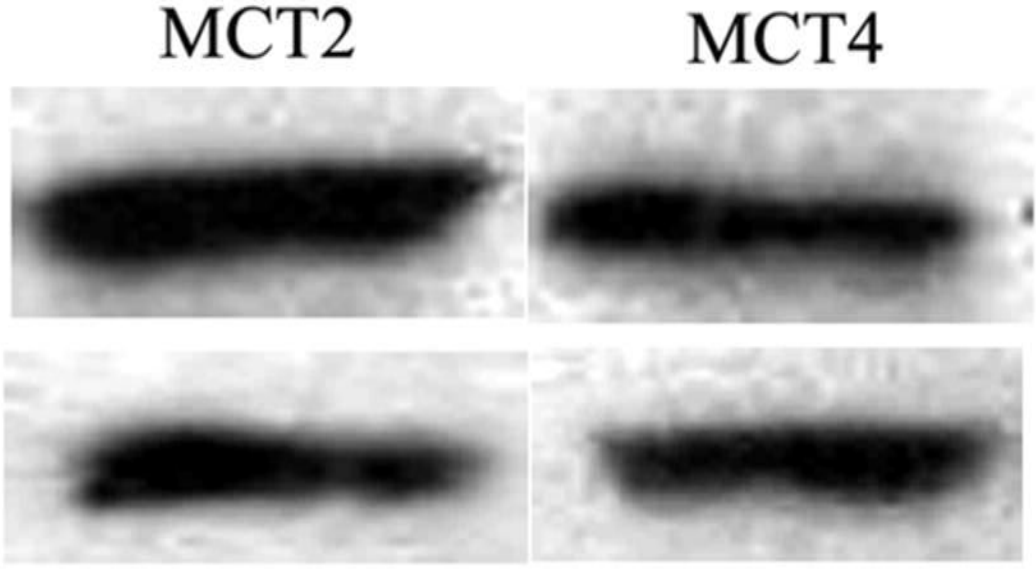


Figure 2

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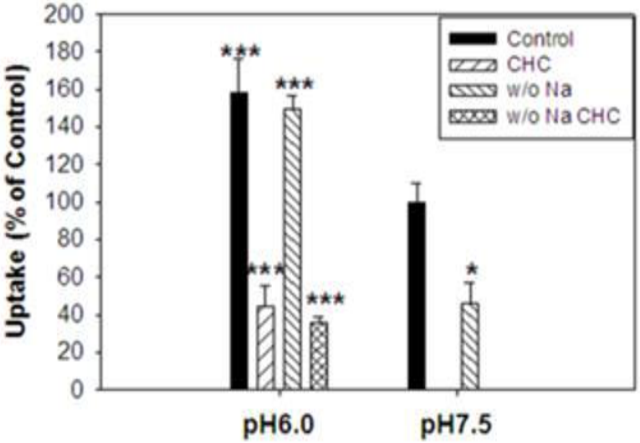
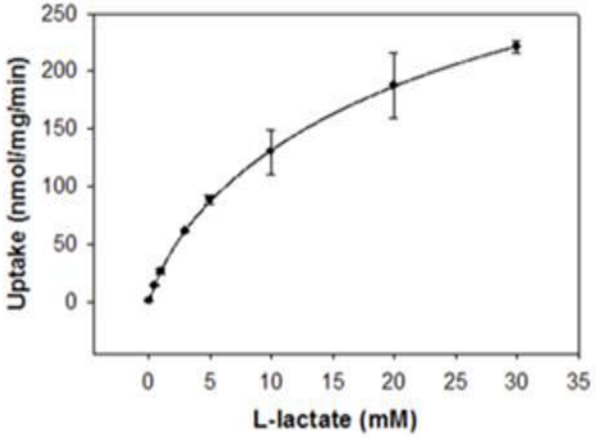

Uptake (% of Control)
200
180
160
140
120
100
80
60
40
20
0
Control
CHC
w/o Na
w/o Na CHC
***
***
***
***
*
pH6.0
pH7.5

B


Uptake (nmol/mg/min)
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200
150
100
50
0
0
5
10
15
20
25
30
35
L-lactate (mM)

C

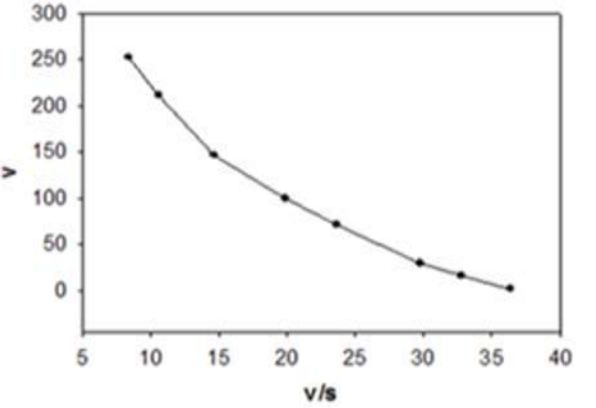
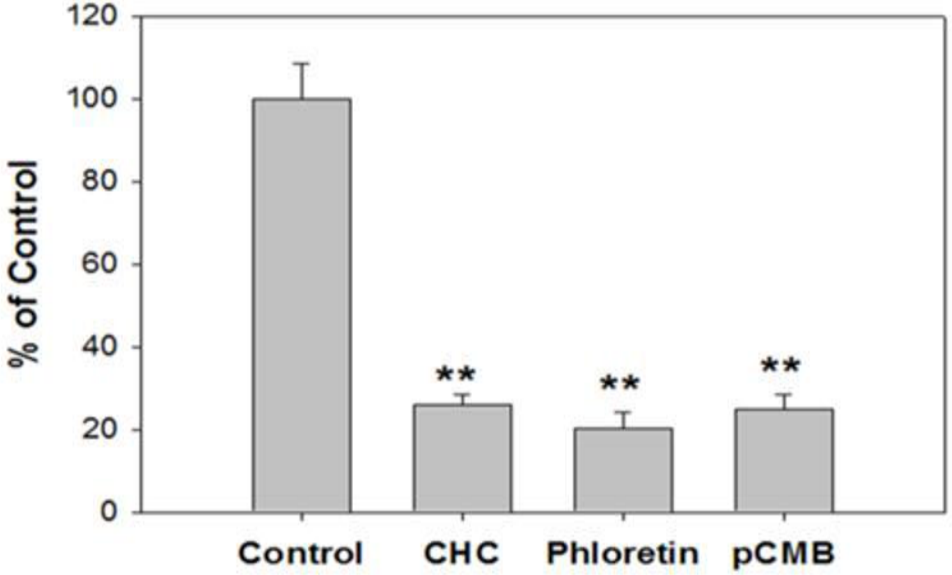

v
300
250
200
150
100
50
0
5
10
15
20
25
30
35
40
v/s

Figure 3

A


120
100
80
60
40
20
0
% of Control
**
**
**
Control
CHC
Phloretin
pCMB

B

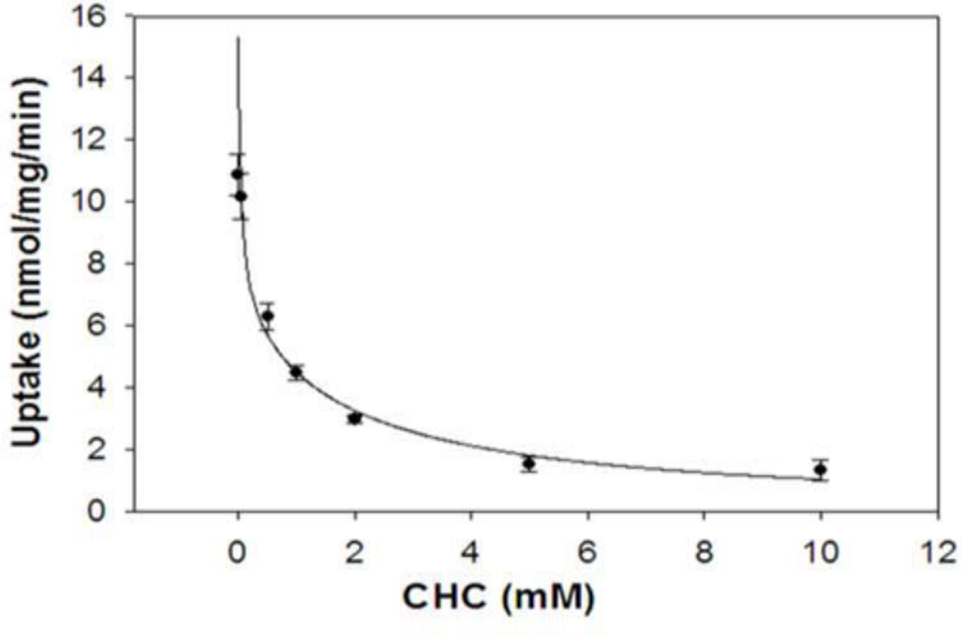
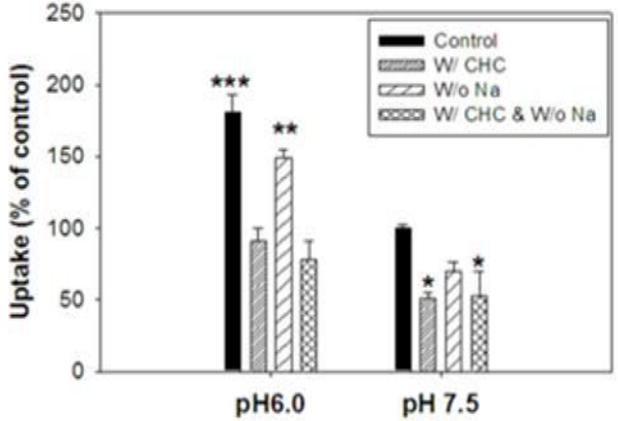
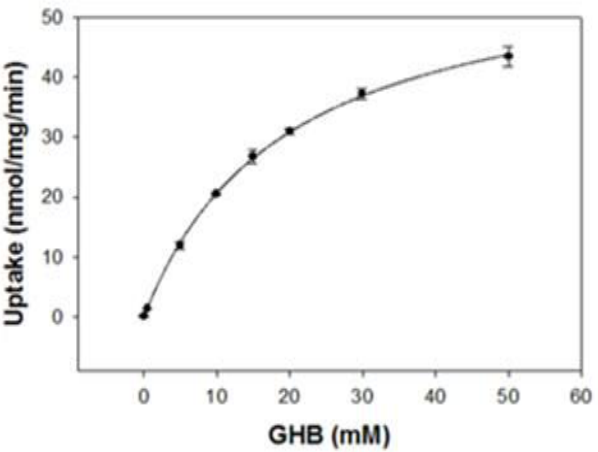

16
14
12
10
8
6
4
2
0
Uptake (nmol/mg/min)
0
2
4
6
8
10
12
CHC (mM)

Figure 4

A


Control
W/ CHC
W/o Na
W/ CHC & W/o Na
Uptake (% of control)
250
200
150
100
50
0
***
**
*
*
pH6.0
pH 7.5

B


Uptake (nmol/mg/min)
50
40
30
20
10
0
0
10
20
30
40
50
60
GHB (mM)

C

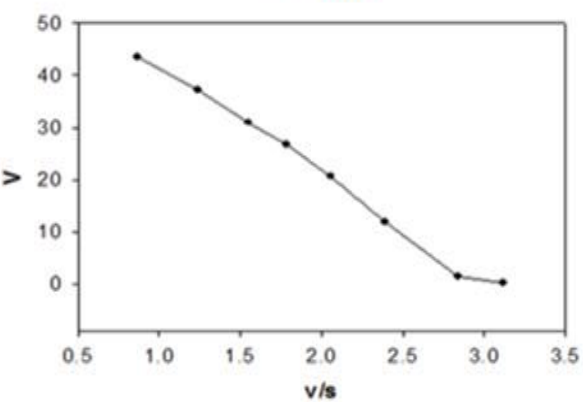
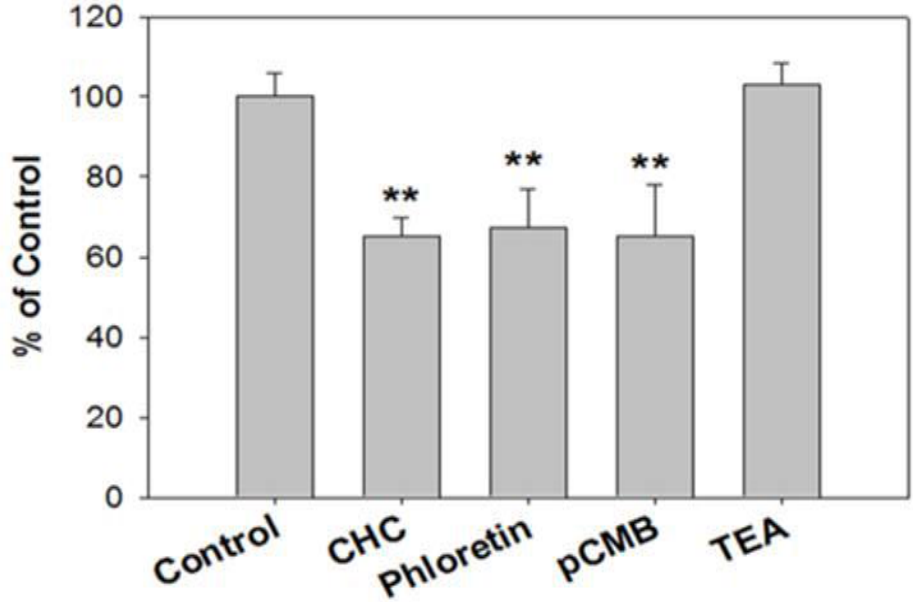

v
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
v/s

Figure 5

A


120
100
80
60
40
20
0
% of Control
**
**
**
Control
CHC
Phloretin
pCMB
TEA

B

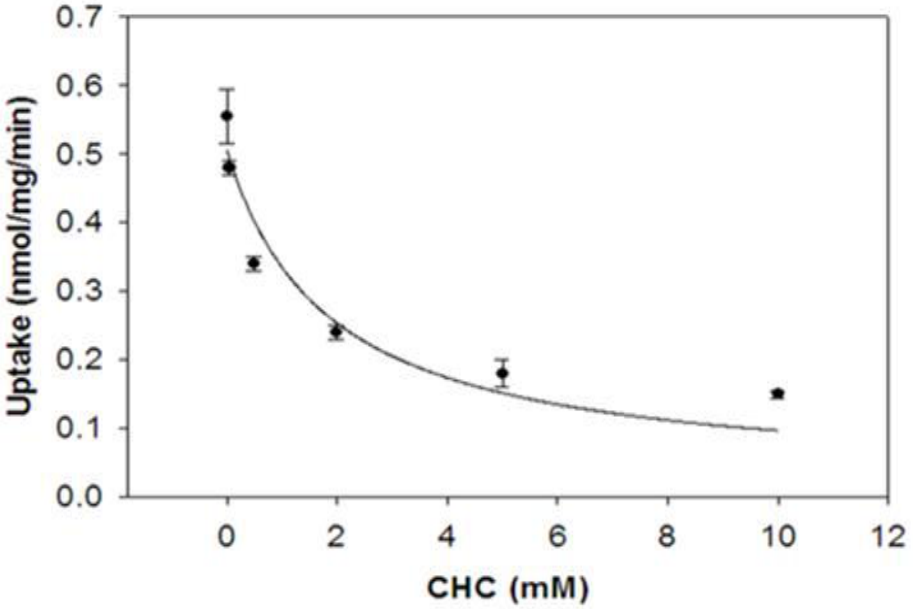

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Uptake (nmol/mg/min)
0
2
4
6
8
10
12
CHC (mM)

Figure 6

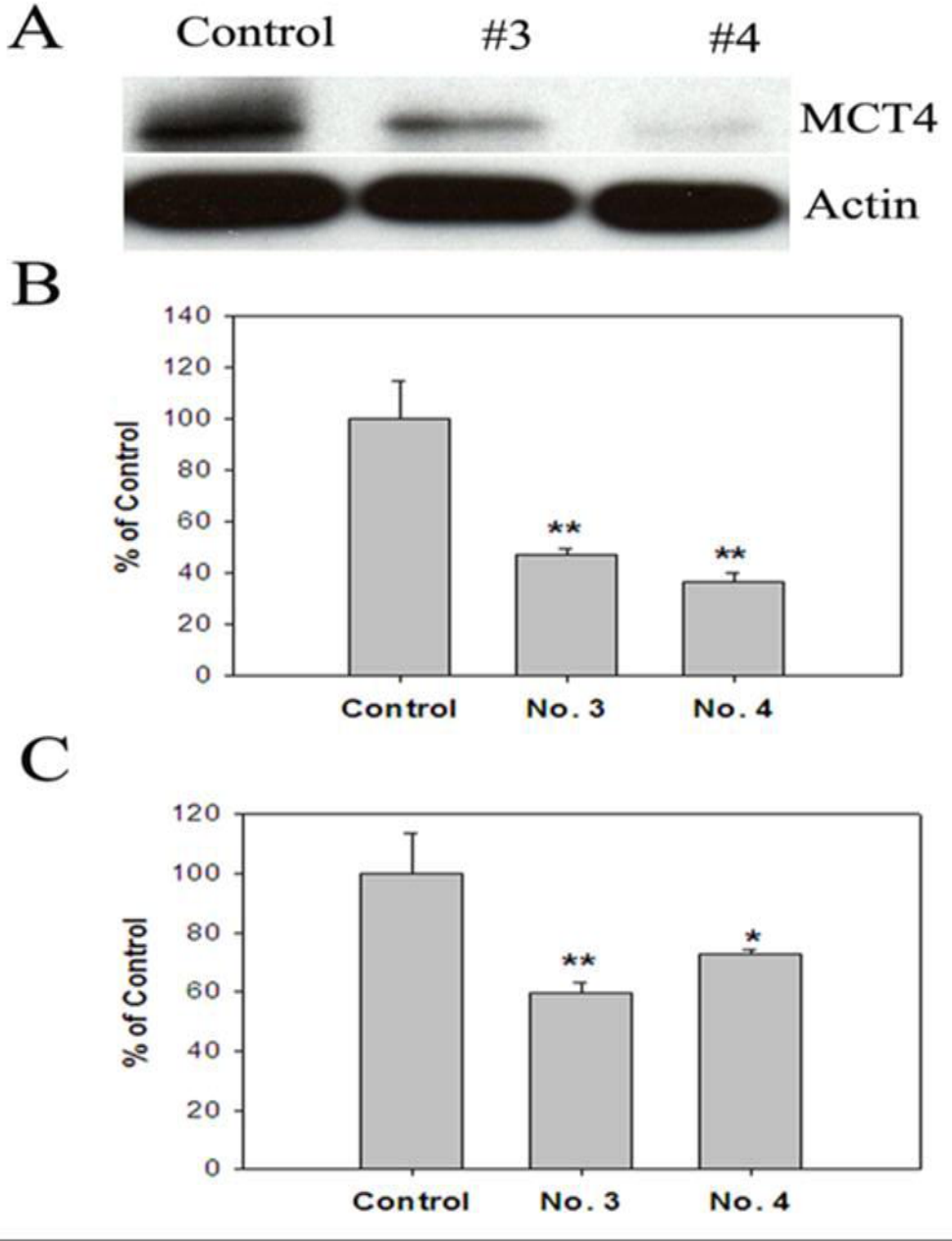
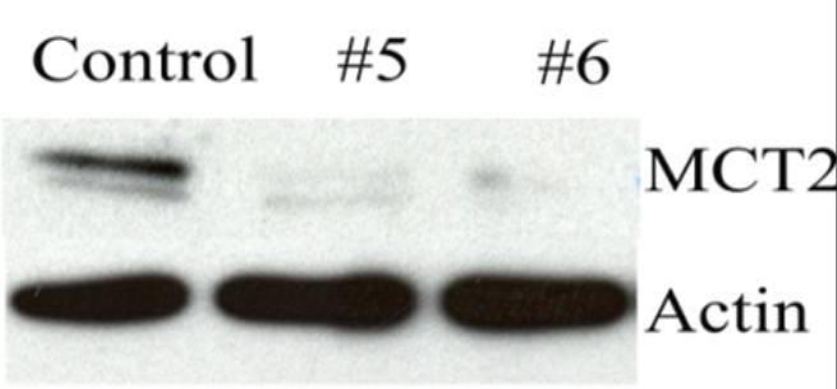

A
Control
#3
#4
MCT4
Actin
B
% of Control
140
120
100
80
60
40
20
0
**
**
Control
No. 3
No. 4
C
% of Control
120
100
80
60
40
20
0
**
*
Control
No. 3
No. 4

Figure 7

A


Control
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MCT2
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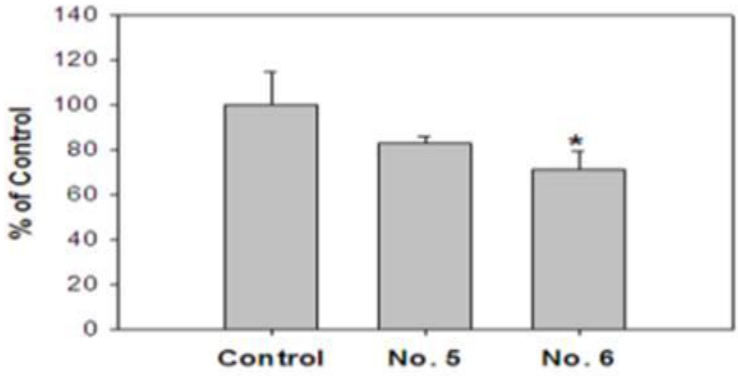
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20
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% of Control
*
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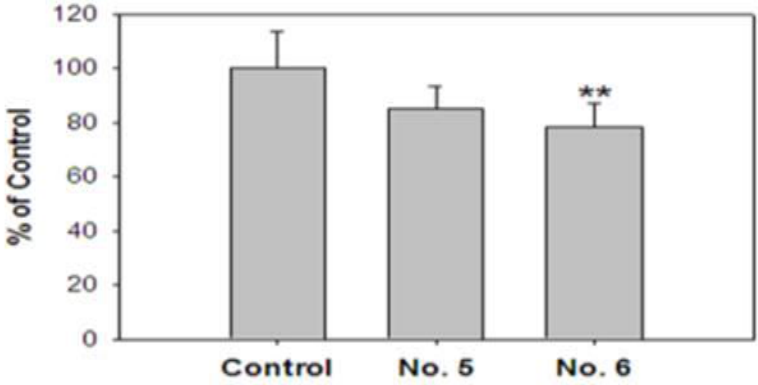
No. 5
No. 6

C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0
% of Control
**
Control
No. 5
No. 6