Cellular uptake properties of lamotrigine in human placental cell lines: Investigation of involvement of organic cation transporters (*SLC22A1–5*)

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Abstract

Lamotrigine (LTG) is an important antiepileptic drug for the treatment of seizures in pregnant women with epilepsy. However, it is not known if the transport of LTG into placental cells occurs via a carrier-mediated pathway. The aim of this study was to investigate the uptake properties of LTG into placental cell lines (BeWo and JEG-3), and to determine the involvement of organic cation transporters (OCTs, *SLC22A1–3*) and organic cation/carnitine transporter (OCTNs, *SLC22A4–5*) in the uptake process. The uptake of LTG at 37°C was higher than that at 4°C. OCT1 and OCTNs were detected in both cell lines. The uptake of LTG was not greatly affected by the extracellular pH, Na^+-free conditions, or the presence of L-carnitine, suggesting that OCTNs were not involved. Although several potent inhibitors of OCTs (chloroquine, imipramine, quinidine, and verapamil) inhibited LTG uptake, other typical inhibitors had no effect. In addition, siRNA targeted to OCT1 had no significant effect on LTG uptake. The mRNA expression in human term placenta followed the order OCTN2 > OCT3 > OCTN1 > OCT1 ≈ OCT2. These observations suggested that LTG uptake into placental cells was carrier-mediated, but that OCTs and OCTNs were not responsible for the placental transport process.

Keywords: antiepileptic drugs, BeWo, JEG-3, lamotrigine, OCTs, OCTNs, placenta

1. Introduction

Epilepsy is one of the most common neurological complications in pregnant women [1]. As epileptic seizures during pregnancy cause a risk to the fetus, some pregnant women continue to use antiepileptic drugs (AEDs) to control seizures. Lamotrigine (LTG), a new-generation AEDs, is used widely for the treatment of focal and generalized seizures. It is also prescribed for the management of bipolar disorder. Recently, LTG has been widely used for the treatment of pregnant woman with epilepsy. Meador *et al*. reported that the most common drug for monotherapy was LTG (42.1% of monotherapy prescriptions) following an analysis of current prescribing patterns for AEDs in pregnant women with epilepsy in the USA [2]. LTG is also frequently prescribed as a component of polytherapy; Meador *et al.* also reported that the most common combination was LTG + levetiracetam (42.9% of polytherapy prescriptions) [2].

Several previous studies have indicated that LTG is transferred from the mother to the fetus via the placenta. Ohman *et al*. estimated that umbilical cord blood-to-maternal LTG concentration ratios ranged from 0.6 to 1.3 (mean = 0.9) from nine pregnant women with epilepsy [3]. Fotopoulou *et al*. reported that the LTG ratio of umbilical cord blood to maternal serum ranged from 0.56 to 1.42 (mean = 1.01) in a study of nine Caucasian pregnant women with epilepsy [4]. Kacirova *et al*. reported that the infant to maternal LTG serum concentration ratios in monotherapy ranged from 0.40 to 1.38 (mean = 0.91) in an analysis of

larger numbers of patients than previous reports [5]. Myllynen *et al*. suggested that LTG penetrates the placenta easily and rapidly in an *ex vivo* placental perfusion study [6].

Exposure to some AEDs during pregnancy is associated with adverse effects to the fetus. For example, the use of valproic acid increases the risks of major congenital malformation, neurodevelopmental delay, and autism spectrum disorder [7]. With regard to LTG, reports have indicated that this AED results in a lower risk of malformation and impaired neuropsychological development [2]. However, it is thought that the developing brain is vulnerable to changes in the maternal environment [8]. The alterations in the fetal blood-brain barrier (BBB) function due to drug exposure from the mother are largely unclear at present. Therefore, improved understanding of the transport of AEDs across placenta may help to identify strategies to protect the fetus from the drugs, thereby obtaining anti-epilepsy medication that is safe for use during pregnancy.

The placenta, which has a variety of functions, including the provision of nutrients to the fetus, waste excretion, gas exchange, and hormone secretion, is an important organ for normal fetal development and an overall healthy pregnancy. The functions involving the transport of substances between the mother and the fetus are mediated by membrane transporters in placental trophoblasts [9]. To avoid potential adverse effects and consider individual variation, it is important to gather information on the role of drug transporters in pharmacokinetic processes. To date, LTG has been shown to be a substrate for the organic

cation transporter 1 (OCT1, *SLC22A1*) in an *in vitro* model of the BBB in hCMEC/D3 cells [10]. As for efflux systems, it has been reported that LTG is also a substrate of mouse and human breast cancer resistance protein (BCRP, *ABCG2*), and P-glycoprotein (P-gp, *ABCB1*) by studies using overexpression systems [11, 12]. OCT1 polymorphisms (rs628031) and BCRP polymorphisms (rs2231142) are associated with normalized LTG plasma concentrations in Chinese patients with epilepsy [13]. Another study also indicated the association between BCRP polymorphisms (rs2231142) and LTG plasma concentrations in the Chinese population [14]. With regard to OCT1, the association between the polymorphisms and LTG pharmacokinetics was not reported to be significant [15]. Although these previous reports focused on the transport process into the central nervous system through the BBB and the involvement of transporters in the efficacy of LTG, little information is available on the transport mechanism of LTG in placental cell models.

In the present study, we characterized the uptake of LTG in human placental choriocarcinoma cell lines (BeWo and JEG-3). As described above, LTG is a substrate of influx transporter OCT1 [10], a member of the SLC22A family. We therefore focused on the major organic cation transporters belonging to the family, including OCT1–3 (*SLC22A1–3*) and organic cation/carnitine transporter (OCTN) 1, 2 (*SLC22A4, 5*), and investigated the possible involvement of these transporters in the influx process of LTG in placental cells.

In the placenta, villous trophoblasts are an important constitution for the functions of the blood-placental barrier [16]. On the villous surface, cytotrophoblasts fuse to form multinucleate syncytiotrophoblasts. It is considered that the syncytiotrophoblasts function as a barrier regulating the transfer of substances between the mother and the fetus. Human placental choriocarcinoma cell lines, BeWo and JEG-3, are widely used as a trophoblast model to investigate the transport mechanisms of xenobiotics through the blood-placental barrier [17]. These cell lines are considered to maintain many characteristics of trophoblasts and express various transporters [17]. Therefore, we used these cell lines in the present study.

2. Materials and methods

2.1 Chemicals

Lamotrigine (LTG) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Verapamil, quinidine, tetraethyl ammonium (TEA), amantadine, metformin, imipramine, chloroquine, lopinavir, and 5-hydroxytriptamine (5-HT) were purchased from Sigma-Aldrich (St. Louis, MO). Choline chloride was purchased from Nacalai Tesque (Kyoto, Japan). L-Carnitine and other chemicals were purchased from Wako (Tokyo, Japan).

2.2 Cell culture

Human placental choriocarcinoma BeWo cells and JEG-3 cells were cultured as

previously described [18]. Human lung adenocarcinoma epithelial A549 cells were maintained in Dulbecco's modified Eagle's medium (D6429, Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich).

2.3 LTG uptake assay

BeWo cells, JEG-3 cells, and human lung adenocarcinoma epithelial A549 cells were seeded in 24-well collagen-coated plastic plates. Once the growth medium was removed, the cells were rinsed with a transport buffer and pre-incubated at 37°C with 0.5 mL of transport buffer. The transport buffer consisted of Hank's balanced salt solution (HBSS) with 25 mM HEPES or 25 mM MES. HEPES was used to provide a buffer between pH 7.4 and 8.5, and MES was used to provide a buffer between pH 5.5 and 6.5. To assess Na^+-free conditions, Na^+ was osmotically substituted by N-methyl-D-glucamine chloride. The uptake was initiated by the addition of the transport buffer containing LTG. The cells were incubated for the indicated time at 37°C or 4°C. After the incubation, the cells were immediately rinsed with ice-cold transport buffer containing 0.1% BSA. The LTG taken up by cells was quantified by using liquid chromatography/electrospray ionization tandem mass spectrometry (LC-MS/MS), as previously described [19]. The uptake of LTG was normalized to the protein concentration of the sample, which was determined by using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

2.4 [^{3}H]-L-Carnitine uptake assay

For the [^{3}H]-L-carnitine uptake assay, BeWo cells were seeded in 24-well collagen-coated plastic plates. Once the culture medium was removed, the cells were washed with transport buffer and pre-incubated at 37°C with 0.5 mL of the transport buffer (pH 7.4). The cells were incubated for 30 min at 37°C with the transport buffer containing 5 nM [^{3}H]-L-carnitine (Amersham Biosciences Corp.). After incubation, the buffer was aspirated, and the cells were immediately rinsed twice with ice-cold transport buffer. To measure the radioactivity of the [^{3}H]-L-carnitine taken up by the cells, the cells were solubilized in 1% SDS/0.2 N NaOH. The samples were mixed with 3 mL of scintillation cocktail and radioactivity was measured by using a liquid scintillation counter. The amount taken up by the cells was normalized to the cell protein concentration that was determined by using a BCA assay.

2.5 OCT1 knockdown by small interfering RNA (siRNA)

OCT1 siRNA (Oligo ID: HSS109969) and negative control (NC) (Stealth™ RNAi Negative Control medium GC Duplex) were purchased from Thermo Fisher Scientific (Waltham, MA). Reverse transfection of siRNAs into the cells was performed by using Lipofectamine^{TM} RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions.

The final RNAi duplex concentration was 10 nM. The cells were incubated for 72 h and used for further experiments.

2.6 Reverse transcription-polymerase chain reaction (RT-PCR) analysis and real-time PCR

To analyze the gene expression of transporters in BeWo cells and JEG-3 cells, the cells were seeded in 12-well plastic plates. Term placentas were donated by five women who had caesarean sections at Hokkaido University Hospital. This study, utilizing placentas, was approved by the Ethics Committee of Hokkaido University Hospital (018-0138) and written informed consent was obtained from the women. In the present study, four placentas were from women with no complications of pregnancy, the other was from a woman with diabetes mellitus. The placenta was obtained within 1 h after delivery, and washed with saline. The placental villous tissue was immediately cut into small fragments with scissors and minced. Total RNA was prepared from the cells and placental villous tissues by using ISOGEN II (Nippon Gene, Japan). Single-strand cDNA was made from 1 μg of total RNA by reverse transcription using ReverTraAce (TOYOBO, Japan) in accordance with the manufacturer's instructions. PCR was performed using Hot Star Taq PCR (QIAGEN) with specific primers, at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primer sequences are shown in Table 1. The PCR products were separated by

electrophoresis on a 2% agarose gel and then visualized by ethidium bromide staining.

Real-time PCR was performed by using an Mx3000™ real-time PCR system (STRATAGENE) with KAPA SYBR® Fast qPCR kit (KAPA Biosystems, Boston, MA) and specific primers (Table 1), at 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 65°C for 20 s. The relative mRNA expression of the target gene was calculated by the comparative cycle time (Ct) method from the following equation: relative mRNA expression = $2^{-(\text{Ct target gene - Ct housekeeping gene})}$. For the housekeeping gene, β-2-microglobulin (*B2M*) was used.

2.7 Statistical analysis and kinetic parameters

Experiments were repeated at least three times. The data were presented as the mean ± S.E of independent experiments. Student's *t*-test was used to determine the significance of differences between the means of two groups. Statistical significance among the means of more than two groups was evaluated by using one-way analysis of variance (ANOVA) followed by Dunnett's test. A value of $p < 0.05$ was considered statistically significant. Nonlinear regression analysis was computed by using SigmaPlot 12.5 (HULINKS). Kinetic parameters were calculated from the following equation:

$$v = V_{max} \times s/(K_m + s)$$

where v was the uptake rate of the compound, s was the compound concentration, K_m was the Michaelis–Menten constant, and V_{max} was the maximum uptake rate.

3. Results

3.1 Uptake of LTG by BeWo cells and JEG-3 cells

First, the time-course of LTG uptake in BeWo cells and JEG-3 cells was investigated. As shown in Fig. 1A and 1B, time-dependent LTG accumulation occurred in both cell lines following incubation with 20 μM LTG at 37°C. The uptake of LTG was linear for up to 30 s in BeWo cells and for up to 20 s in JEG-3 cells. Therefore, subsequent LTG uptake experiments in BeWo cells and JEG-3 cells were performed with a 15 s incubation period. The concentration-dependent uptake of LTG in BeWo cells and JEG-3 cells is shown in Fig. 1C and 1D. The uptake of LTG at 37°C was higher than that at 4°C in both cell lines. The uptake of LTG after the subtraction of the value at 4°C was saturated. The value of the parameters K_m and V_{max} were calculated as 904.5 ± 438.7 μM and 26.6 ± 6.2 nmol/mg protein/15 s for BeWo cells and 1289 ± 366.4 μM and 24.6 ± 3.8 nmol/mg protein/15 s for JEG-3 cells, respectively. In addition, the effects of extracellular pH and Na^+-free conditions on LTG uptake in BeWo cells and JEG-3 cells were investigated (Fig. 1E and 1F). When the transport buffer was replaced with Na^+-free buffer (NMDG-Cl) (pH 7.4), the uptake of LTG was not significantly altered in either cell line. The variation in pH had no remarkable effect on LTG uptake in either cell line, although the uptake at pH 5.5 was statistically lower compared with that at pH 7.4 in BeWo cells.

3.2 Effects of various inhibitors on LTG uptake in BeWo cells and JEG-3 cells

The inhibitory effects of various compounds that are inhibitors or substrates of OCTs and OCTNs [20-25] were investigated in both cell lines (Table 2). TEA (a substrate of OCT1,2 and OCTN1,2 [20]), metformin (a substrate of OCT1–3 [21, 22]), 5-HT (a substrate of OCT2 [21, 22]), choline (a substrate of OCT2 and OCTN2 [20]), L-carnitine (a substrate of OCTN1-2 [23]), and amantadine (a substrate/inhibitor of OCT1, 2 [20]) had no effect on LTG uptake. Chloroquine, which has inhibitory effects against OCT2 [24, 25], and imipramine, which has inhibitory effects against OCT2, 3 [20, 24] significantly decreased the carrier-mediated uptake of LTG in BeWo cells and JEG-3 cells. The inhibitory effect of quinidine, which can inhibit OCT1–3 and OCTN1, 2 [20-23], was also statistically significant. Verapamil, which can inhibit OCT1-3 and OCTNs [20], decreased the uptake of LTG in BeWo cells.

3.3 Expression of OCTs and OCTNs in BeWo cells, JEG-3 cells, and human term placentas

RT-PCR analysis indicated that OCTN1 and OCTN2 were detected at the mRNA level in both BeWo cells and JEG-3 cells (Fig. 2A and 2B), as we have reported previously [16]. Among organic cation transporters (OCTs), OCT1 was detected in both cell types. OCT2 was not detected in BeWo cells, but was detected at low levels in JEG-3 cells. OCT3

was not detected in either cell line. In human term placentas, OCT3, OCTN1, and OCTN2 were detected (Fig. 2C). The band intensities of OCT1 and OCT2 were weak in human placenta.

The expression of these transporters in human term placentas from five donors was also investigated by using real-time PCR (Fig. 3). OCTN2 was highly expressed, followed by OCT3, and then OCTN1. OCT1 and OCT2 were expressed at low levels. In one in five placentas, OCT1 was not detected.

3.4 Effect of OCT1 knockdown on LTG uptake

As discussed in the *Introduction*, a previous report suggested that OCT1 contributes to the influx of LTG into human brain endothelial (hCMEC/D3) cells [10]. To evaluate the specific role of OCT1, the effect of siRNA-mediated OCT1 knockdown was investigated (Fig. 4). In BeWo cells and JEG-3 cells, RT-PCR analysis indicated that OCT1 mRNA expression tended to be reduced 72 h after siRNA transfection. Although treatment with OCT1 siRNA tended to reduce LTG uptake at 37°C, the effects were not statistically significant in both cell lines.

4. Discussion

LTG is an important drug for the treatment of seizures in pregnant women or women of childbearing age with epilepsy [2]. However, it has not been evaluated if the transport of LTG into placental cells occurs via a carrier-mediated pathway. In the present study, we therefore determined the uptake properties of LTG into placental cell lines, and investigated whether organic cation transporters (*SLC22A1–5*) were involved in the transport process.

We previously reported on the accumulation levels of new-generation AEDs, including LTG, gabapentin, levetiracetam, and topiramate, in placental cell lines [18]. In particular, gabapentin accumulation was high, and the L-type amino acid transporter was involved in the transport process [18]. Following gabapentin, LTG was also accumulated 30 min after treatment within the therapeutic concentration range [18]. The intracellular concentrations of LTG in BeWo and JEG-3 cells were estimated from our reported values of intracellular volumes in these cell lines [18] and the uptake values after 10 min incubation with 20 μM LTG (Fig. 1A and 1B). The estimated intracellular concentration of LTG in BeWo and JEG-3 cells was 205 μM and 132 μM, respectively. In addition, the uptake of LTG at 37°C was higher than that at 4°C, and uptake in these cell lines reached saturation (Fig. 1C and D). These findings suggest that LTG was taken up in placental cells by a carrier-mediated process.

The distribution coefficient (Log*D*, pH 7.4) of LTG has been experimentally

determined to be 0.0 [10]. As mentioned in the *Introduction* section, LTG transport into brain endothelial cells is mediated by OCT1 (*SLC22A1*). In the present study, OCT1 and closely related cation transporters, namely OCT2 (*SLC22A2*), OCT3 (*SLC22A3*), OCTN1 (*SLC22A4*), and OCTN2 (*SLC22A5*), were considered as candidates.

OCT1–3 play important roles in the transport of cationic drugs. These transporters also transport neutral compounds [20]. In the present study, OCT1 was detected in human term placentas (Fig. 2), as previously reported [26], but with low expression: it was not detected in one in five placentas (Fig. 3). With regard to placental cell lines, OCT1 expression was the strongest among the OCTs (Fig. 2). However, real-time PCR analysis indicated that OCT1 expression in BeWo and JEG-3 cells was low (Ct value > 30, data not shown). We also previously reported that OCT1 expression in BeWo cells was low by using PCR array analysis of 84 transporters [27]. The results suggested that OCT1 function in these cell lines was low. Although several inhibitors of OCT1 (e.g., verapamil and quinidine) inhibited LTG uptake, other potent inhibitors (e.g., TEA, amantadine, and metformin) had no effect. In addition, OCT1 knockdown had no significant effects on LTG uptake in either cell line. The K_m values of the LTG uptake rate were calculated as 904.5 μM in BeWo cells and 1289.0 μM in JEG-3 cells (Fig. 1C, 1D). It has been reported that the K_m value of OCT1-mediated LTG uptake in hCMEC/D3 cells was estimated as 62 μM [10], which was very different to the values in the present study. These findings suggest that LTG uptake by placental cell lines was

not mediated by OCT1.

Although OCT2 was expressed in all the human placentas we investigated, low expression was observed (Fig. 3). As for placental cell lines, OCT2 was not detected or only expressed at very low levels (Fig. 2). In contrast, several potent inhibitors of OCT2 (verapamil, quinidine, chloroquine, and imipramine) inhibited LTG uptake in placental cells. These results suggested that the effects of these inhibitors on LTG uptake were mediated through the inhibition of other factors than OCT2.

As previously reported [26], the expression of OCT3 mRNA was the strongest among OCTs in human term placentas (Fig. 3). However, RT-PCR analysis suggested that the function of OCT3 was absent in placental cell lines (Fig. 2). As a preliminary study, we investigated the possibility of involvement of OCT3 in LTG uptake in A549 cells endogenously expressing OCT3 (Supplemental Fig. 1). Corticosterone, which is a potent inhibitor of OCT3 [10], had no effect on LTG uptake in A549 cells. The results suggested that the involvement of OCT3 in LTG uptake was not reasonable.

OCTN1 and OCTN2 are physiologically crucial transporters for L-carnitine disposition [23]. It was proposed that OCTN2 has a major role in L-carnitine transport from the mother to the fetus across the placenta [28]. OCTNs also are involved in various zwitterionic and cationic drugs [23]. In human term placentas, OCTN2 showed notably high expression compared with OCTN1 (Fig. 3). The result was in accordance with previous

reports [28]. The human placental cell lines, BeWo and JEG-3, also expressed OCTN1 and OCTN2 (Fig. 2), and the expression patterns and levels (Ct values) of these cells showed a similar trend to those in human term placentas. LTG uptake in placental cell lines was independent of the existence of Na^+ or the change in extracellular pH (Fig. 1E, F). In addition, L-carnitine and other potent inhibitors of OCTNs (TEA and choline) had no effect on LTG uptake in either cell line. Although verapamil and quinidine, which can inhibit OCTNs, tended to inhibit LTG uptake, these compounds can affect multiple transporters. As shown in Supplemental Fig. 2, the uptake of [^{3}H]-L-carnitine in BeWo cells was not inhibited by LTG (0.5–2 mM). In addition, Wu *et al*. reported that LTG had the lowest inhibitory potency of [^{3}H]-L-carnitine uptake into human placental brush-border membrane vesicles among AEDs [29]. These results supported that OCTNs were not involved in the LTG transport process, and that LTG had no effect on placental L-carnitine disposition.

In addition to *SLC22A* family, multidrug and toxin extrusion (MATEs, *SLC47A1, 2*) and plasma membrane monoamine transporter (PMAT, *SLC29A4*) are known as organic cation transporters. Therefore, we investigated these transporters as the other potential mechanisms. The RT-PCR analysis showed that MATE1 mRNA was only slightly detected in the human term placenta (Supplemental Fig. 3A). In the placental cell lines (BeWo and JEG-3), MATEs were not detected. The results suggest that the contribution of MATEs to LTG uptake is deniable. In regards to PMAT, the mRNA expression was detected in both the cell

lines and human placenta (Supplemental Fig. 3A). Although several inhibitors of PMAT (e.g. imipramine, verapamil, and quinidine) [30-32] inhibited LTG uptake in both the BeWo and JEG-3 cells, the other potent inhibitors (e.g. amantadine, 5-HT, and metformin) [30-32] did not have the effects. Furthermore, an additional experiment indicated that lopinavir, a potent and selective inhibitor of PMAT [32], did not affect LTG uptake (Supplemental Fig. 3B). The results suggest that the involvement of PMAT can be excluded.

As described above, OCT1 contributed to LTG uptake in the BBB model [10]. Given the low expression of OCT1 in the human placenta and the results utilizing several inhibitors and siRNA, we concluded that OCT1 does not appear to be involved in placental transfer. Although we were not able to identify the carrier(s) involved in LTG uptake, we revealed that the carrier was sensitive to chloroquine, imipramine, quinidine, and verapamil. Dicken *et al*. reported the possibility of other influx transporters in addition to OCT1, because mock-transfected cells exhibited a high accumulation of LTG compared with TEA, another typical substrate of OCT1 [10] Chloroquine, imipramine, quinidine, and verapamil were able to inhibit other transporters [33-35]. Besides, several groups have reported novel organic cation transport systems that were not sensitive to L-carnitine, choline, metformin, and TEA in various experimental models. In the inner blood-retinal barrier (BRB) model, verapamil transport mediated by a pH- and Na^+-independent system was sensitive to imipramine, quinidine, amantadine, and L-carnitine, but not to TEA, choline, and 5-HT [36]. Furthermore,

the research group reported that propranolol transport in the BRB model was sensitive to pH, imipramine, quinidine, verapamil, and amantadine, but not to Na+, TEA, choline, and L-carnitine [37]. It has been suggested that nicotine transport in various cell models, including lung cells, HepG2, MCF-7, BRB, and JAR cells was mediated by a novel transport system [38−41]. The transport system was pH-dependent, Na^+-independent, and inhibited by verapamil, quinidine, imipramine, but not by L-carnitine, TEA, 5-HT, and choline. In addition, pH-dependent clonidine transport systems that were inhibited by quinidine, verapamil, imipramine, and amantadine, but not by L-carnitine, TEA, choline, and 5-HT, have been suggested in the BRB model and lung cell lines [30, 42]. Although not completely identical, the properties of LTG uptake as indicated in the present study share in common with those of the previous findings.

Furthermore, passive diffusion or adsorption of LTG were high in cell lines. The clinical plasma concentration of LTG has been reported to be approximately between 10–60 μM [43]. In the present study, the K_m values were calculated as approximately 1 mM. Therefore, the K_m values were greater than the clinical ranges. In addition, we estimated the contribution of the carrier at the clinical concentration of LTG from the data in Fig. 1 (C) and (D). The cellular uptake values at 50 μM of LTG at 37°C (carrier process + passive diffusion) were 1.24 ± 0.4 nmol/mg protein/15 sec for BeWo cells and 1.69 ± 0.2 for JEG-3 cells. The values at 4°C (passive diffusion) were 0.68 ± 0.1 nmol/mg protein/15 sec for BeWo cells and

0.42 ± 0.01 for JEG-3 cells. Therefore, we estimated the contributions of the carrier process under the present experimental condition as 45% for BeWo cells and 75% for JEG-3 cells. Besides, we could not exclude the possibilities that plural processes were involved in LTG uptake in the present study. Future studies are required to provide a comprehensive investigation of the putative pathways.

Several experimental models, such cell lines, human placental primary cells, placental membrane vesicles, pregnant animals (*in vivo*), and placental perfusion (*ex vivo*), have been utilized to investigate placental drug transport [17]. Each model has advantages and limitations. In the present study, we characterized the uptake of LTG in BeWo and JEG-3 cells, which have been used widely to examine the transport mechanisms of drugs in the placenta. Although these cell lines have characteristics of human trophoblasts, there are several transporters with different expression from normal trophoblasts [17]. This is one of the limitations of these models. With regard to the organic cation transporters we investigated in this study, the expression of OCTNs and OCT1 in BeWo cells and JEG-3 cells was similar to that in normal human placentas; however, these cell lines were not suitable for the investigation of OCT3 function. Therefore, various models should be comprehensively employed to evaluate the mechanisms of placental transport, as suggested by other groups [44].

In summary, the present study was undertaken to evaluate the uptake properties of

LTG and the involvement of organic cation transporters (*SLC22A1–5*) in the choriocarcinoma cells lines, BeWo and JEG-3. Our findings suggested that the transport of LTG into these cells can occur via a carrier-mediated pathway. OCT1, which contributed to the LTG uptake in brain endothelial cells, did not contribute to the process in placental cell lines. Furthermore, other related cation transporters were not involved. Although we were unable to identify the carrier(s) involved in LTG uptake, we determined that the carrier was sensitive to chloroquine, imipramine, quinidine, and verapamil. The molecular structures and several physicochemical properties of LTG and the inhibitors are shown in Supplemental Table 1. As for the common properties of these four inhibitors, they have tertiary amines in their structures, are predicted to exist in the cationic form predominantly at pH 7.4, and are lipophilic compounds. To the best of our knowledge, this is the first study to investigate the uptake properties of LTG into placental cells. Although the extrapolation of findings observed in choriocarcinoma cells to normal cells should be made with caution, the results of this study may form a basis for safe AED therapy during pregnancy. Further comprehensive studies are required to elucidate the involvement of the carrier in the transfer of LTG across the placenta.

Authorship contributions

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Conflicts of Interest

The authors report no conflicts of interest.

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Figure legends

Figure 1. Uptake properties of LTG in human placental choriocarcinoma cell lines. (A, B) Time course of LTG uptake into BeWo (A) and JEG-3 cells (B). The cells were incubated with transport buffer containing 20 μM LTG at 37°C. Each point represents the mean ± S.E. of three to six independent experiments, each performed in triplicate. **(C, D)** Concentration dependence of LTG uptake in BeWo (C) and JEG-3 cells (D). Cells were incubated with transport buffer containing 10–2000 μM LTG for 15 s at 37°C (closed circles) or 4°C (open circles). The LTG uptake was obtained by subtracting the uptake at 4 °C from that at 37 °C (*Inset*). Each point represents the mean ± S.E. of three or four independent experiments, each performed in triplicate. **(E, F)** Effects of extracellular pH and Na^+ withdrawal on LTG uptake. The cells were incubated with transport buffer at various pH values or Na^+-free (NMDG) buffer containing 50 μM LTG for 15 s at 37°C. Each column represents the mean ± S.E. of three independent experiments, each performed in triplicate. *; significantly different from control (at pH 7.4) at $p < 0.05$.

Figure 2. RT-PCR analysis of mRNA expression of organic cation transporters (*SLC22A1–5*) in BeWo, JEG-3 cells, and human term placentas. PCR was performed by using specific primers, as described in Table 1, with the detection of β-actin as a loading control.

Figure 3. Quantitative analysis of the mRNA expression of organic cation transporters (*SLC22A1–5*) in human term placentas. Five placentas were donated by volunteers and gene expression was investigated by using real-time PCR. Each point represents an individual placenta. The inset figure shows the data for *SLC22A1–4* on an enlarged scale. The relative mRNA expression of target gene was calculated by using the comparative cycle time (Ct) method, with β-2-microglobulin (*B2M*) used as a housekeeping gene.

Figure 4. Effects of OCT1 siRNA on LTG uptake in choriocarcinoma cells. BeWo cells (A) and JEG-3 cells (B) were transfected with negative-control siRNA or OCT1 siRNA (HSS109969). At 72 h after siRNA transfection, the cells were incubated with transport buffer (pH 7.4) containing 50 μM LTG at 37°C for 15 s. Each column represents the mean ± S.E. of four to five independent experiments, each performed in triplicate. The reduction of OCT1 expression was confirmed by RT-PCR.

Supplemental Figure 1.

A549 cells were incubated with transport buffer (pH 7.4) containing 50 μM LTG in the presence or absence of corticosterone (1–50 μM). Each column represents the mean ± S.E. of three independent experiments, each performed in triplicate. The mRNA expression of organic cation transporters (*SLC22A1–3*) in A549 cells was investigated by using RT-PCR.

Supplemental Figure 2.

BeWo cells were incubated with transport buffer (pH 7.4) containing 5 nM [^{3}H]-L-carnitine at 37ºC for 30 min. Each column represents the mean ± S.E. of three independent experiments, each performed in triplicate.

Supplemental Figure 3. Investigations for the involvement of MATEs and PMAT in LTG uptake.

(A) RT-PCR analysis of the mRNA expression of MATEs and PMAT in BeWo, JEG-3 cells, and human term placentas. (B) Effects of lopinavir on LTG uptake in the placental choriocarcinoma cell lines. The BeWo cells and JEG-3 cells were incubated with the transport buffer (pH 7.4) containing 50 μM LTG in the presence or absence of lopinavir (10 μM) for 15 s at 37ºC or 4ºC. The LTG uptake was obtained by the subtraction of the uptake at 4ºC from that at 37ºC. Each column represents the mean ± S.E. of three to seven independent experiments each performed in triplicate.

Table 1. Primer sequences

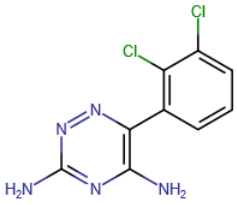
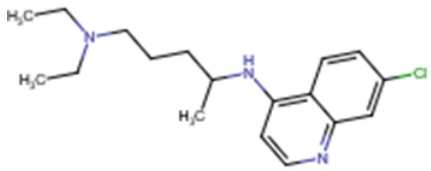
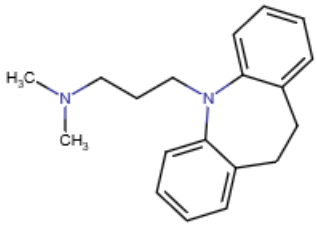
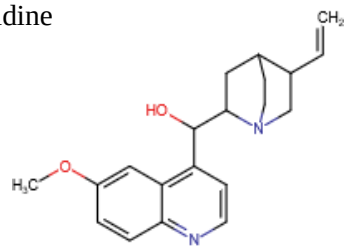
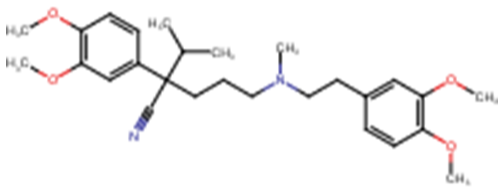
Name		Primer sequence	Product size (bp)
OCT1 (*SLC22A1*)	Forward	5’-TAATGGACCACATCGCTCAA-3’	190
	Reverse	5’-AGCCCCTGATAGAGCACAGA-3’	
OCT2 (*SLC22A2*)	Forward	5’-TTGGTTGCTGGAGGTCTGGT-3’	255
	Reverse	5’-TGGTTGAGTTGTATGGGCTTTGT-3’	
OCT3 (*SLC22A3*)	Forward	5’-CAGCAGACAGGTATGGCAGG-3’	111
	Reverse	5’-GGAAGATCACAAACACAGGGAAGT-3’	
OCTN1 (*SLC22A4*)	Forward	5’-GCCCCCTACTTTGTTTACCTC-3’	129
	Reverse	5’-CCTTGGGATTTTCTTCTGTCTCC-3’	
OCTN2 (*SLC22A5*)	Forward	5’-CAGCCATCCTCACCTTGTTTC-3’	146
	Reverse	5’-TGTGGGCCTTTCTTGACCATC-3’	
MATE1 (*SLC47A1*)	Forward	5’-TCCATCGTGTATGAACTGGC-3’	186
	Reverse	5’-ACAGCTTAACAGCAGGACAC-3’	
MATE2 (*SLC47A2*)	Forward	5’-TTCATTCCAGGACTTCCGGTG-3’	143
	Reverse	5’-AGGTGTGAGTGAGATGGATGG-3’	
PMAT (*SLC29A4*)	Forward	5’-TGCTGCCATACAACAGCTTC-3’	80
	Reverse	5’-CATGTCAAACACGATGGAGG-3’	
β-2-microglobulin (*B2M*)	Forward	5’-CAGCATCATGGAGGTTTGAA-3’	178
	Reverse	5’-TGGAGACAGCACTCAAAGTA-3’	
β-Actin (*ACTB*)	Forward	5’-TGGCACCCAGCACAATGAA-3’	186
	Reverse	5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’	

Table 2. Effects of various compounds on LTG uptake in BeWo cells and JEG-3 cells

Compounds	BeWo				JEG-3			
	Concentration (mM)	Carrier-mediated uptake (% of control)			Concentration (mM)	Carrier-mediated uptake (% of control)		
Amantadine	1	85.5	±	7.4	1	97.3	±	7.2
L-Carnitine	1	89.7	±	10.4	1	108.0	±	14.8
Chloroquine	1	17.9	±	4.0 **	1	42.8	±	2.5 **
Choline	1	98.2	±	12.2	1	108.1	±	14.7
5-HT	1	94.1	±	9.2	1	96.2	±	9.2
Imipramine	1	28.4	±	12.2 **	0.5	56.4	±	3.4 **
Metformin	1	121.6	±	16.1	1	109.2	±	5.6
Quinidine	1	51.7	±	10.1 **	1	51.0	±	9.0 *
TEA	1	74.0	±	14.1	1	113.0	±	12.9
Verapamil	1	58.5	±	7.4 *	1	78.5	±	9.3

BeWo cells and JEG-3 cells were incubated with transport buffer (pH 7.4) containing 50 μM LTG in the presence or absence of compounds (0.5 or 1 mM) for 15 s at 37ºC or 4ºC. Each column represents the mean ± S.E. of three to seven independent experiments, each performed in triplicate. *; significantly different from control at $p < 0.05$. **; significantly different from control at $p < 0.01$. LTG uptake was obtained by the subtraction of the uptake at 4ºC from that at 37ºC.

Supplemental Table 1. Inhibitors of LTG uptake in BeWo and JEG-3 cells.

LTG and inhibitors	Molecular weight	Predicted Log *P*	Predicted pKa
Lamotrigine 	256.1	2.17	5.89
Chloroquine 	319.9	4.4	10.32
Imipramine 	280.4	4.35	9.2
Quinidine 	324.4	2.68	8.98
Verapamil 	454.6	4.63	9.68

The predicted log P values were calculated by ALOGPS 2.1. The predicted pKa values were calculated by Marvin Sketch.

Fig. 1

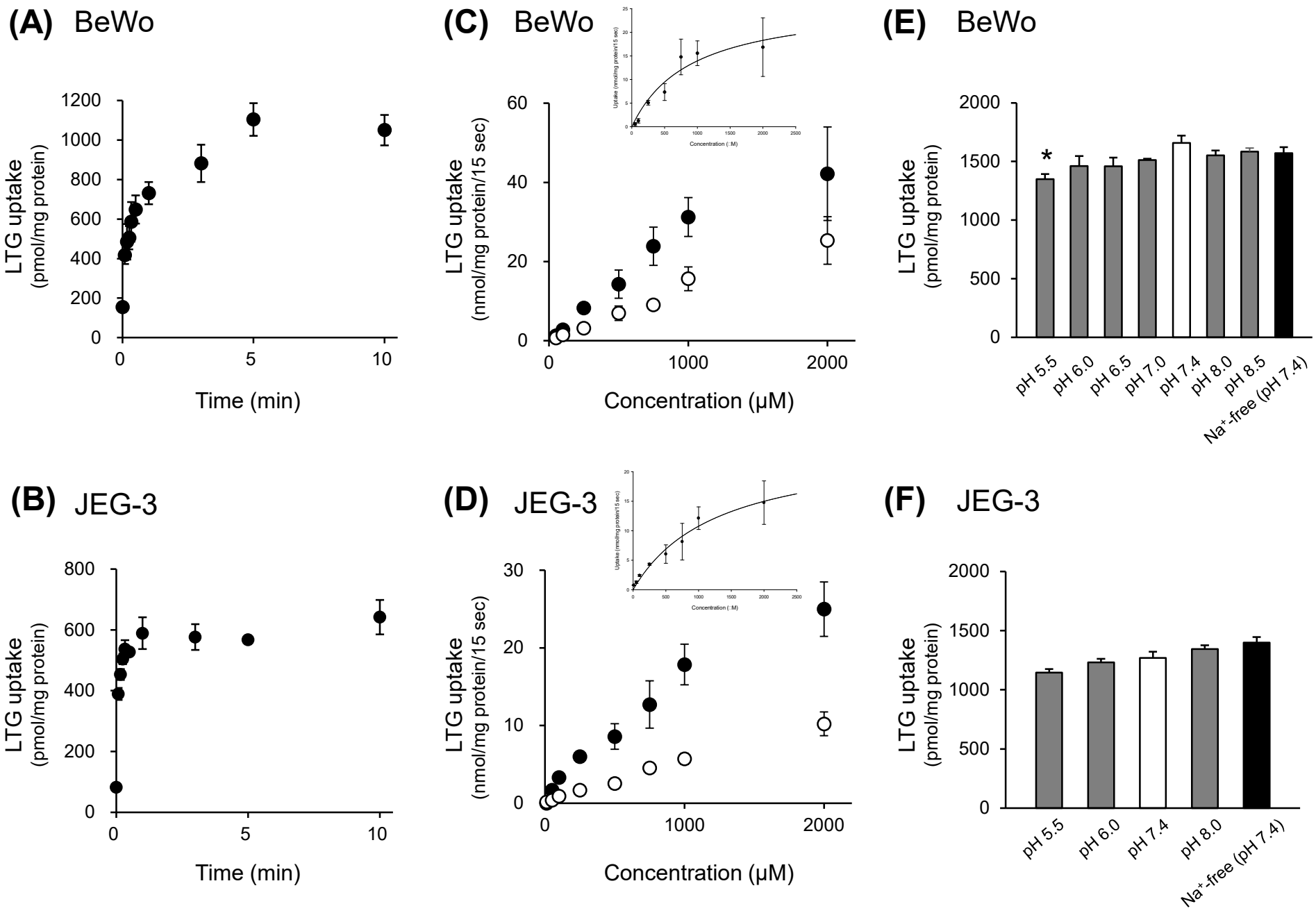

(A) BeWo
LTG uptake (pmol/mg protein)
Time (min)
(B) JEG-3
LTG uptake (pmol/mg protein)
Time (min)
(C) BeWo
LTG uptake (nmol/mg protein/15 sec)
Concentration (μM)
(D) JEG-3
LTG uptake (nmol/mg protein/15 sec)
Concentration (μM)
(E) BeWo
LTG uptake (pmol/mg protein)
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.4
pH 8.0
pH 8.5
Na+-free (pH 7.4)
(F) JEG-3
LTG uptake (pmol/mg protein)
pH 5.5
pH 6.0
pH 7.4
pH 8.0
Na+-free (pH 7.4)

Fig. 2

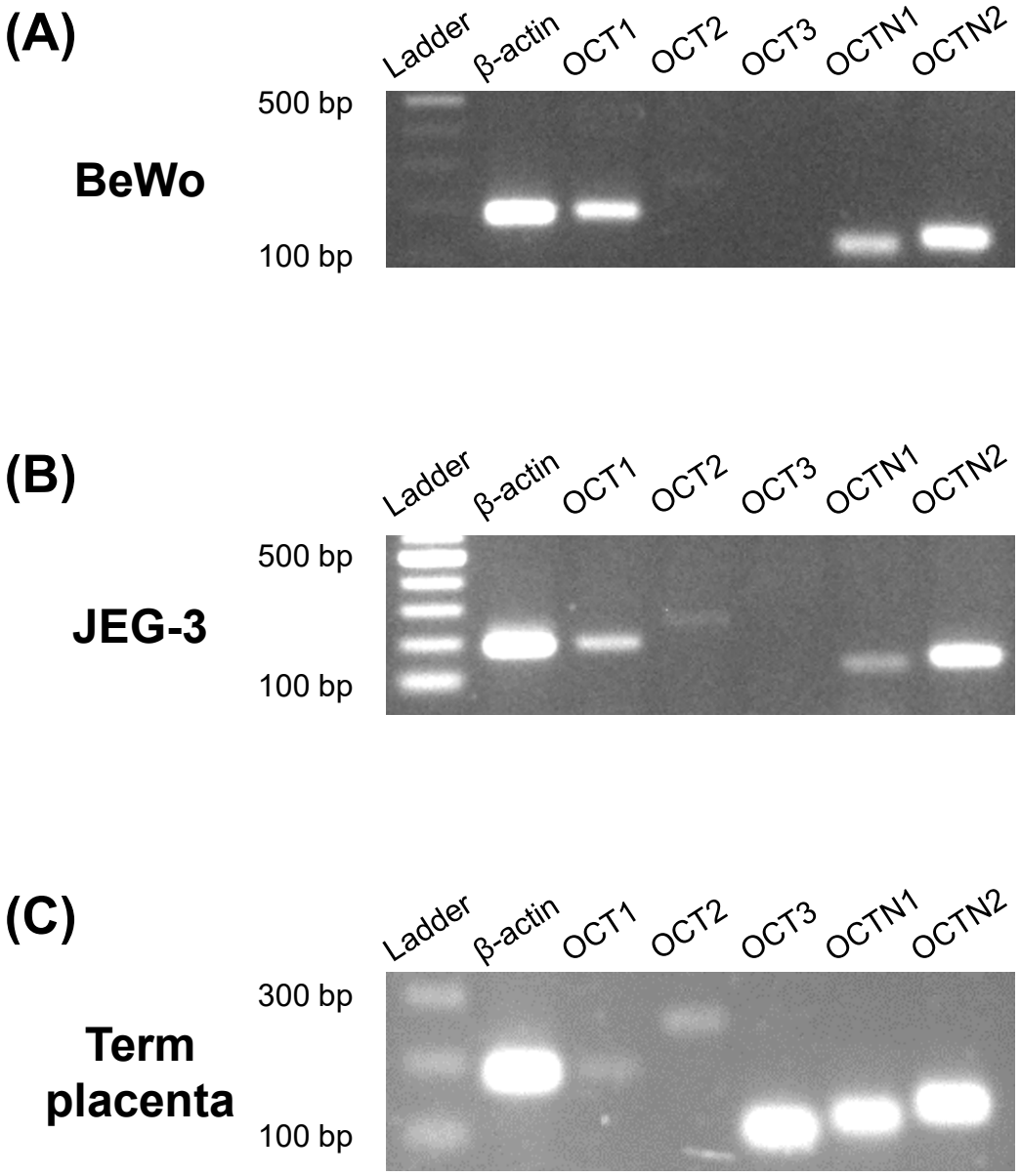

(A)
Ladder
β-actin
OCT1
OCT2
OCT3
OCTN1
OCTN2
500 bp
100 bp
BeWo
(B)
Ladder
β-actin
OCT1
OCT2
OCT3
OCTN1
OCTN2
500 bp
100 bp
JEG-3
(C)
Ladder
β-actin
OCT1
OCT2
OCT3
OCTN1
OCTN2
300 bp
100 bp
Term placenta

Fig. 3

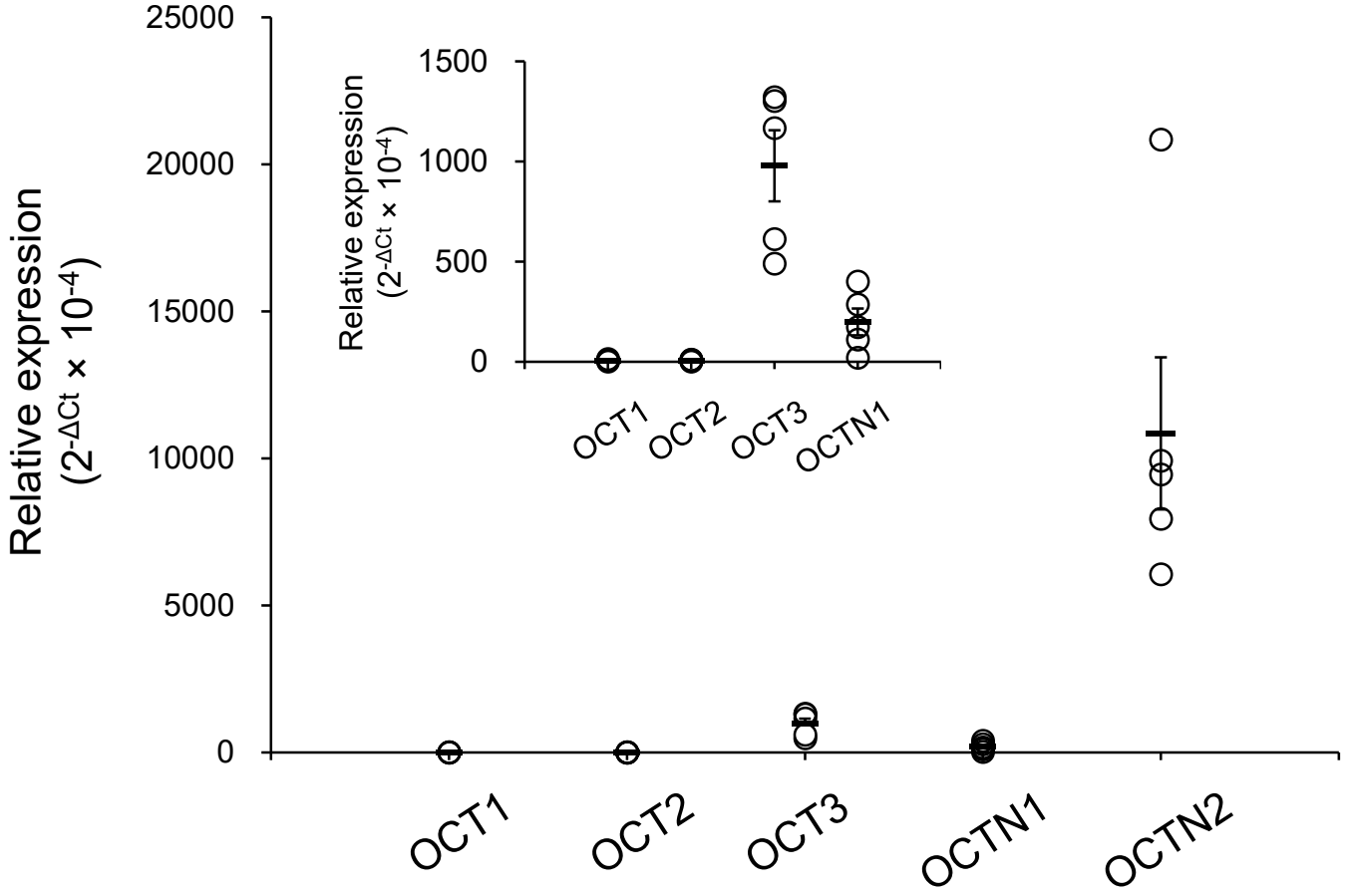
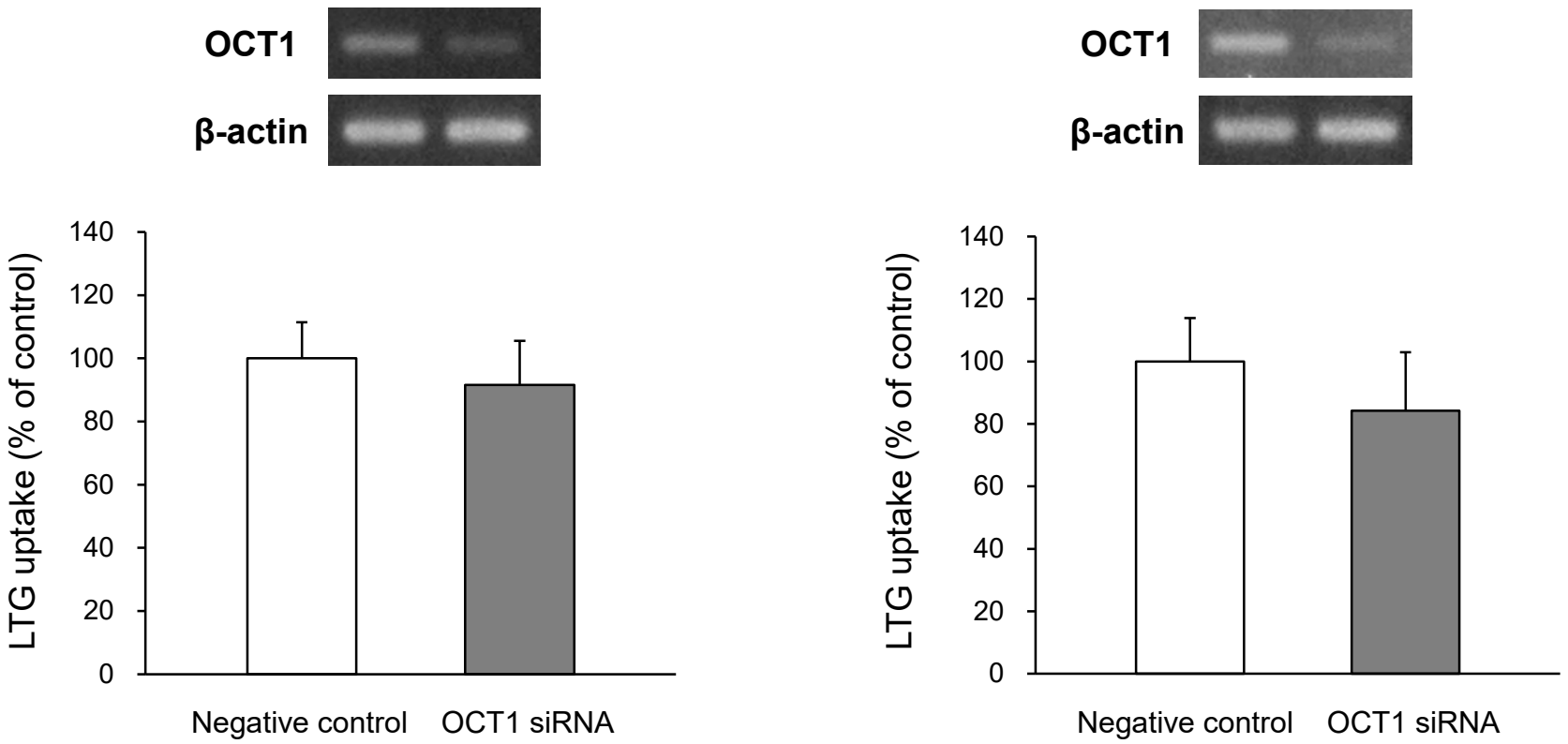
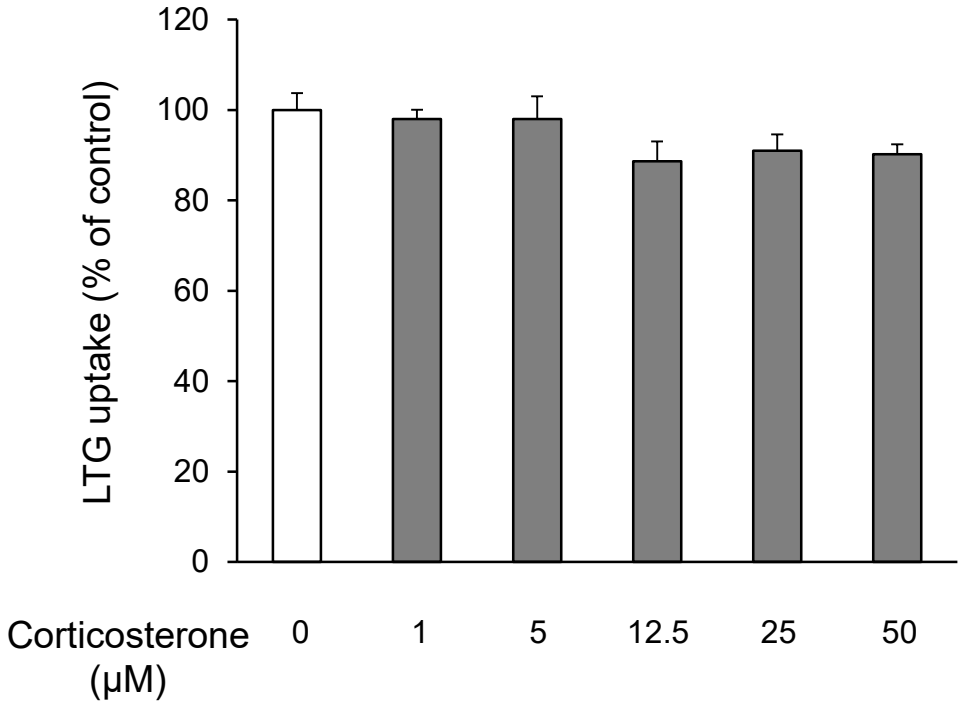

Relative expression
(2-ΔCt × 10-4)
25000
20000
15000
10000
5000
0
OCT1
OCT2
OCT3
OCTN1
OCTN2
Relative expression
(2-ΔCt × 10-4)
1500
1000
500
0
OCT1
OCT2
OCT3
OCTN1

Fig. 4

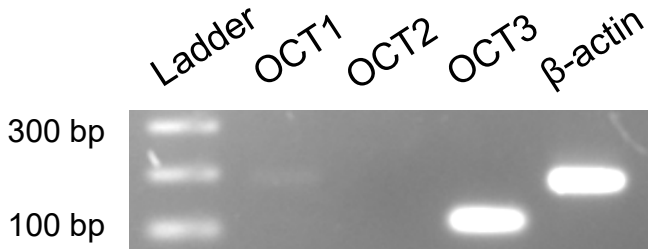

(A) BeWo
OCT1
β-actin
LTG uptake (% of control)
140
120
100
80
60
40
20
0
Negative control
OCT1 siRNA
(B) JEG-3
OCT1
β-actin
LTG uptake (% of control)
140
120
100
80
60
40
20
0
Negative control
OCT1 siRNA

Supplemental Fig. 1

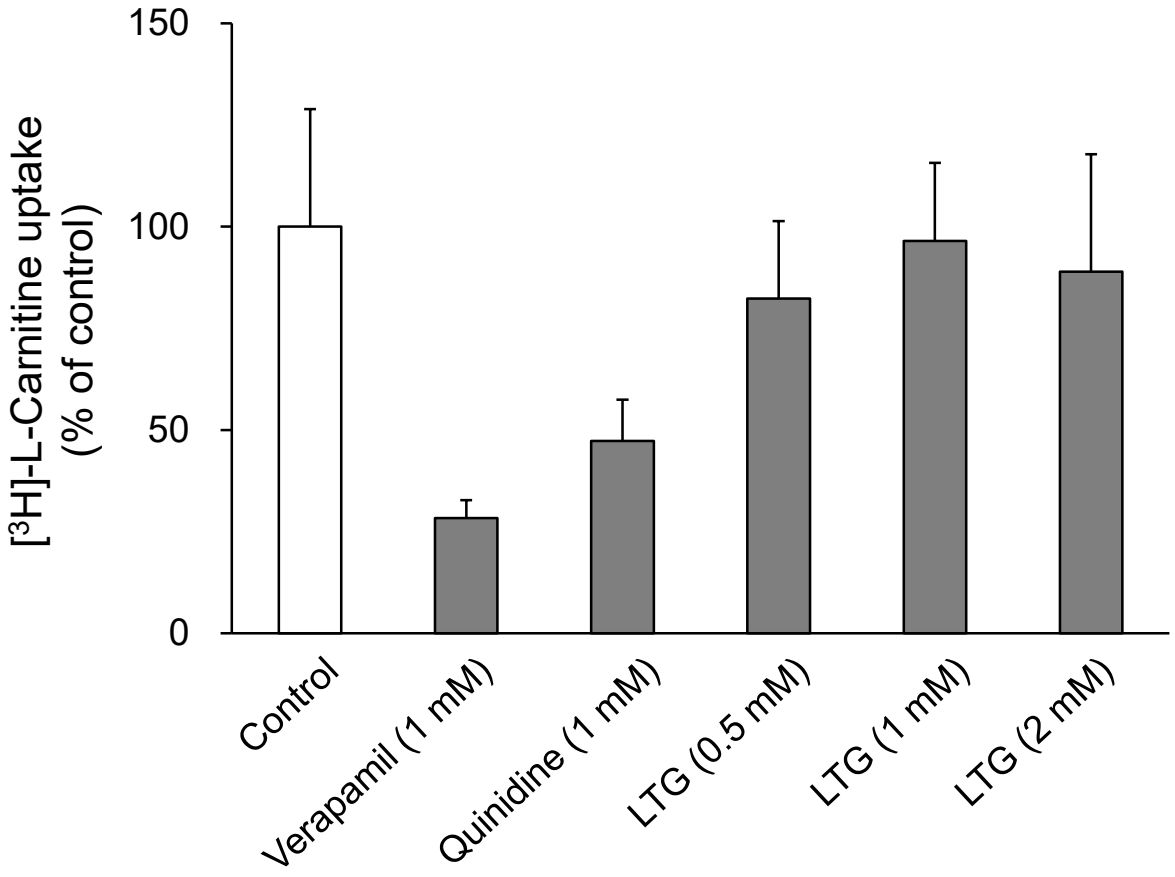
(A)



(B)

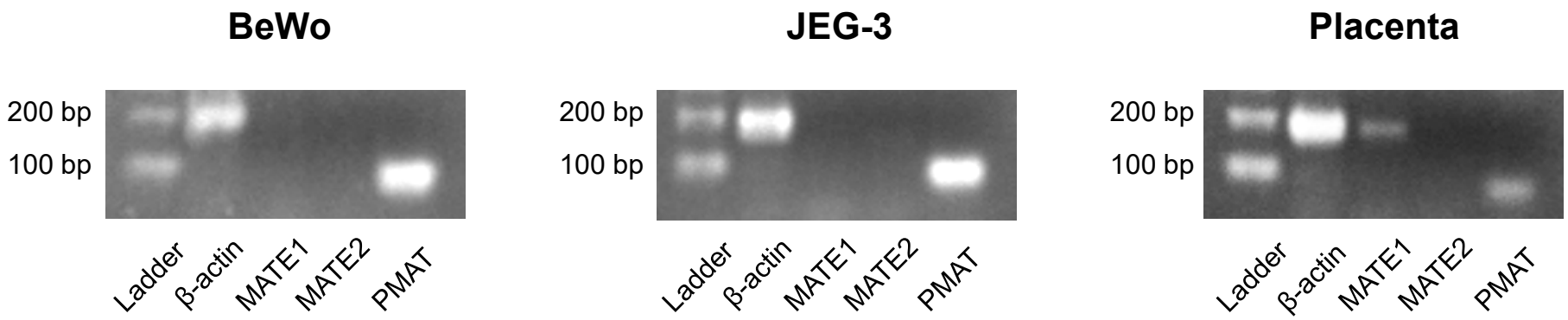


Supplemental Fig. 2



Supplemental Fig. 3

(A)



(B)