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Evaluation of the effects of antiepileptic drugs on folic acid uptake by human placental choriocarcinoma cells

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Abbreviations: BM, basal plasma membrane; CBZ, carbamazepine; FR, folate receptor; GBP, gabapentin; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; MVM, microvillous plasma membrane; OXC, oxcarbazepine; PB, phenobarbital; PCFT, proton-coupled folate transporter; PER, perampanel; PGB, pregabalin; PHT, phenytoin; RFC, reduced folate carrier; RUF, rufinamide; STR, stiripentol; TPM, topiramate; VGB, vigabatrin; VPA, valproic acid; ZNS, zonisamide

Abstract

Folate status during pregnancy is important for fetal development and health. The placenta plays an important role in supplying the fetus with folate. Most women with epilepsy continue their medication during pregnancy. In the present study, we aimed to evaluate the effects of 16 antiepileptic drugs, clinically used for treatment of epilepsy, on folic acid uptake in two *in vitro* placental models, BeWo and JEG-3 cells. Short-term exposure to antiepileptic drugs had no effects on [³H]-folic acid uptake by BeWo cells. However, long-term exposure (24 h) to valproic acid (VPA) increased [³H]-folic acid uptake by BeWo and JEG-3 cells. VPA treatment for 24 h increased folate receptor- α (FR α) and proton-coupled folate transporter (PCFT) mRNA expression; however, it did not affect reduced folate carrier expression. These results suggested that the increase in folic acid uptake after exposure to VPA can be attributed to the induction of FR α and PCFT expression. Furthermore, the present study showed that exposure to clinical concentrations of oxcarbazepine and stiripentol reduced the viability of BeWo cells. Therefore, the findings of the present study may contribute to better understanding of the mechanisms of toxicity of antiepileptic drugs, and estimation of their potential risk to fetus.

Keywords: antiepileptic drug, folic acid, placenta, folate receptor- α , reduced folate carrier, proton-coupled folate transporter

1. Introduction

Folates, a group of essential water-soluble B vitamins (B9), are involved in the one-carbon metabolism, such as the synthesis of nucleic acids and amino acids and regulation of gene expression (Djukic *et al.*, 2007). Folate is a collective term for both the naturally occurring food folate and the synthetic form, folic acid (pteroylglutamate). Folic acid is used for fortification and as a nutritional supplement. During gestation, folates are critical for the development of fetus and placenta. The fetal needs for folates increase during pregnancy (Antony, 2007). It has been reported that fetal and placental accumulation of folic acid increases as gestation progresses (Yasuda *et al.*, 2008b). Folate deficiency during pregnancy is associated with a risk of fetal malformations; thus, folic acid supplements are recommended during the periconceptual period to prevent fetal neural tube defects (Lumley *et al.*, 2001). Furthermore, it has been reported that maternal folate status during pregnancy affects fetal growth (Feketa *et al.*, 2012; van Uitert and Steegers-Theunissen, 2013).

Most women with epilepsy continue their medication during pregnancy because epileptic seizures pose a risk to both the mother and fetus. However, *in utero* exposure to certain antiepileptic drugs is associated with increased health risks to the fetus. Administration of valproic acid (VPA) during the periconceptual period dose-dependently increased the risk of fetal malformations (Tomson *et al.*, 2011). Folic acid supplement during the periconceptual period is also recommended for women with epilepsy (Yerby, 2003). Besides malformation risk, maternal VPA administration is believed to be associated with brain-related conditions in the offspring. It was

reported that exposure to high-dose VPA during pregnancy was associated with an increased risk of impaired cognitive function, compared to other commonly used antiepileptic drugs (Meador *et al.*, 2013). Furthermore, this study reported that maternal use of folate was associated with a higher intelligence quotient of the child. Christensen *et al.* (2013) reported that VPA use during pregnancy was associated with an increased risk of autism spectrum disorders. Besides older antiepileptic drugs, such VPA, numerous new antiepileptic drugs have been developed and approved in recent years. Newer antiepileptic drugs are used with a high frequency even during pregnancy (Vajda *et al.*, 2014). Although several epidemiological studies have addressed the risks of antiepileptic drugs, the safety of these drugs during pregnancy is not entirely clear.

It has been known that several folate carriers, including folate receptor- α (FR α), reduced folate carrier (RFC), and proton-coupled folate transporter (PCFT), contribute to the uptake of folates by cells (Zhao *et al.*, 2009). FR α (*FOLR1*) is a high-affinity folate receptor, which transports folates *via* receptor-mediated endocytosis at a neutral to mildly acidic pH. FR α has a higher affinity to the oxidized form of folate than to the reduced form. RFC (*SLC19A1*) transports the reduced form of folate into the cells coupled with the exchange of organic phosphate. Uptake mediated by this carrier is optimum at pH 7.4. However, with high-level expression, activity can be detected at low pH (Zhao *et al.*, 2011). PCFT (*SLC46A1*) is a proton symporter that efficiently transports the oxidized and reduced forms of folate at acidic pH. The placenta plays a crucial role in supplying nutrients, including folates, to the fetus. These three carriers are expressed in human placenta. It has

been reported that FR α and PCFT are detected in the microvillous plasma membrane (MVM), whereas RFC is detected in both the basal plasma membrane (BM) and MVM (Solanky *et al.*, 2010).

Several researchers have reported that some compounds and pregnancy-related disorders affect the activity and expression of folate carriers. Keating *et al.* (2008) reported that dietary bioactive compounds, such as polyphenols, affected the uptake of folic acid by BeWo cells. In addition, they reported alterations in folic acid transport by antihypertensive drugs and drugs of abuse in primary human trophoblasts (Keating *et al.*, 2009). Araújo *et al.* (2009) reported that cannabinoids slightly changed the transport activity of folic acid in BeWo cells. Hutson *et al.* (2012) showed that chronic alcohol exposure during pregnancy impaired folate transport to the fetus. In addition, Williams *et al.* (2012) reported a decrease in the expression of folate carriers in the placenta of women with pre-eclampsia. Furthermore, it has been reported that folic acid uptake by syncytiotrophoblasts is modulated by gestational diabetes mellitus (Araújo *et al.*, 2013).

Although folates play important roles in fetal development and health, there is limited information on the effects of antiepileptic drugs, which are possibly administered to pregnant woman, on the transport of folic acid *via* the placenta. In the present study, we aimed to investigate whether antiepileptic drugs directly inhibit/stimulate the transport of folic acid in placental cells. Because antiepileptic drugs are administered on chronic basis, their long-term effects on the transport of folic acid, cell viability, and certain gene expressions were also investigated. The 16 antiepileptic drugs investigated were carbamazepine (CBZ), gabapentin (GBP), lacosamide (LCM),

lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), perampanel (PER), phenobarbital (PB), phenytoin (PHT), pregabalin (PGB), rufinamide (RUF), stiripentol (STR), topiramate (TPM), valproic acid (VPA), vigabatrin (VGB), and zonisamide (ZNS). Human placental choriocarcinoma cell lines, BeWo and JEG-3, were used as *in vitro* placental cell models. These cell lines have been widely used to examine the transport mechanisms of compounds by the placenta (Myllynen and Vähäkangas, 2013).

2. Materials and methods

2.1. Chemicals

[3',5',7,9,³H]- Folic acid diammonium salt (³H-folic acid) was purchased from Moravek, Inc. (Brea, CA). CBZ, PB sodium salt, PHT, and ZNS were purchased from Wako (Tokyo, Japan). GBP, LTG, LEV, OXC, TPM, RUF, and STR were purchased from Tokyo Chemical Industry (Tokyo, Japan). LCM, PER, and PGB were purchased from Toronto Research Chemicals (North York, ON, Canada). VPA and VGB were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Dojindo (Tokyo, Japan).

2.2. Cell culture

Human placental choriocarcinoma cells, BeWo and JEG-3, were cultured as previously described (Furugen *et al.*, 2017). Briefly, BeWo cells were cultured in Ham's F-12K (Kaighn's) medium (Wako), supplemented with 15 % fetal bovine serum and 1 % penicillin-streptomycin, at 37 °C under 5 % CO₂. JEG-3 cells were cultured in Eagle's minimum essential medium (Wako), supplemented with 10 % fetal bovine serum, 1 % MEM non-essential amino acids, 1 mM sodium pyruvate, and 1 % penicillin-streptomycin.

2.3. Treatment of human placental choriocarcinoma cells with antiepileptic drugs

Antiepileptic drugs were dissolved in dimethyl sulfoxide (DMSO), methanol, or distilled water. They were further diluted with the cell culture medium (final concentration ≤ 0.2 %) or

transport buffer (final concentration ≤ 1 %). For examining the effects of increasing concentrations of the drugs on cell viability, OXC and STR were diluted with the cell culture medium to a final concentration of 0.5 %. As a control, the same concentration of the respective solvent was included. To assess the short-term effects of antiepileptic drugs on the transport of [3 H]-folic acid, cells were incubated for 10 min in the transport buffer containing each antiepileptic drug, as described in *section 2.4*. To assess the long-term effects of antiepileptic drugs, cells were treated with cell culture media containing different concentrations of the drugs for 24 h. The drug concentrations were selected based on the therapeutic plasma levels of each drug (Jacob and Nair, 2016; Krasowski, 2010; Patsalos *et al.*, 2008). After treatment, the cells were used in the uptake experiment, MTT assay, and real-time polymerase chain reaction (PCR).

2.4. Uptake experiment

BeWo cells (1×10^5 cells/well) and JEG-3 cells (5×10^4 cells/well) were seeded onto 24-well collagen-coated plastic plates. Once the culture medium was removed, cells were washed with the transport buffer and pre-incubated at 37 °C with 0.5 mL of the transport buffer. The transport buffer consisted of Hank's balanced salt solution (HBSS) containing 10 mM HEPES, adjusted to a pH of 7.4. For the pH-dependent study, 10 mM HEPES (pH 7-8) or 10 mM MES (pH 6-6.5) was used. Uptake was initiated by applying the transport buffer containing 14 nM [3 H]-folic acid with or without the tested compounds to the cells. The cells were incubated for the indicated time at 37 °C. After incubation, the applied buffer was aspirated, and the cells were immediately

rinsed with ice-cold transport buffer. To measure the radioactivity of [³H]-folic acid taken up by the cells, the cells were solubilized in 1 % sodium dodecyl sulfate (SDS)/0.2 N NaOH. The samples were mixed with 3 mL of a scintillation cocktail to measure the radioactivity using a liquid scintillation counter. The amount taken up by the cells was normalized to the cell protein level. The protein concentration was determined using a Pierce[®] bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA), in accordance with the manufacturer's instructions.

2.5. MTT assay

Cell viability was assessed by the MTT assay. BeWo cells (5×10^3 cells/well) were seeded onto 96-well plastic plates. After growth of the cells, various antiepileptic drugs were added for 24 h, as described in *section 2.3*. Before the end of treatment, 10 μ L of the MTT solution dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL was added to the cells. The cells were then incubated for 30 min, and the culture medium was aspirated. The MTT formazan was dissolved by 200 μ L of DMSO, and the absorbance was read at 570 nm.

2.6. RT-PCR analysis and quantitative real-time PCR

RT-PCR analysis was carried out as previously described (Furugen *et al.*, 2017). Total RNA was extracted from BeWo cells and JEG-3 cells using ISOGEN II (Nippon Gene, Japan), in accordance with the manufacturer's instructions. Single-strand cDNA was prepared from 1 μ g of total RNA by reverse transcription using ReverTraAce (TOYOBO, Japan). The ratios of A260/A280 and A260/A230 of RNA isolated from BeWo cells were 1.91 ± 0.01 and 2.09 ± 0.02 , respectively,

and those of JEG-3 cells were 1.85 ± 0.01 and 2.17 ± 0.02 , respectively. PCR was performed using HotStarTaq DNA polymerase (QIAGEN) and specific primers through 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 10 s. The primer sequences are summarized in Table 1. The PCR products were subjected to electrophoresis on 2 % agarose gel and then visualized by ethidium bromide staining.

Quantitative real-time PCR was performed using an Mx3000™ real-time PCR system (STRATAGENE) with KAPA SYBR® Fast qPCR kit (KAPA Biosystems, Boston, MA) and specific primers (Table 1) through 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 15 s. The relative mRNA levels of target gene were normalized to β -actin.

2.7. Statistical analysis

All experiments were repeated at least three times. Data are presented as the means \pm standard error of the mean (S.E.) of independent experiments. Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's test. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Activities of folic acid carriers in BeWo cells

It has been reported that the transport of folic acid by BeWo cells is carrier-mediated (Keating *et al.*, 2008; Takahashi *et al.*, 2001; Yasuda *et al.*, 2008a). We investigated the involvement of carriers in folic acid uptake under our experimental conditions. Unlabeled folic acid (5 μ M and 500 μ M) significantly decreased the uptake of [3 H]-folic acid at pH 7.4 by 40 and 28 %, respectively (Fig. 1A). Furthermore, in accordance with previous reports (Keating *et al.*, 2008; Yasuda *et al.*, 2008a), FR α , RFC, and PCFT mRNA were detected in BeWo cells (Fig. 1B).

3.2. Short-term effects of antiepileptic drugs on folic acid uptake by BeWo cells

The effects of 16 antiepileptic drugs on [3 H]-folic acid uptake at physiological pH (pH 7.4) were investigated. As shown in Table 2, exposure to the antiepileptic drugs, CBZ, GBP, LCM, LEV, LTG, OXC, PB, PHT, RUF, STR, TPM, PER, PGB, VGB, VPA, and ZNS had no significant effects on [3 H]-folic acid uptake by BeWo cells.

3.3. Long-term effects of antiepileptic drugs on folic acid uptake by BeWo cells

Then, the effects of long-term antiepileptic drug exposure on [3 H]-folic acid uptake were investigated. The tested concentrations were determined based on the relevant therapeutic concentrations (Jacob and Nair, 2016; Krasowski, 2010; Patsalos *et al.*, 2008). Before investigating the long-term effects of antiepileptic drugs on [3 H]-folic acid uptake, cell viability was assessed after exposure to antiepileptic drugs by the MTT assay. As shown in Table 3, CBZ, GBP, LCM, LEV, LTG,

PB, PHT, RUF, TPM, PER, PGB, VGB, VPA, and ZNS had no significant effects on cell viability. However, OXC (130 μ M) and STR (90 μ M) significantly decreased the viability of BeWo cells. Figure 2 shows the concentration-dependent effects of OXC and STR on cell viability. OXC reduced the cell viability to approximately 40 %, compared to the control. Exposure to STR concentration-dependently decreased the cell viability to approximately 20 %, compared to the control.

Treatment with CBZ, GBP, LCM, LEV, LTG, OXC, PB, PHT, RUF, STR, TPM, PER, PGB, VGB, and ZNS had no significant effects on the uptake of [3 H]-folic acid. Exposure to OXC and STR for 24 h did not alter [3 H]-folic acid uptake per protein although the two drugs affected the cell viability. Exposure of BeWo cells to VPA for 24 h significantly increased the uptake of [3 H]-folic acid (Table 4).

3.4. The effect of VPA exposure on folic acid uptake in JEG-3 cells

Subsequently, the effects of VPA on folic acid uptake by other choriocarcinoma cell lines were examined. Because the transport mechanism of folic acid by JEG-3 cells is not clear, the uptake mechanism of [3 H]-folic acid by JEG-3 cells was first investigated. As shown in Figure 3A, the uptake tended to increase at low pH (pH 6-8). The time-dependent uptake of [3 H]-folic acid by JEG-3 cells at physiological pH (pH 7.4) and acidic pH (pH 6) is shown in Figure 3B. The uptake of [3 H]-folic acid at both pH 7.4 and 6 was linear during the first 30 min. In subsequent experiments, the uptake of [3 H]-folic acid by JEG-3 cells was investigated for 10 min. Unlabeled folic acid (5 μ M)

significantly decreased the uptake of [³H]-folic acid at pH 7.4 and 6 to 25 and 16 % of the control values, respectively (Fig. 3C). RT-PCR analysis showed that FR α , RFC, and PCFT were expressed at the mRNA level in JEG-3 cells (Fig. 3D). VPA treatment of JEG-3 cells for 24 h increased the uptake of [³H]-folic acid to 142 % at physiological pH (Table 4). Although the uptake of [³H]-folic acid at acidic pH tended to increase, the difference was not statistically significant.

3.5. Effects of VPA exposure on the expression of folic acid carriers in choriocarcinoma cells

To investigate the mechanism of increase in folic acid uptake after 24-hour exposure to VPA, the mRNA expression levels of folic acid carriers, including FR α , RFC, and PCFT, were assessed. As shown in Figure 4, VPA exposure significantly increased FR α mRNA level to 171 and 225 % of the control value in BeWo and JEG-3 cells, respectively. PCFT mRNA levels in BeWo and JEG-3 cells were significantly increased by VPA treatment to 175 and 234 % of the control values, respectively. RFC mRNA level was not significantly changed by VPA treatment.

4. Discussion

Folate status during pregnancy is important for fetal development and health. Most pregnant women with epilepsy need to use antiepileptic drugs. Besides older antiepileptic drugs, such as CBZ, PB, PHT, and VPA, newer antiepileptic drugs, such as GBP, LCM, LEV, LTG, OXC, PER, PGB, RUF, STR, TPM, VGB, and ZNS have become available for clinical use since 1990 (Reimers, 2014). For better understanding of the mechanisms of developmental toxicity of antiepileptic drugs and estimation of their potential risk to fetus, it is important to investigate the effects of antiepileptic drugs on folic acid transport *via* the placenta. Therefore, the current study was carried out to evaluate the effects of antiepileptic drugs on folic acid uptake using *in vitro* placental models.

BeWo and JEG-3 cells are continuous cell lines originating from human placenta, which are widely used in toxicology research. These cell lines are also used to study the mechanisms of placental transport of compounds and the role of transporters in these processes (Myllynen and Vähäkangas, 2013). Several studies have shown that folic acid uptake by BeWo cells is carrier-mediated (Keating *et al.*, 2008; Takahashi *et al.*, 2001; Yasuda *et al.*, 2008a). In accordance with previous reports, FR α , RFC, and PCFT mRNA were detected in BeWo cells. Unlabeled folic acid significantly decreased the uptake of [³H]-folic acid at pH 7.4 (Fig. 1A). These results suggest that a folic acid carrier is active at physiological pH in BeWo cells. It has been reported that [³H]-folic acid uptake by BeWo cells is pH-dependent, increasing with the decrease in the extracellular pH (Yasuda *et al.*, 2008a). The uptake of [³H]-folic acid by JEG-3 cells was found to be

pH-dependent (Fig. 3A). In JEG-3 cells, unlabeled folic acid significantly decreased the uptake of [³H]-folic acid at pH 6 and 7.4 (Fig. 3C). Similar to BeWo cells, FR α , RFC, and PCFT mRNA were detected in JEG-3 cells (Fig. 3D). These results suggest that the transport of folic acid by JEG-3 cells at physiological and acidic pH is also a carrier-mediated process.

As shown in Table 2, exposure to antiepileptic drugs had no significant effect on [³H]-folic acid uptake by BeWo cells. These results suggest that the 16 antiepileptic drugs tested in the present study had no direct effects on the activity of folate carriers in the placental cells. However, exposure of BeWo and JEG-3 cells to VPA for 24 h significantly increased the uptake of [³H]-folic acid at physiological pH (Table 4). As shown in Figure 4, FR α and PCFT expression was induced by VPA. These results imply that induction of FR α and PCFT expression contributes to the increase in [³H]-folic acid uptake after exposure to VPA. The use of VPA during pregnancy has been associated with several fetal risks, including neurodevelopmental delay, autism spectrum disorders, and malformations (Christensen *et al.*, 2013; Meador *et al.*, 2013; Vajda *et al.*, 2014). Although it is not clear whether long-term VPA-induced increase in the expression of folate carriers and uptake of folic acid is involved in the toxicological aspects of VPA, our results suggest that chronic VPA treatment affects intracellular folate behavior in placental cells.

At acidic pH, VPA exposure tended to increase the uptake of [³H]-folic acid to 130 %, compared to the control. However, the difference was not statistically significant (Table 4). However, the reason underlying the less evident effects at acidic pH compared to that at physiological pH is not

clear. It has been reported that FR α and RFC are involved in the transport of folate by placental cells at physiological pH, whereas PCFT and RFC are involved in folate transport at acidic pH (Keating *et al.*, 2009). FR α is a high-affinity folate carrier (in nanomolar range), whereas PCFT has a low affinity (micromolar range). In the present study, the uptake of [^3H]-folic acid was investigated at a nanomolar range because plasma folate concentration was reported to range from 7.05 to 17 nM (Kiekens *et al.*, 2015). Therefore, the effect of PCFT induction by VPA on the uptake of folic acid might be difficult to observe at acidic pH. However, the conclusions regarding the contribution of different carriers in placental cells to folic acid transport were based on the results of studies using inhibitors that are not specific to each carrier. Therefore, further investigations are needed to clarify the detailed contribution of each folate carrier by using gene knockdown or knockout.

Rubinchik-Stern *et al.* (2015) recently showed that FR α mRNA expression increased after 2- or 5-day treatment of BeWo cells with VPA (166 $\mu\text{g}/\text{mL}$). In line with the results of this study, FR α mRNA expression was induced after 24-hour exposure of both BeWo and JEG-3 cells to VPA (Fig. 4). Rubinchik-Stern *et al.* (2015) showed that RFC expression was reduced by VPA treatment. Although RFC expression tended to decrease by VPA treatment in BeWo cells in the present study, the reduction was not statistically significant. The disagreement between the results of the prior study and our present study might be owing to the difference in exposure time of BeWo cells to VPA. Besides the change in FR α expression, we showed that PCFT mRNA expression was induced after treatment with VPA. Although the mechanisms of induction of FR α and PCFT expression by VPA

are not clear at present, three potential mechanisms may be involved. First, deficiency of intracellular folate induced by VPA might lead to increased expression of folate carriers to stimulate folic acid uptake by the cells. It was reported that VPA reduced serum folate level (Karabiber *et al.*, 2003), and folate deficiency increased the expression of PCFT (Zhao *et al.*, 2017). Second, the function of VPA as a histone deacetylase (HDAC) inhibitor might alter gene expression. HDAC inhibition leads to acetylation of histone tails and regulation of the expression of many genes (Göttlicher *et al.*, 2001). Third, VPA might affect some transcriptional factors. At the molecular level, it was shown that PCFT expression was regulated by several factors, such as nuclear respiratory factor-1, vitamin D, and Kruppel-like factors (Zhao *et al.*, 2017). Future studies are needed to address the molecular mechanisms responsible for the induction of folate carrier expression in placental cells by VPA.

The present study showed that OXC and STR significantly decreased the viability of BeWo cells at clinical concentrations (Table 3). STR at high concentration markedly inhibited the cell viability (Fig. 2), whereas high concentrations of OXC reduced the cell viability to approximately 40 %, compared to the control. However, 24-hour exposure to OXC and STR did not significantly alter [³H]-folic acid uptake per protein, compared to the control (Table 4). OXC was shown to reduce the viability of hippocampal neurons (Morte *et al.*, 2013), glioblastoma (Lee *et al.*, 2016), and several cancer cell lines, such as MCF-7, HeLa, and HepG2 cells (El Sharkawi *et al.*, 2014). The present study suggests that administration of OXC and STR during pregnancy may affect the placental development. Further investigations are needed to clarify the effects of OXC and STR on

placental and fetal development.

In conclusion, our results showed that short-term exposure to 16 antiepileptic drugs had no effect on folic acid uptake. However, long-term exposure to VPA increased folic acid uptake in human placental cell lines, which was associated with induction of FR α and PCFT mRNA expression. In addition, our results showed that OXC and STR might affect placental cell viability. To the best of our knowledge, this is the first study to comprehensively investigate the effects of antiepileptic drugs on folic acid transport in human placental cell lines. Although choriocarcinoma cell lines, including BeWo and JEG-3 cells, have been widely used in toxicology research in the placenta, these cell lines differ from the normal placental trophoblasts. Extrapolation of the findings observed in cell lines to normal trophoblasts should be made with caution. Therefore, further studies using normal trophoblasts and *in vivo* models are needed to understand the mechanisms of toxicity of antiepileptic drugs and estimate their potential risk to fetus.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Figure captions

Figure 1. Activities of folic acid carriers in BeWo cells. (A) Effect of unlabeled folic acid on [³H]-folic acid uptake by BeWo cells. BeWo cells were incubated with a transport buffer containing 14 nM [³H]-folic acid in the absence or presence of unlabeled folic acid (5 or 500 μM) at physiological pH (pH 7.4). Each column represents the mean with S.E. of three independent experiments. (B) RT-PCR analysis of mRNA expression in BeWo cells. PCR was performed using specific primers as described in the materials and methods.

Figure 2. Concentration-dependent effects of OXC (A) and STR (B) on the viability of BeWo cells. BeWo cells were treated for 24 h with a culture medium containing OXC (1-500 μM) or STR (1-800 μM). After exposure to drugs, cell viability was assessed by the MTT assay. Each point represents the mean ± S.E. of three to four independent experiments.

Figure 3. Uptake properties of folic acid by JEG-3 cells. (A) pH dependence of [³H]-folic acid uptake. JEG-3 cells were incubated with a transport buffer containing 14 nM [³H]-folic acid at various pH (pH 6-8) for 10 min. Each point represents the mean ± S.E. of three independent experiments. (B) Time dependence of [³H]-folic acid uptake. JEG-3 cells were incubated with a transport buffer containing 14 nM [³H]-folic acid at pH 7.4 (closed square) or pH 6 (closed circle). Each point represents the mean ± S.E. of three to four independent experiments. (C) Effect of

unlabeled folic acid on [³H]-folic acid uptake. JEG-3 cells were incubated with a transport buffer containing 14 nM [³H]-folic acid in the absence or presence of unlabeled folic acid (5 μM) at pH 7.4 (left) or pH 6 (right). Each column represents the mean with S.E. of three to four independent experiments. **p* < 0.05, ***p* < 0.01 compared to the control. (D) RT-PCR analysis of mRNA expression in JEG-3 cells.

Figure 4. Effects of VPA exposure on expression of folate carriers in choriocarcinoma cell lines. BeWo (A) and JEG-3 cells (B) were treated with 1000 μM VPA for 24 h. Expression of folate carriers, including FR α , RFC, and PCFT, was assessed by real-time PCR. Each column represents the mean with S.E. of three independent experiments. **p* < 0.05, ***p* < 0.01 compared to the control.

Table 1. Primer sequences

Name		Primer sequence	Product size (bp)	Reference
FR α (<i>FOLR1</i>)	Forward	5'-GCA TTT CAT CCA GGA CAC	146	Yasuda <i>et al.</i> , 2008a
	Reverse	CT-3' 5'-GAC AAT CTT CCC ACC ATT		
RFC (<i>SLC19A1</i>)	Forward	5'-GCA CAT CTG GCT GTG CTA TG-3'	161	Yasuda <i>et al.</i> , 2008a
	Reverse	5'-TGA TGG TCT TGA CGA TGG		
PCFT (<i>SLC46A1</i>)	Forward	5'-GCA ACT AAG CAC ACC CCT CT-3'	182	Yasuda <i>et al.</i> , 2008a
	Reverse	5'-CAA AGG CAA AGA CCA CCA TC-3'		
β -Actin (<i>ACTB</i>)	Forward	5'-TGG CAC CCA GCA CAA TGA A-3'	186	Sugatani <i>et al.</i> , 2014
	Reverse	5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'		

Table 2. Short-term effects of antiepileptic drugs on [³H]-folic acid uptake by BeWo cells

Antiepileptic drug	Concentration (μ M)	Uptake (% of control)
CBZ	500	81 \pm 12
GBP	500	111 \pm 11
LCM	500	92 \pm 6
LEV	500	110 \pm 10
LTG	500	102 \pm 9
OXC	500	95 \pm 3
PB	500	113 \pm 11
PHT	500	97 \pm 17
RUF	200	95 \pm 5
STR	500	84 \pm 6
TPM	500	98 \pm 9
PER	50	100 \pm 5
PGB	500	103 \pm 3
VGB	500	94 \pm 5
VPA	500	107 \pm 13
ZNS	500	110 \pm 15

BeWo cells were incubated with a transport buffer containing 14 nM [³H]-folic acid for 10 min in the presence or absence of antiepileptic drugs, carbamazepine (CBZ), gabapentin (GBP), lacosamide (LCM), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), perampanel (PER), phenobarbital (PB), phenytoin (PHT), pregabalin (PGB), rufinamide (RUF), stiripentol (STR), topiramate (TPM), valproic acid (VPA), vigabatrin (VGB), and zonisamide (ZNS). Data represent the mean \pm S.E. of three independent experiments.

Table 3. Effects of antiepileptic drugs on the viability of BeWo cells

Antiepileptic drug	Concentration (μM)	Cell viability (% of control)
CBZ	50	93 \pm 22
GBP	100	90 \pm 19
LCM	40	100 \pm 7
LEV	170	98 \pm 24
LTG	50	91 \pm 6
OXC	130	43 \pm 1**
PB	130	82 \pm 5
PHT	80	92 \pm 18
RUF	120	83 \pm 3
STR	90	69 \pm 5**
TPM	50	86 \pm 20
PER	1.2	81 \pm 3
PGB	50	97 \pm 6
VGB	270	93 \pm 6
VPA	1000	91 \pm 13
ZNS	140	84 \pm 0.2

BeWo cells were treated for 24 h with culture media containing different concentrations of carbamazepine (CBZ), gabapentin (GBP), lacosamide (LCM), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), perampanel (PER), phenobarbital (PB), phenytoin (PHT), pregabalin (PGB), rufinamide (RUF), stiripentol (STR), topiramate (TPM), valproic acid (VPA), vigabatrin (VGB), and zonisamide (ZNS). After exposure to drugs, cell viability was assessed by the MTT assay. Data represent the mean \pm S.E. of three to five independent experiments. ** $p < 0.01$ compared to the control.

Table 4. Long-term effects of antiepileptic drugs on [³H]-folic acid uptake by BeWo cells

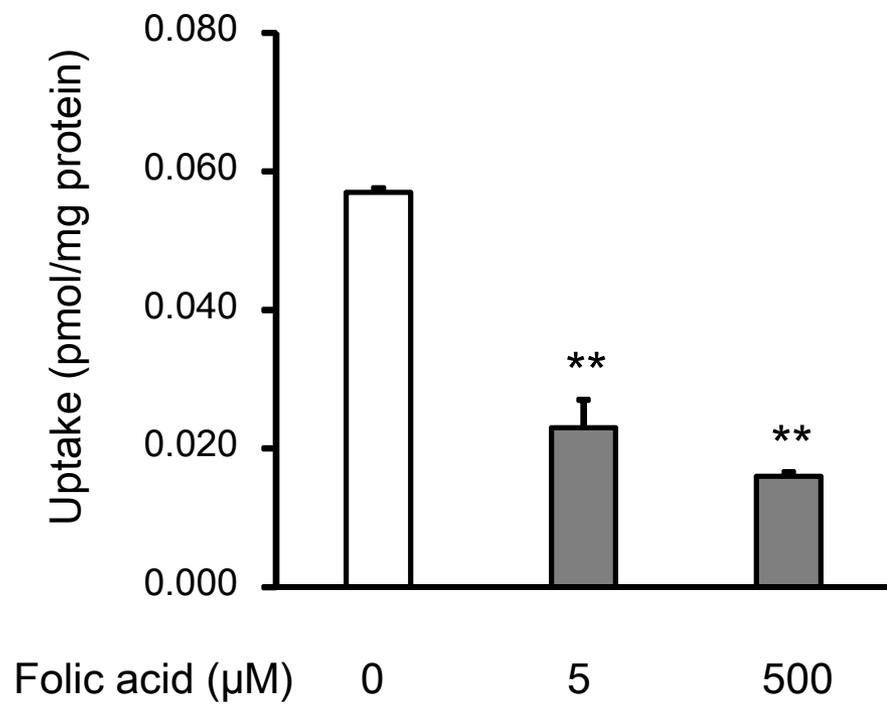
Antiepileptic drug	Concentration (μM)	Uptake (% of control)
BeWo cells (pH 7.4)		
CBZ	50	121 ± 14
GBP	100	109 ± 14
LCM	40	112 ± 19
LEV	170	119 ± 11
LTG	50	112 ± 15
OXC	130	110 ± 14
PB	130	119 ± 12
PHT	80	120 ± 13
RUF	120	112 ± 15
STR	90	118 ± 14
TPM	50	113 ± 11
PER	1.2	117 ± 13
PGB	50	113 ± 15
VGB	270	119 ± 13
VPA	1000	144 ± 6**
ZNS	140	109 ± 14
JEG-3 cells		
VPA (pH 7.4)	1000	142 ± 10*
VPA (pH 6)	1000	130 ± 12

BeWo or JEG-3 cells were treated for 24 h with culture media containing different concentrations of carbamazepine (CBZ), gabapentin (GBP), lacosamide (LCM), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), perampanel (PER), phenobarbital (PB), phenytoin (PHT), pregabalin (PGB), rufinamide (RUF), stiripentol (STR), topiramate (TPM), valproic acid (VPA), vigabatrin (VGB), and zonisamide (ZNS). After exposure to drugs, the transport activity of f [³H]-folic acid was investigated. BeWo cells were incubated with a transport buffer containing 14 nM [³H]-folic acid for

10 min at pH 7.4. JEG-3 cells were incubated with a transport buffer containing 14 nM [³H]-folic acid for 10 min at pH 7.4 or pH 6. Data represent the mean ± S.E. of three to four independent experiments. **p* < 0.05, ***p* < 0.01 compared to the control.

Fig. 1

(A)



(B)

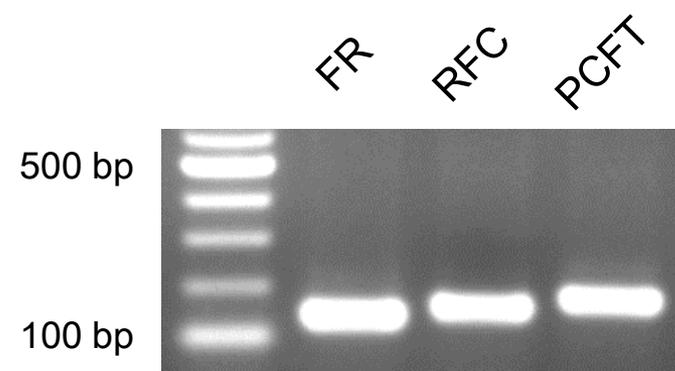
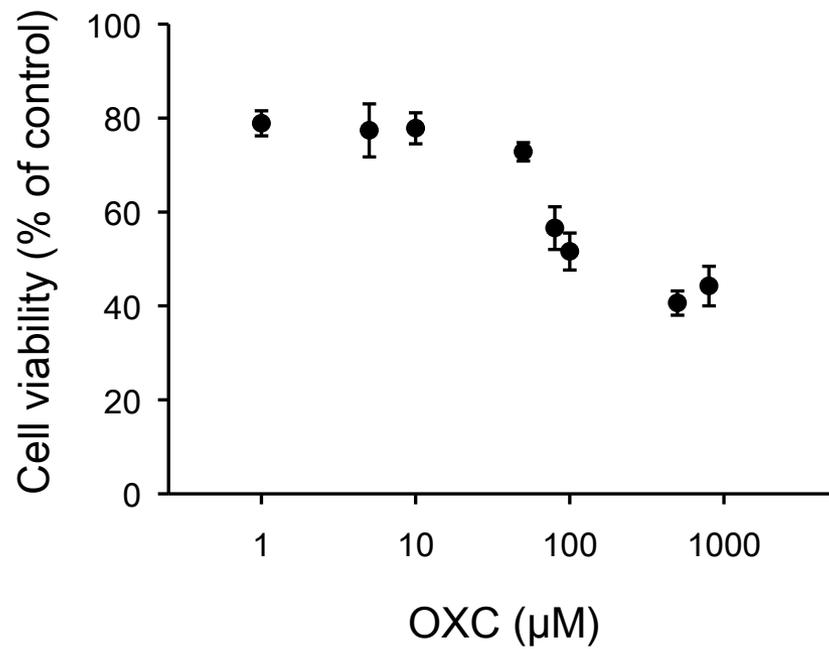


Fig. 2

(A)



(B)

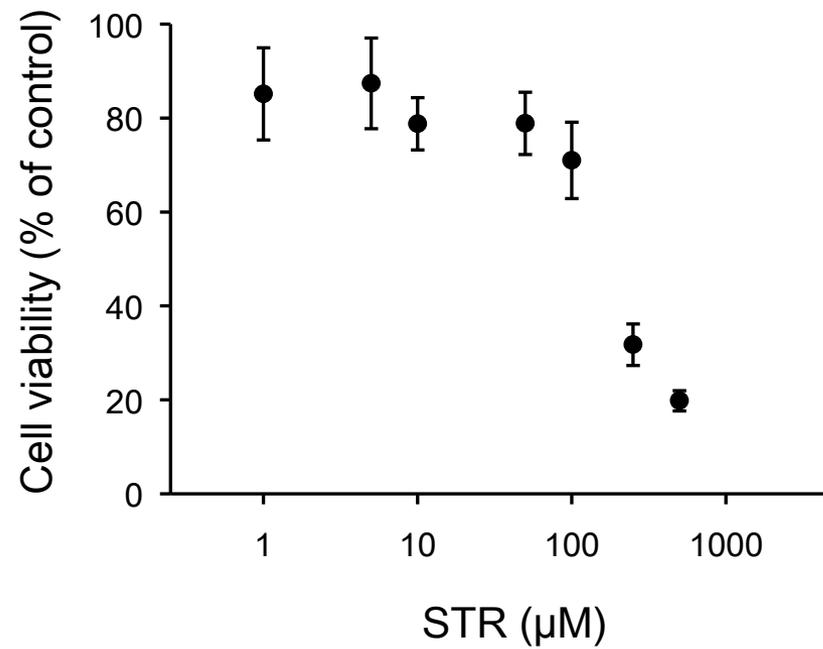
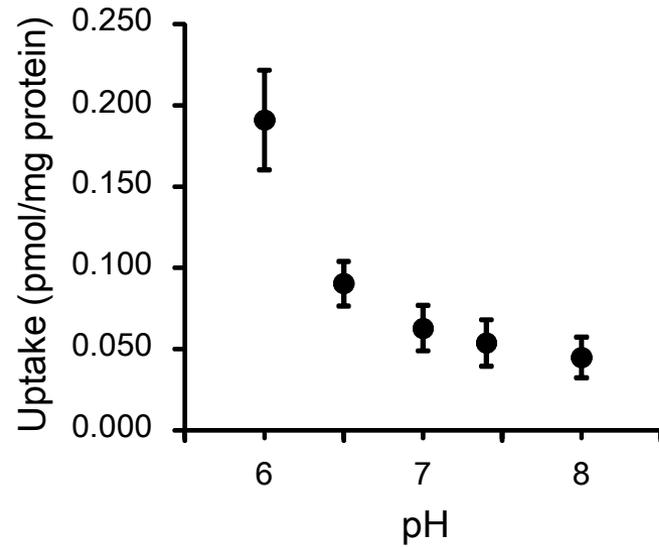
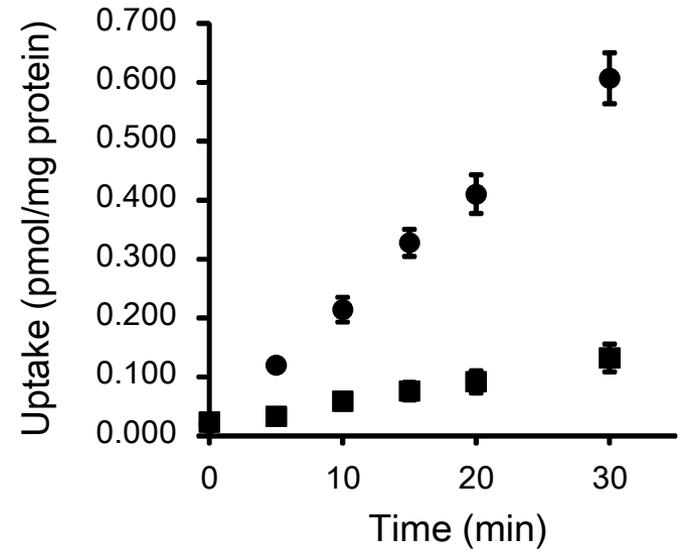


Fig. 3

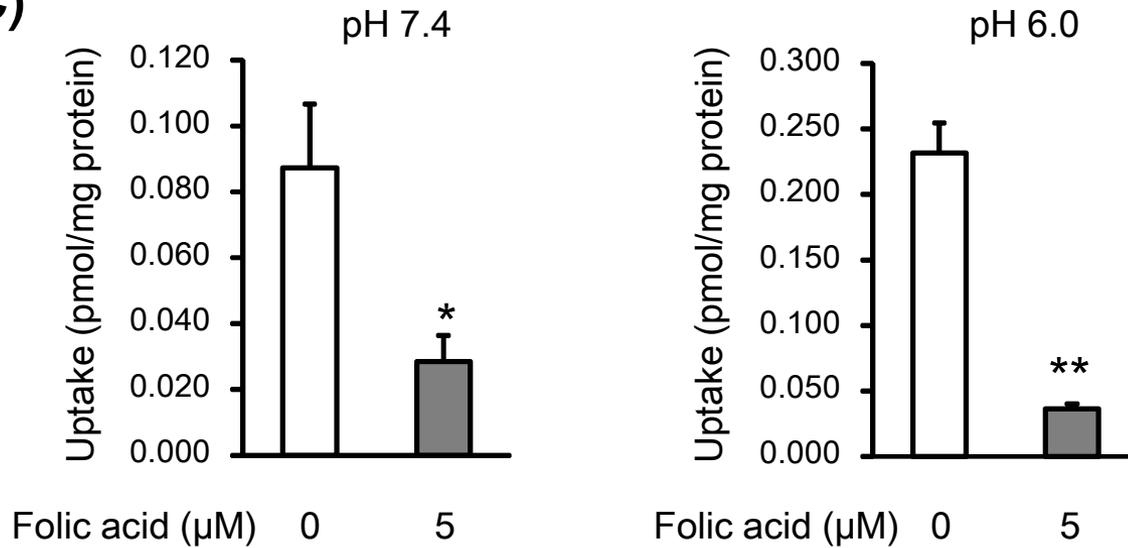
(A)



(B)



(C)



(D)

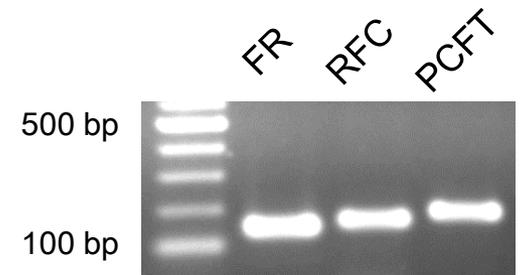
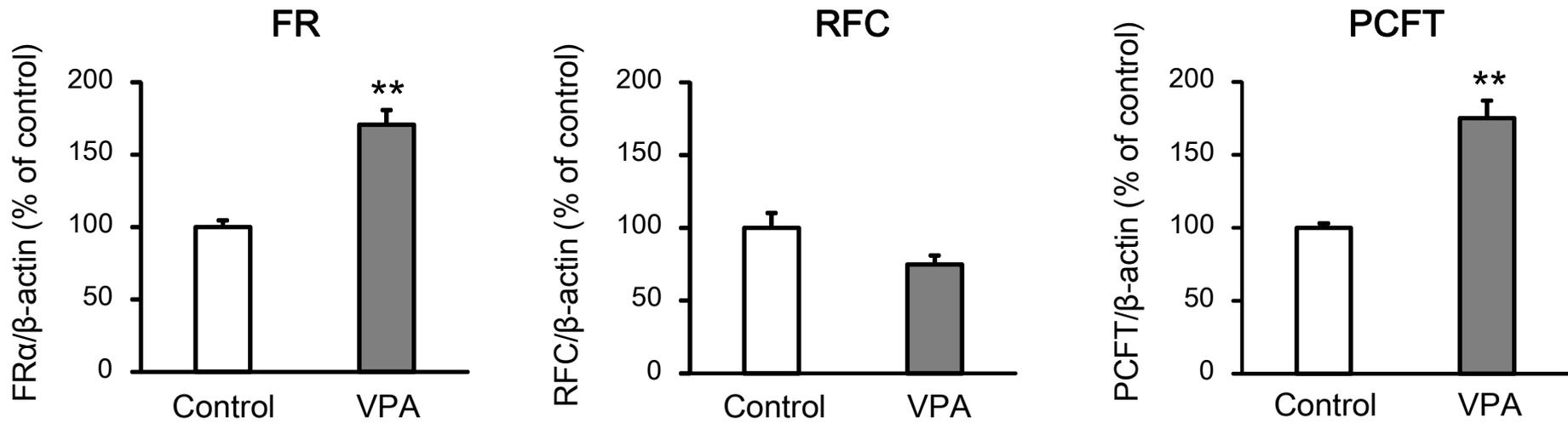
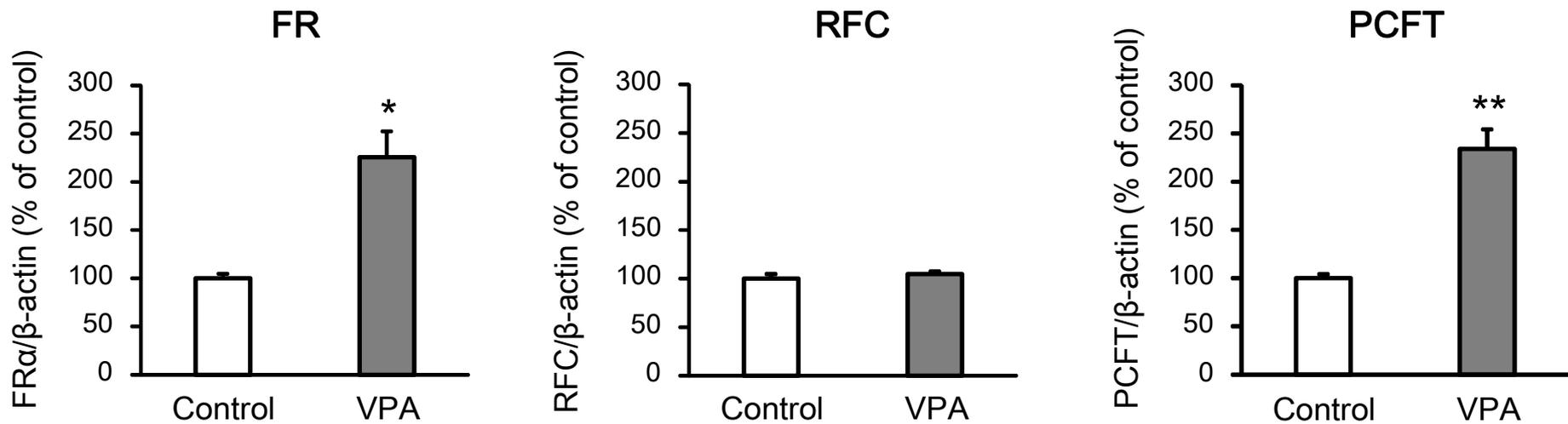


Fig. 4

(A)



(B)



Highlights

- Short-term exposure to 16 antiepileptic drugs had no effect on folic acid uptake
- Long-term exposure to VPA increased folic acid uptake by human placental cells
- Exposure to VPA increased FR α and PCFT mRNA expression
- Exposure to OXC and STR reduced cell viability