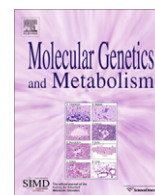




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## Effect of antiepileptic drugs and reactive oxygen species on folate receptor 1 (FOLR1)-dependent 5-methyltetrahydrofolate transport

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### ABSTRACT

Metabolic breakdown of valproate (VPA), carbamazepine (CBZ) and phenytoin (PHT) by the cytochrome P450 pathway generates toxic drug intermediates and reactive oxygen species (ROS). This mechanism has been suspected to play a role in the pathogenesis of secondary cerebral folate deficiency (CFD). Using KB-cell cultures, highly expressing the folate receptor 1 (FOLR1), the effect of antiepileptic drugs (AEDs) and reactive oxygen species (ROS) on the FOLR1 dependent 5-methyltetrahydrofolate (MTHF) uptake was studied. MTHF uptake is time and concentration dependent and shows saturation kinetics. At physiological MTHF concentrations the high-affinity FOLR1 represents the predominant mechanism for cellular incorporation, while at high MTHF concentrations other transport mechanisms participate in folate uptake. Exposure to PHT for more than 8 h led to a higher MTHF uptake and decreased cell count, whereas MTHF uptake remained unaltered by VPA and CBZ. However, exposure to superoxide and hydrogen peroxide radicals significantly decreased cellular MTHF uptake. By specific elimination and downregulation of FOLR1 using phosphatidyl-inositol-specific phospholipase C (PIPLC) and siRNA silencing, it was shown that ROS not only inhibited FOLR1 mediated MTHF uptake but also affected all other mechanisms of membrane-mediated MTHF uptake. Generation of ROS with the use of AED might therefore provide an additional explanation for the disturbed folate transfer across the blood–CSF barrier in patients with CFD.

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### Introduction

5-Methyltetrahydrofolate (MTHF) is the predominant form of folate in plasma and cerebrospinal fluid (CSF). MTHF and its other reduced folates are involved in various one-carbon exchange reactions like the *de novo* synthesis of purines and thymidylate. MTHF is also co-factor of methionine synthase catalyzing the conversion of homocysteine to methionine and is the precursor for the activated methyl-group donor S-adenosyl-methionine which acts as an important co-factor used in more than 100 different methyl-transfer reactions including methylation of DNA, proteins and phospholipids [1].

Folates are required by all cell types. Folate transport across cell membranes is facilitated by at least three different mechanisms: folate receptors (FOLR1 and FOLR2) with high affinity for MTHF, the reduced folate carrier (RFC) having low affinity for MTHF and, by passive MTHF diffusion at extremely high extracellular

concentrations [2]. The glycosyl phosphatidyl-inositol (GPI) membrane-anchored folate receptors (FOLR1 and FOLR2) possess high affinity for folate at concentrations within the nanomolar range and is therefore able to bind physiological levels of folate [3,4]. FOLR1 shows a higher affinity for MTHF compared to FOLR2. The FOLR proteins are crucial for the assimilation, distribution and retention of food folates while intestinal absorption depends also on intact function of the co-transporter called the proton-coupled folate transporter (PCFT) [5,6]. FOLR proteins have been identified in a soluble form (FOLR3) within extracellular fluids or attached to various cell membranes (including KB-cells) with a tissue-specific distribution. FOLR1 is predominantly expressed by epithelial cells whereas FOLR2 is expressed by mesenchymal cells.

Normal brain development and function depend on active MTHF transport by membrane-attached FOLR protein localized at the basolateral membrane surface of choroid epithelium. After binding to FOLR proteins, plasma MTHF is incorporated into choroid epithelial cells against a concentration gradient by FOLR-mediated endocytosis and is subsequently delivered to the CSF compartment [7,8]. By using this active vectorial transport CSF folate levels are 1.5–2 times higher compared to blood folate levels [9–11].

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Recently, a novel clinical condition described as cerebral folate deficiency (CFD) with low MTHF in spinal fluid (CSF) was characterized in pediatric patients [12]. Following high dose folinic acid supplementation the group of youngest patients, diagnosed and treated before the age of 6 years, showed a favorable and sometimes dramatic response with marked neurological recovery and cessation of seizures, whereas the group of older children beyond the age of 6 years tended to show a more delayed response with incomplete neurological recovery. However, treatment with folinic acid was able to prevent further deterioration [12]. The various etiologies explaining low spinal fluid MTHF levels have not been completely identified. In some patients the cause of CFD could be attributed to circulating serum auto-antibodies against the membrane-attached FOLR1 of the choroid plexus epithelial cells, preventing the passage of MTHF into the spinal fluid compartment [13]. In addition, genetic defects of the FOLR1 gene have been found recently in patients where autoimmunity was excluded [14,15]. In the latter patients CSF folates are undetectable, thus much lower than in other forms. Further monogenic causes of CFD include 5,10-methylene-tetrahydrofolate reductase (MTHFR) deficiency [16], 3-phosphoglycerate dehydrogenase (PGDH) deficiency dihydropteridine reductase (DHPR) deficiency [17], as well as with Rett syndrome [18], Aicardi–Goutières syndrome [19], Alpers disease [20], Kearns–Sayre syndrome [21] and hereditary folate malabsorption [22]. Unfortunately, in a minority of patients suffering from CFD the etiology could not be identified. Different factors interfering with MTHF binding or with the process of FOLR1 mediated endocytosis of choroid plexus have been suggested [23]. One hypothesis is that the use of antiepileptic drugs (AED) in CFD patients with epilepsy interferes with folate metabolism and transport across the blood–CSF barrier.

This hypothesis is based on the observation that the use of AED (valproate; VPA, carbamazepine; CBZ) during pregnancy increased the risk of neural tube defects (NTD) and congenital malformations in the offspring of women suffering from epilepsy [24,25]. Folic acid supplementation before and during pregnancy markedly reduced the risk of neural tube defects [26]. This led to the hypothesis that NTDs due to AED are developmental disorders secondary to folate deficiency *in utero*. Unlike in phenytoin (PHT) where impaired intestinal deconjugation of dietary folates [27] or increased catabolism of folates result in low folates in plasma, erythrocyte and CSF and thus provided an explanation for the development of congenital malformations, the pathophysiological mechanisms in VPA or CBZ are not well understood [28].

To investigate the potential influence of different AED and/or ROS on cellular FOLR1 dependent folate uptake a cell culture model with KB-cells was created.  $^{14}\text{C}$  labeled MTHF uptake was determined under different experimental conditions.

## Materials and methods

KB-cells (human nasopharyngeal epidermoid carcinoma cells) and Minimum Essential Medium Eagle were obtained from the American Type Culture Collection (ATCC),  $^{14}\text{C}$ -5MTHF (specific activity of 58.0 Ci/mol) from Amersham Biosciences, folic acid-free medium and phosphate buffered saline (PBS) from GIBCO, Invitrogen-Cooperation. Trypsin–EDTA (0.5%/0.2%), fetal calf serum (FCS), OpiMEM and penicillin/streptomycin (10,000 IU/ml/10,000  $\mu\text{g}/\text{ml}$ ) were obtained from BioConcept's AMIMED, Switzerland. Xanthine, xanthineoxidase, valproic acid sodium salt, carbamazepine, 5,5-diphenylhydantoin, folic acid and trypan-blue were purchased from Sigma–Aldrich, monoclonal antibody to folate receptor 1 (Mov18) from Alexis Biochemicals, FITC-conjugated AffniPure Anti-mouse IgG from Milan Analytica, La Roche, Coelenterazine from Molecular Probes, Inc. (Eugene, Oregon, USA) and Ultima Gold

solution for liquid scintillation counter from Perkin Elmer. For scintillation count a Packard liquid Scintillation Counter 1900 CA and for detection of oxidative radicals a Turner Luminometer TD 20/20 were used.

### $^{14}\text{C}$ -MTHF uptake

Cellular  $^{14}\text{C}$ -MTHF uptake experiments were performed according to the following general procedure: KB-cells were cultured in minimal essential medium, containing 10% FCS and 5000 IU penicillin and streptomycin. Sub-cultivation was performed every 4–5 days at a ratio 1:3. Twenty-four hours before each experiment 70–80% confluent cell dishes (60.1  $\text{cm}^2$ ) were incubated over 5 min with 0.15 M Sodium chloride solution (pH 3.5) to strip membrane-bound folates. After washing with PBS, cells were divided in equal shares in new cell culture dishes and incubated overnight in folic acid-free medium, containing 10% FCS and 5000 U penicillin/streptomycin. Prior to the experiment the medium was exchanged and fresh folic acid-free medium was added (10 ml). Depending on the particular experimental setting  $^{14}\text{C}$ -MTHF was added together with PIPLC, unlabeled MTHF, ROS or different AED's. After incubation cells were washed three times with PBS and harvested with 1:10 diluted trypsin. Viable cells were determined by trypan-blue and counted. Dishes containing more than  $1.5 \times 10^6$  cells were discarded. Subsequently cells were lysed by three cycles of freezing and thawing.

To assess the uptake of  $^{14}\text{C}$ -MTHF centrifugation was performed (3 min at 1090 U/m Hettich Rotina 46) and the supernatant containing the cytosolic  $^{14}\text{C}$ -MTHF fraction was separated, 7.5 ml Ultima Gold solution was added and radioactivity was counted. From the resulting counts per minute the background counts, achieved from one milliliter washing solution was subtracted. All experiments were done as triplicates and were expressed as pmol MTHF/ $10^6$  cells.

### Time- and concentration-dependent $^{14}\text{C}$ -MTHF uptake

For the time-dependent uptake 10 nmol/L  $^{14}\text{C}$ -MTHF was added and incubated over 0.5, 1, 1.5, 2, 4, 8, 12 h at 37 °C and 5%  $\text{CO}_2$ . To investigate concentration-dependent uptake  $^{14}\text{C}$ -MTHF was added to the extracellular medium at concentrations of 10, 25, 40, 50, 75 and 100 nmol/L and measured after a period of 2 h.

### $^{14}\text{C}$ -MTHF uptake in presence of non-labeled MTHF and PIPLC

Cells were incubated over 2 h both with 10–100 nmol/L  $^{14}\text{C}$ -MTHF and in a second experiment together with 1000-fold increased concentrations (10–100  $\mu\text{mol}/\text{L}$ ) of non-labeled MTHF (gift from EPROVA, Switzerland), solved in an aqueous solution containing 1:2000 diluted 0.05% ascorbic acid solution. Pre-incubation with 1000 U phosphatidyl-inositol-specific phospholipase C (PIPLC) is known to cleave the FOLR1 from its GPI anchor. To investigate the influence of PIPLC, cells were pre-incubated with 500 or 1000 IU PIPLC (approximately 40–50 mU/ $10^5$  cells) during 1 h.  $^{14}\text{C}$ -MTHF was added and after another incubation over 2 h the cells were harvested, their viability determined and counted while radioactivity was measured from the cell supernatant.

### Small interference RNA (siRNA) silencing of FOLR1

To evaluate the impact of the FOLR1 and to exclude unspecific effects of PIPLC specific small interference RNA (siRNA) silencing was performed. For gene transfection KB cells were plated equally into 6 cm wells and grown overnight until they were 60–80% confluent. One hour before transfection growth medium was changed to OpiMEM without antibiotics and fetal calf serum. SiRNA was

transfected into KB cells by Lipofectamine 2000 (Invitrogen, Life technology) as recommended from the manufacturer's protocol (125 pmol/L pre-designed siRNA (Ambion, Inc., Woodward, USA) in 250  $\mu$ l OptiMEM and 2.5  $\mu$ l Lipofectamine 2000). OptiMEM was exchanged to folic acid-free MEM after 8–12 h. The result of siRNA silencing was evaluated by Western blot and gene expression analysis.

#### *FOLR1 gene expression*

To analyze *FOLR1* gene expression levels under siRNA inhibition total RNA was extracted from cell lysate at different time points after transfection (QIAamp, RNA blood mini kit). Total RNA concentration was quantified and single stranded cDNA synthesized by reverse transcription according protocol (Promega Corporation: Reverse transcription reaction). Quantitative gene *FOLR1* gene expression was studied by TaqMan<sup>®</sup> Gene expression assay: Primers and a probe specific for the *FOLR1* receptor gene were obtained as Gene Expression Assay from Applied Biosystems. The 25  $\mu$ l reaction mixture contained in addition 50 ng cDNA, 12.5  $\mu$ l TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems) and RNase-free water. 18S was used as controls. The reaction mixture was transferred to a MicroAmp optical 96-well reaction plate, incubated at 95 °C for 10 min and then run for 40 cycles at 95 °C for 15 s and 60 °C for 60 s on the Applied Biosystems GeneAmp 7700 sequence Detection System. The PCR results were analyzed with the GeneAmp SDS software (Applied Biosystems).

#### *FOLR1 protein expression and <sup>14</sup>C-MTHF uptake during siRNA silencing*

*FOLR1* expression studies were done by Western blot. Cells were planted as usual in a 10-cm Petri dish. Cells were washed in ice-cold PBS and lysated with 400  $\mu$ l lyses buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100 and Complete<sup>®</sup> Protease inhibitor Cocktail (Roche)). Nucleic fraction was separated by centrifugation at 4000g for 15 min. Afterwards membranes were prepared by centrifugation of protein lysates at 200,000g during 1 h. Supernatant (cytosolic fraction) was separated and pellet resuspended in 50  $\mu$ l membrane buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, Complete<sup>®</sup> Protease inhibitor Cocktail (Roche)). Samples were frozen at –20 °C. Protein concentration was determined directly before doing Western blot analysis (Bio-Rad Protein Assay). For protein separation equal amount of cytosolic and membrane protein were loaded on NuPAGE<sup>®</sup> 4–12% Bis–Tris Gel with MES running buffer (Invitrogen). Result was controlled with Commassie blue staining. During Western blotting gel was transferred to a nitrocellulose transfer membrane (Protan<sup>®</sup> Schleicher & Schuell, Germany). The blotting was incubated with primary antibody against *FOLR1* (1:500, Mov18, mouse derived) followed by incubation with peroxidase conjugated secondary antibody (1:5000 goat anti-mouse IgG labeled dilution, Sigma–Aldrich). The specific protein was detected using an ECL Western blot Detection Kit (Amersham bioscience). <sup>14</sup>C-MTHF uptake to transfected cells was measured as described above at 48, 72, 96, 120 and 144 h.

#### *Uptake after exposure to oxygen radicals (ROS)*

To analyze the influence of oxygen radicals, incubation with <sup>14</sup>C-MTHF was performed in 10 ml Hanks balanced salt solution (HBSS) containing 25 mg bovine serum albumin (Fluka, Switzerland) 100  $\mu$ g transferrin and 20  $\mu$ g insulin. Three millimolar xanthine and 0.2 U/L xanthine-oxidase were added. Xanthine is metabolized by xanthine-oxidase to uric acid generating the production of superoxide anions and hydrogen peroxide. Previous tests had shown that this mixture is able to produce these ROS over

60 min, without obvious harm to the cells. Before and 1 h after adding 10 nmol/L or 50 nmol/L <sup>14</sup>C-MTHF the amount of produced reactive oxygen species was measured by Luminometer counting after addition of 10  $\mu$ l Coelenterazine. To exclude the possible oxidation of the added <sup>14</sup>C-MTHF itself by these free radicals, in a second experiment cells were exposed to oxygen radicals before adding <sup>14</sup>C-MTHF. Only after confirmation of the disappearance of oxygen radicals, <sup>14</sup>C-MTHF was added.

#### *<sup>14</sup>C-MTHF uptake after exposure to oxygen radicals (ROS) in presence of PIPLC and during siRNA silencing*

To assess if the influence of oxygen radicals on <sup>14</sup>C-MTHF transport is *FOLR1* dependent, cells were pre-incubated with 1000 IU PIPLC as described above and afterwards exposed to xanthine and xanthine-oxidase as described before. Intracellular <sup>14</sup>C-MTHF was determined. In a second step <sup>14</sup>C-MTHF uptake in siRNA transfected cells after exposure to oxygen radicals was analyzed. Untreated cells and cells 72 h after siRNA transfection cells were exposed to xanthine and xanthine-oxidase as described before. <sup>14</sup>C-MTHF uptake in both cell types was measured. To confirm that changes of intracellular <sup>14</sup>C-MTHF concentration were due to oxidations stress, in a third experiment ascorbic acid at 30  $\mu$ M was added to neutralize reactive oxygen species. Previous experiments had shown that ascorbic acid at this concentration has no side effect regarding cellular <sup>14</sup>C-MTHF uptake or viability.

#### *Uptake in presence of valproic acid, carbamazepine or phenytoin*

<sup>14</sup>C-MTHF (10 nmol/L) was added together with either physiological concentrations of 1000  $\mu$ mol/L valproic acid, 40  $\mu$ mol/L carbamazepine (physiological concentrations) or 5–40  $\mu$ g/ml phenytoin. VPA was dissolved in water, CBZ and phenytoin was solved in 1:10 dimethyl sulfoxide (DMSO, Sigma–Aldrich)/water solution. Uptake and oxygen radicals were determined at time points 1, 2, 4, 8, 12 and 24 h.

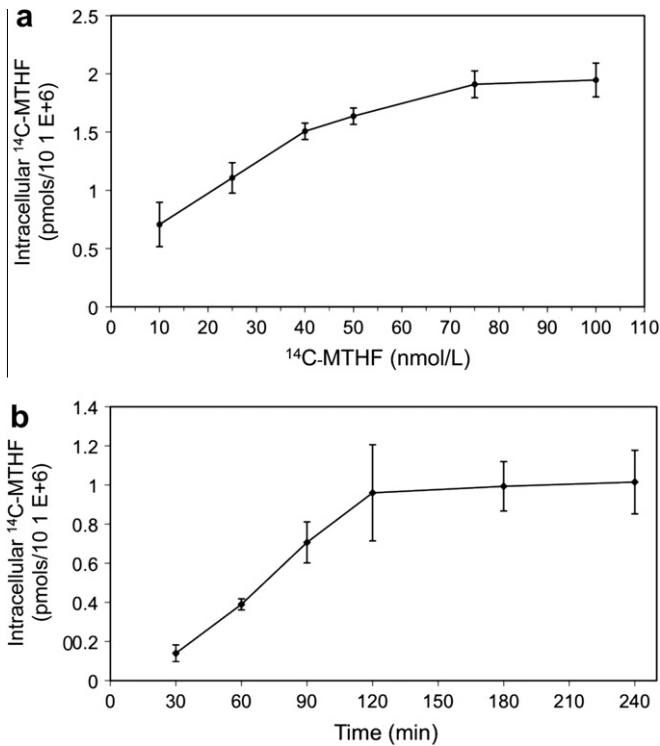
#### *FACS analysis*

Cells were incubated during 1 h with ROS or 250, 500 and 1000 U PIPLC, as well as with physiological concentrations of VPA, CBZ and phenytoin. Cells were solved from the plate by Versene solution (GIBCO, Invitrogen-Cooperation) to protect the membrane-bound receptor. Cells were washed and resuspended in ice-cold 2% FCS in PBS (washing solution). Anti-*FOLR1* antibodies (MoV18 1:100, derived from mouse) were added and incubated during 45 min. After washing cells three times with washing solution secondary antibody (goat anti-mouse IgG, Fluorescein Isothiocyanate (FITC) conjugated, 1:200) for 45 min. After three washing cycles fluorescence was analyzed with flow cytometry (FACS Calibur BD Biosciences) and CellQuest Software (BD Biosciences).

## **Results**

#### *<sup>14</sup>C-MTHF uptake characteristics*

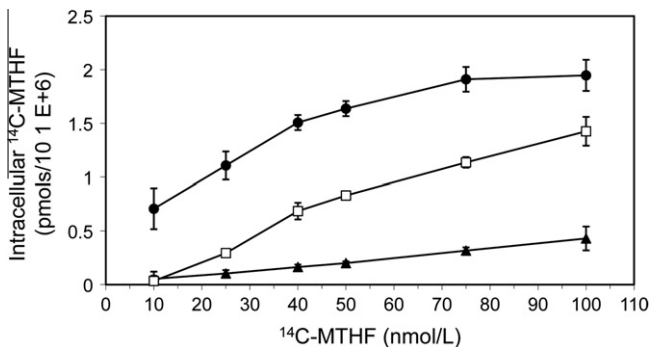
Kinetics of <sup>14</sup>C-MTHF uptake by KB-cells was found to be both concentration- and time-dependent. At constant extracellular <sup>14</sup>C-MTHF concentrations of 10 nmol/L, a time-dependent saturation plateau was reached after 120 min (Fig. 1a). At extracellular <sup>14</sup>C-MTHF concentrations between 0 and 100 nmol/L, <sup>14</sup>C-MTHF uptake after 2 h incubation increased linearly between 0 and 50 nmol/L and showed saturation kinetics at concentrations from 75 to 100 nmol/L (Fig. 1b).



**Fig. 1.** (a) Concentration-dependent uptake of MTHF after incubation with increasing 5MTHF concentration during 2 h. (b) Time-dependent uptake of 10 nM 5MTHF.

FOLR1 was identified as the exclusive high-affinity MTHF transport system operating at low extracellular concentrations, which could be demonstrated by pre-incubation of KB-cells with 1000 U PIPLC, known to cleave the FOLR1 from its GPI anchor (Fig. 2). Nearly complete inhibition of uptake for PIPLC pre-incubated cells was noticed at 10 nmol/L <sup>14</sup>C-MTHF (96% inhibition = 0.03 pmol/10<sup>6</sup> cells versus 0.71 pmol/10<sup>6</sup> cells). However, at progressively higher extracellular <sup>14</sup>C-MTHF concentrations PIPLC induced inhibition became less effective (40 nmol/L = 72% inhibition (0.43 pmol/10<sup>6</sup> cells versus 1.51 pmol/10<sup>6</sup> cells) and 27% inhibition at 100 nmol/L (1.43 pmol/10<sup>6</sup> cells versus 1.95 pmol/10<sup>6</sup> cells)). Thus, in order to obtain a specific model focusing on FOLR1 mediated <sup>14</sup>C-MTHF uptake, experiments were performed at an extracellular concentration of 10 nmol/L <sup>14</sup>C-MTHF (physiological concentration in humans).

To prove that PIPLC specifically decreases FOLR1 on KB cells, flow-cytometric analysis with monoclonal antibodies to FOLR1



**Fig. 2.** Decreased intracellular 5MTHF after incubation with increasing MTHF concentration for 2 h (●–●) in comparison with incubation in presence of 1000 mU (□–□) phosphatidyl-inositol-specific phospholipase C and in presence of 1000-fold concentration non-labeled 5MTHF (▲–▲) (minus background CPM, triplicates).

was performed after pre-incubation with different concentrations of PIPLC. Increasing PIPLC concentrations ranging from 250, 500 to 1000 mU resulted in a continuous loss of the percentage of cells with positive monoclonal antibody binding against FOLR1 (Table 1).

To rule out non-specific effects of PIPLC on cellular folate transport specific FOLR1 silencing was performed with small interference RNA (siRNA). The level of messenger RNA of FOLR1 showed a time dependent decrease. Already after 24 h *FOLR1* gene expression was reduced to 20% of controls and at 48 h it reached its minimal percentage of 10% (Fig. 3a). Subsequently, it increased again to reach normal values at 168 h after transfection. Protein expression followed similar kinetics, but compared to the mRNA expression downregulation of FOLR1 protein was delayed (Fig. 3b). It showed a more moderate decrease, reached the minimal value of around 16% at 96 h and was at 144 h still at 40% of untreated controls. <sup>14</sup>C-MTHF uptake by KB-cells after siRNA silencing was also time dependent. At 48 h nearly a normal uptake was observed, followed by continuous decrease to around 40% of control level after 120 h (Fig. 3c).

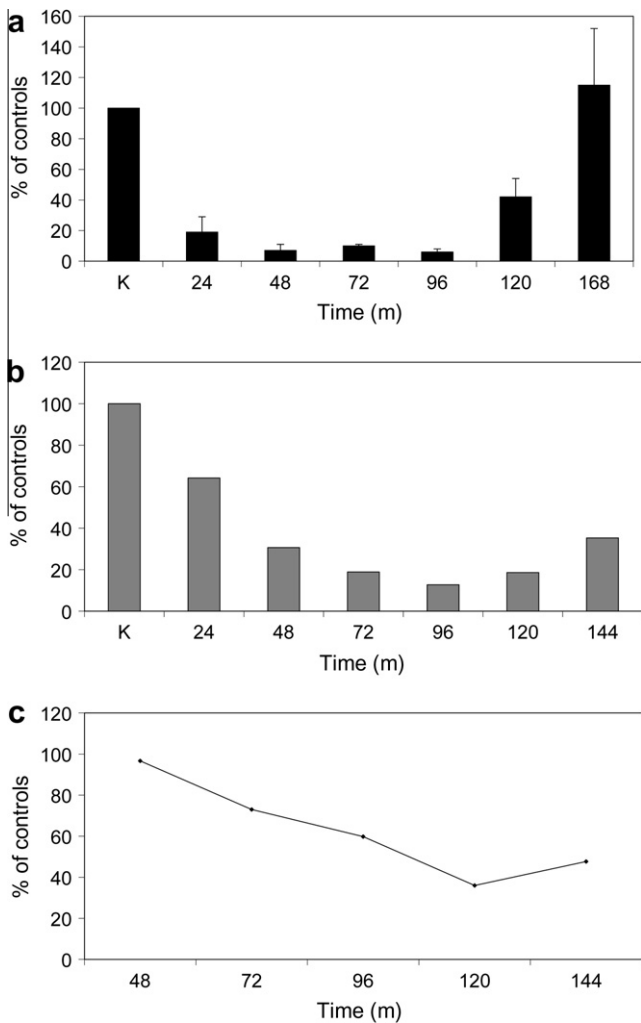
*<sup>14</sup>C-MTHF uptake after exposure to ROS*

To evaluate <sup>14</sup>C-MTHF uptake after exposure to reactive oxygen radicals (ROS), the superoxide radical and hydrogen peroxide were produced enzymatically. ROS significantly reduced <sup>14</sup>C-MTHF uptake by KB-cells (Fig. 4) to an equal extent both at low concentrations of 10 nmol/L (45%, 0.51 pmol/10<sup>6</sup> cells versus 0.28 pmol/10<sup>6</sup> cells; *p* = 0.014) and at higher extracellular concentrations of 50 nmol/L MTHF (44%, 1.59 pmol/10<sup>6</sup> cells versus 0.89 pmol/10<sup>6</sup> cells; *p* = 0.002). It should be stressed that the exposure to ROS did not have any impact on cell count, viability or the binding of monoclonal antibodies (Mov18) against FOLR1 as determined by FACS analysis. To exclude the possibility of <sup>14</sup>C-MTHF degradation itself by these oxygen radicals, <sup>14</sup>C-MTHF was added to the cell medium after oxygen radicals had become undetectable.

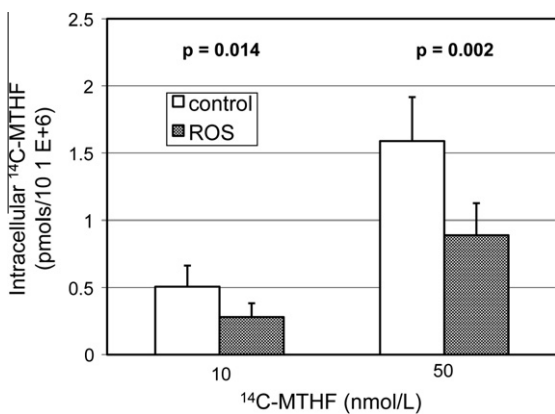
To access whether loss of FOLR1 protein function is responsible for the decreased uptake under exposure to ROS an experiment using of 50 nmol/L extracellular <sup>14</sup>C-MTHF concentration was performed. Firstly, the FOLR1 was removed by PIPLC pre-incubation, leading up to 76% reduction of expressed FOLR1 (see FACS analysis). As consequence intracellular <sup>14</sup>C-MTHF uptake was reduced to 51% of control value (1.495 pmol/10<sup>6</sup> cells versus 0.73 pmol/10<sup>6</sup> cells). Additional incubation of PIPLC pre-treated cells with ROS resulted again in up to 90% decreased uptake, showing that FOLR1 contributes only partially to the loss of <sup>14</sup>C-MTHF uptake after ROS exposure (Fig. 5). These findings could be confirmed and secondary effects of PIPLC could be excluded by <sup>14</sup>C-MTHF uptake studies in siRNA transfected cells. <sup>14</sup>C-MTHF uptake was reduced to 50% in siRNA treated cells (see above). Under the influence of ROS the uptake was further reduced both in the siRNA treated group as well as in the control group (Fig. 6). Interestingly, percent <sup>14</sup>C-MTHF uptake between controls and FOLR1 silenced cells remained the same, indicating again, that uptake reduction was not due to specific loss of FOLR1 protein function. By adding

**Table 1**  
PIPLC decreases concentration-dependent binding of monoclonal anti-FOLR1 antibody (for details see 'Materials and methods').

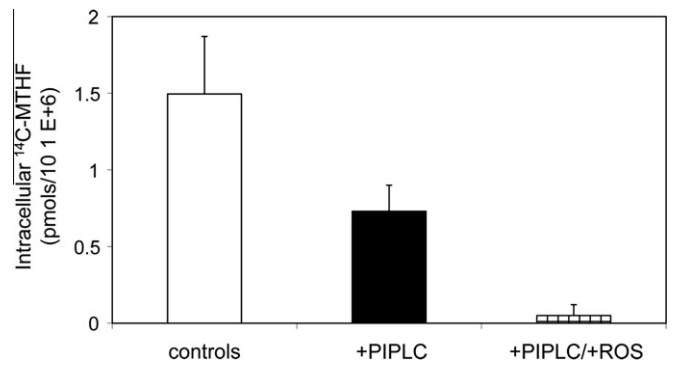
	Antibody 1	Antibody 2	PIPLC (mU)	% Binding/ Controls
Controls	Anti-FOLR1 (Mov18)	Goat anti-mouse IgG	–	100
PIPLC 250	Anti-FOLR1 (Mov18)	Goat anti-mouse IgG	250	60
PIPLC 500	Anti-FOLR1 (Mov18)	Goat anti-mouse IgG	500	33
PIPLC 1000	Anti-FOLR1 (Mov18)	Goat anti-mouse IgG	1000	24



**Fig. 3.** Downregulation of FOLR1 by siRNA interference technique. (a) Gene expression is downregulated already after 24 h to 20% of control level. Expression level stays on this level until 96 h and increases again after 120 h (Taqman analysis, siRNA transfected cells versus controls, %). (b) FOLR1 expression level is also downregulated. Compared to gene expression, protein expression is delayed and reaches maximum downregulation after 96 h. (Western blot analysis, siRNA transfected cells versus controls, %). (c) MTHF uptake decreases after 48 h continuously to minimal level of 40% of controls after 120 h.



**Fig. 4.** ROS exposure leads to significantly decreased  $^{14}\text{C-MTHF}$  uptake in balanced salt solution (HBSS) containing bovine serum albumin, transferrin and insulin (1 h incubation).



**Fig. 5.** PIPLC decreases MTHF uptake and coexposure with ROS and PIPLC leads to an additional decrease, indicating an FOLR1 unrelated effect of ROS on 5MTHF uptake 1 h incubation.

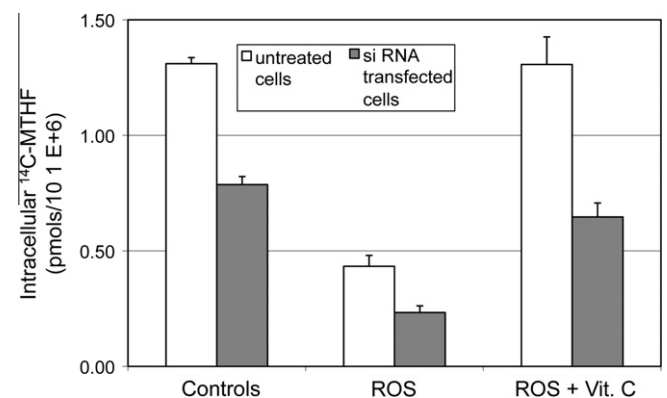
the radical scavenger vitamin C, which was able to restore the original state, it was confirmed that the reduced  $^{14}\text{C-MTHF}$  uptake was caused by oxidative stress (data not shown).

#### $^{14}\text{C-MTHF}$ uptake in presence of valproic acid (VPA), carbamazepine (CBZ) and phenytoin (PHT)

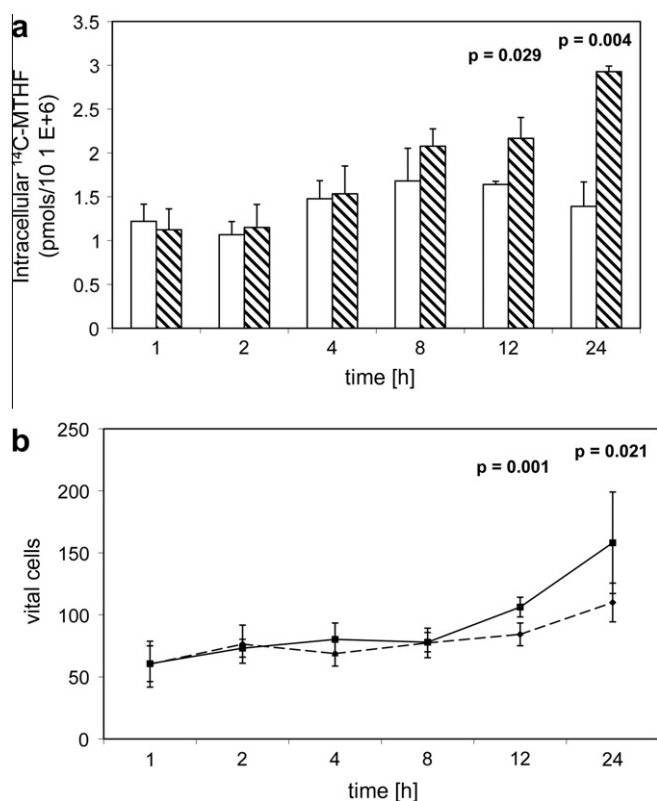
$^{14}\text{C-MTHF}$  uptake in the presence of therapeutic concentration of VPA, CBZ and PHT was measured. No significant difference with respect to intracellular  $^{14}\text{C-MTHF}$  uptake or cell counts was noted between the experiments performed with or without VPA or CBZ (data not shown). In contrast exposure to 20  $\mu\text{g/ml}$  PHT led to an increased  $^{14}\text{C-MTHF}$  uptake after 8, 12 and 24 h (120%, 132% and 176%, respectively; Fig. 7a). Simultaneously, the cell count was reduced by 79% after 12 and by 70% after 24 h exposure to PHT (Fig. 7b). The observed effect of PHT on the cell count was depending on the concentration of added PHT (data not shown). FACS analysis did not reveal any direct effect of AED on anti-FOLR1 antibodies binding to KB-cells.

## Discussion

The etiology of cerebral folate deficiency (CFD) is only partly understood. Apart from high titers of auto-antibodies and genetic defects of the FOLR1 gene further unknown factors interfering with MTHF binding or with the process of FOLR1 mediated endocytosis have to be discussed. [23]. Antiepileptic treatment during early



**Fig. 6.** Uptake of MTHF in presence of ROS in siRNA transfected and untreated cells: MTHF uptake is reduced in siRNA treated cells. ROS lead to further reduction of  $^{14}\text{C-MTHF}$  uptake both in the siRNA treated group as well as in the control group. After addition of vitamin C 5MTHF uptake is restored to the normal level again. K: control.



**Fig. 7.** (a) Phenytoin leads to significant higher intracellular MTHF after incubation of 12 h White boxes control group (all results minus background CPM). (b) Similar exposure to phenytoin results in a decreased cell count after incubation longer than 12 h. (20 µg/ml phenytoin (dashed line, ●–●) in comparison with control group (continuous line, ■–■)).

pregnancy predisposes to NTD [24,25]. Although the exact mechanism explaining development of a NTD during use of AED in pregnancy remains not entirely clear it appears to be linked to *in utero* folate deficiency during embryonic neural tube closure. A comparable mechanism can be discussed in CFD patients with epilepsy. Here a direct impact of AED or of intermediary drug metabolites or of reactive oxygen species generated during their metabolic breakdown is possible. To study the effect of AED and ROS on folate homeostasis, intracellular uptake and transport across intestinal, placental and blood–brain barriers we used an *in vitro* model of KB-cells highly expressing the FOLR1.

First, <sup>14</sup>C-MTHF cellular incorporation under normal conditions was studied. Initial kinetics studies confirmed earlier data showing that at physiological serum concentrations (10–30 nmol/L) the <sup>14</sup>C-MTHF uptake is time- and concentration dependent with saturation kinetics [29]. For each individual concentration a saturation plateau was reached after 2 h and a plateau of maximum uptake occurred for concentrations exceeding 75 nmol/L <sup>14</sup>C-MTHF. Comparison of MTHF uptake with or without PIPLC proved that at low physiologic extracellular concentrations, MTHF uptake is only mediated by the FOLR1. To focus our investigations on the FOLR1, our first experiments were performed at the 10 nmol/L <sup>14</sup>C-MTHF concentration.

Exposure to VPA and CBZ did not alter MTHF uptake significantly whereas exposure to PHT resulted in direct inhibition of cell growth and increased folate uptake. An increased MTHF uptake under the influence of PHT might explain the decreased serum folate levels in patients receiving PHT treatment [30] but is not concordant with the findings where PHT inhibits the uptake of folic acid into isolated chick intestinal epithelial cells. The latter results are likely to be explained by a direct effect on intestinal folic acid up-

take mechanisms [31]. The reduced cell count is due to cytotoxicity by PHT, already observed in various cell culture models [32–34]. The effect of PHT on folate uptake by different tissues still needs further clarification, while earlier findings suggesting enhanced folate catabolism by PHT need to be considered as well. Nevertheless, folic acid supplementation should be an essential part of preventive pregnancy care particularly among women suffering from epilepsy.

However, during degradation of the other antiepileptic drugs VPA and CBZ by the cytochrome P450 system, ROS are generated. Our studies showed decreased <sup>14</sup>C-MTHF uptake after exposure to the *in vitro* generated ROS superoxide anions and hydrogen peroxide. To investigate the role of FOLR1 with respect to the reduced folate uptake after ROS exposure, first the receptor was removed by pre-incubation of KB-cells with PIPLC. Under exposure to ROS was still a significant decreased uptake notable. In a second more specific step FOLR1 was eliminated by siRNA silencing which confirmed the results. Therefore, the inhibition of <sup>14</sup>C-MTHF uptake cannot only be attributed to the interference of ROS with the FOLR1. ROS are believed to lead to a more general functional loss of all membrane-coupled folate uptake mechanisms including also the RFC or other unidentified folate transporters. The radical scavenger vitamin C is able to alleviate the inhibitory effect of ROS on cellular folate incorporation.

Our data might suggest alternative mechanisms for patients with the CFD syndrome, in whom genetic FOLR1 abnormalities or FOLR1 autoimmunity have been excluded. CFD is a newly identified clinical syndrome with post-natal onset of irritability and insomnia, followed by head growth deceleration, hypotonia and ataxia, psychomotor retardation, spastic paraplegia, and in one-third of patients epilepsy and dyskinesia [12]. The only detectable biochemical abnormality is low MTHF in spinal fluid, suggesting a disorder of folate transport across the blood–CSF barrier. In the majority of CFD patients, circulating FOLR1 auto-antibodies of the blocking type have been identified in serum and have been suspected to prevent the binding of MTHF to the membrane-attached FOLR1 of choroid plexus [13]. Recently, in patients without FOLR1 autoimmunity a genetic defect of the FOLR1 gene has been found [14,15]. Unfortunately, in a number of patients the cause remains unknown, while one-third of these patients suffer from epilepsy and receive AED. In the latter patients the generation of ROS with or without the use of AED, might provide an additional explanation being at the origin of disturbed folate transfer across the blood–CSF barrier or aggravating brain folate deficiency.

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