

# The Antiepileptic Drugs Phenobarbital and Carbamazepine Reduce Transport of Methotrexate in Rat Choroid Plexus by Down-Regulation of the Reduced Folate Carrier<sup>[S]</sup>

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Received March 22, 2011; accepted July 7, 2011

## ABSTRACT

Intrathecal methotrexate (MTX) has been associated with severe neurotoxicity. Because carrier-associated removal of MTX from the cerebrospinal fluid (CSF) into blood remains undefined, we determined the expression and function of MTX transporters in rat choroid plexus (CP). MTX neurotoxicity usually manifests as seizures requiring therapy with antiepileptic drugs (AEDs) such as phenobarbital (PB). Because we have demonstrated that PB reduces activity of MTX influx carrier reduced folate carrier (Rfc1) in liver, we investigated the influence of the AEDs PB, carbamazepine (CBZ), or gabapentin on Rfc1-mediated MTX transport in CP. Reverse transcriptase-polymerase chain reaction and Western blot analysis showed similar expression of the MTX influx carrier Rfc1 and organic anion transporter 3 or efflux transporter multidrug resistance-associated protein 1 (Mrp1) and breast cancer resistance protein (Bcrp) in rat CP tissue and choroidal epithelial Z310 cells. Confocal

microscopy revealed subcellular localization of Rfc1 and Bcrp at the apical and of Mrp1 at the basolateral CP membrane. Uptake, efflux, and inhibition studies indicated MTX transport activity of Rfc1, Mrp1, and Bcrp. PB and CBZ but not gabapentin significantly inhibited Rfc1-mediated uptake of MTX in CP cells. Studies on the regulatory mechanism showed that PB significantly inhibited Rfc1 translation but did not alter carrier gene expression. Altogether, removal of intrathecal MTX across the blood-CSF barrier may be achieved through Rfc1-mediated uptake from the CSF followed by MTX extrusion into blood, particularly via Mrp1. Antiepileptic treatment with PB or CBZ causes post-transcriptional down-regulation of Rfc1 activity in CP. This mechanism may result in enhanced MTX toxicity in patients with cancer who are receiving intrathecal MTX chemotherapy by reduced CSF clearance of the drug.

## Introduction

In patients with acute lymphoblastic leukemia (ALL) or lymphoma, the folate antagonist methotrexate (MTX) is the most common chemotherapeutic drug used in central nervous system (CNS) prophylaxis and treatment and is associ-

ated with a reduction in CNS relapse (Kwong et al., 2009). Despite its clinical success, intrathecal MTX has been shown to involve significant neurotoxicity (Shuper et al., 2002), the pathogenic mechanism of which remains poorly understood. The antifolate MTX mainly inhibits the enzyme dihydrofolate reductase, resulting in inhibition of DNA synthesis and cell death through depletion of reduced folate cofactors, including 5-methyltetrahydrofolate (5-MTHF) (Shuper et al., 2002). Moreover, lack of the carbon donor 5-MTHF reduces the conversion of homocysteine to methionine, resulting in hyperhomocysteinemia, an established risk factor for vascular disease (Shuper et al., 2002). These MTX-induced bio-

This work was supported by the Deutsche Forschungsgemeinschaft [Grant HO-2103/2-1].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.111.072421.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

**ABBREVIATIONS:** ALL, acute lymphoblastic leukemia; AED, antiepileptic drug; Bcrp, breast cancer resistance protein; CAR, constitutive androstane receptor; CBZ, carbamazepine; CP, choroid plexus; CSF, cerebrospinal fluid; FOLR, folate receptor; 5-MTHF, 5-methyltetrahydrofolate; MTX, methotrexate; Mrp, multidrug resistance-associated protein; Oat, organic anion transporter; Oatp, organic anion transporting polypeptide; PAH, *para*-aminohippurate; PCR, polymerase chain reaction; PB, phenobarbital; PCFT, proton-coupled folate transporter; ZO-1, zonula occludens-1; RT-PCR, reverse transcription-polymerase chain reaction; ABC, ATP-binding cassette; MAb, monoclonal antibody; AP, alkaline phosphatase; DAPI, 4',6'-diamidino-2-phenylindole; aCSF, artificial cerebrospinal fluid; MK571, 3-([(3-(2-[7-chloro-2-quinolinyl]ethenyl) phenyl)-[(3-dimethylamino-3-oxopropyl)-thio-methyl]thio]propanoic acid; Ko143, 3-[(3S,6S,12aS)-6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydro-pyrazino[1',2':1,6]pyrido[3,4-b]indol-3-yl)-propionic acid *tert*-butyl ester.

chemical alterations are suggested to cause demyelination and endothelial cell injury, resulting in leukoencephalopathy (Shuper et al., 2002).

Besides the blood-brain barrier, the blood-cerebrospinal fluid barrier protects the brain from potential harmful xenobiotics, including drugs such as MTX. The blood-cerebrospinal fluid barrier is composed of tight-junctioned choroid plexus (CP) epithelial cells with an apical side facing the cerebrospinal fluid (CSF) and a basolateral side facing the CP fenestrated capillaries (Spector and Johanson, 2010). The choroid plexus generally limits drug disposition of the brain parenchyma through the prevention of entry of systemically administered compounds into the CSF or excretion of intrathecally applied drugs from the CSF into blood. The latter is achieved by several plasma membrane proteins expressed at the luminal side of the CP that mediate uptake of drugs from the CSF into CP cells and carrier proteins located at the basolateral membrane mediating drug efflux from the CP into blood (Spector and Johanson, 2010).

The chemotherapeutic drug MTX is a known substrate of the reduced folate carrier (Rfc1, Slc19a1) (Matherly and Goldman, 2003) and members of the organic anion transporter (Oat) (Anzai et al., 2006) as well as of ABC export carrier, including multidrug resistance-associated protein (Mrp) and breast cancer resistance protein (Bcrp) (Nies, 2007). Until now, expression and subcellular localization has only been shown for rodent Rfc1 and Oat3 (Slc22a8) at the apical CP membrane and for Mrp1 (Abcc1) and Mrp4 (Abcc4) at the basolateral membrane of human and rodent CP (Nies 2007; Hinken et al., 2011). Oat1 (Slc22a6) was also found in rat and human CP epithelial cells, but the exact membrane localization is not clear (Nies 2007). Presence of Bcrp in the blood-CSF barrier is currently unknown (Nies 2007). It has been shown previously that uptake of MTX in CP epithelial cells is concentrative and sodium-dependent (Breen et al., 2004). However, the involvement of particular influx or efflux carrier proteins in MTX transport across the blood-CSF barrier remains unclear (Spector and Johanson, 2010).

Several studies indicate that sodium-dependent Rfc1 provides the major route for cellular uptake of MTX at neutral pH (Zhao et al., 2008; Biswal and Verma, 2009). We have demonstrated that hepatocellular Rfc1-mediated MTX influx is significantly reduced after treatment with a clinically relevant concentration of the antiepileptic drug (AED) PB or CBZ as a result of post-transcriptional down-regulation of the carrier protein (Halwachs et al., 2007). In childhood ALL, seizures represent a frequent clinical manifestation of MTX neurotoxicity particularly related to intrathecal MTX (Maytal et al., 1995). Besides, seizures due to metabolic disorders, cerebral infarction, or CNS infection have been observed (Maytal et al., 1995). As in these patients, comedication with AEDs is regularly required (Tibussek et al., 2006), we examined the contribution of Rfc1 to MTX transport at the blood-CSF barrier and investigated the effect of AEDs on Rfc1 uptake activity on the functional, transcriptional, and post-transcriptional level using the rat CP-like *in vitro* model Z310 (Zheng and Zhao, 2002; Shi et al., 2008).

In this study, we showed the expression of important MTX transporters in choroidal epithelial Z310 cells analog to rat CP tissue. Moreover, we first illustrate subcellular localization of Rfc1 and Bcrp at the apical CP membrane. Furthermore, our data suggested that vectorial transport of MTX

across the CP is mainly achieved by Rfc1-mediated MTX uptake followed by drug efflux that very likely particularly involved Mrp1. Antiepileptic treatment with clinical relevant concentrations of the constitutive androstane receptor (CAR) agonists PB or CBZ but not non-CAR agonist gabapentin resulted in a significant reduction of Rfc1-mediated MTX uptake because of an inhibition of Rfc1 mRNA translation.

## Materials and Methods

**Materials.** All chemicals, including media and supplements, were obtained from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

**Cell Culture.** The immortalized Z310 rat choroidal epithelial cell line was kindly provided by Dr. Wei Zheng (Purdue University, West Lafayette, IN) and has been described previously (Zheng and Zhao, 2002). Cells were maintained in Dulbecco's modified Eagle's medium (PAA Laboratories GmbH, Coelbe, Germany) containing 10% (v/v) fetal calf serum (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 40  $\mu$ g/ml gentamicin (all from PAA Laboratories). Polarized cell monolayers were obtained by culture on Polyester Transwell-Clear inserts (1.0 cm<sup>2</sup> growth area, 0.4  $\mu$ m pore size; Corning Life Sciences, Wiesbaden, Germany) coated with 0.01% rat-tail collagen overnight. Z310 cells ( $2 \times 10^5$ ) were suspended in 1 ml of complete growth medium supplemented with 1  $\mu$ M dexamethasone (Shi and Zheng, 2005) and added to the inner chamber, which was inserted into the outer chamber containing 1.3 ml of culture medium. Growth medium was exchanged daily. Formation of cell monolayer was followed by measurement of transepithelial electrical resistance by using a low-impedance volt-ohm meter equipped with a Chopstick electrode (Millipore, Schwalbach, Germany).

**Choroid Plexus Tissue.** Samples of the lateral and fourth ventricle CP were from male Sprague-Dawley rats (200–250 g). Rats were euthanized by asphyxiation with CO<sub>2</sub>, brains were removed, and CPs were then excised. These procedures were conducted in the central animal facility of the Medical Faculty of the Universität Leipzig according to institutional guidelines for ethical care and use of animals for experimental and other scientific purposes.

**RT-PCR.** Total RNA was prepared from CP and  $5 \times 10^6$  Z310 cells using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Total RNA (1  $\mu$ g) was used to synthesize cDNA under standard conditions using the First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, St. Leon-Rot, Germany) with 20 U of reverse transcriptase (Moloney murine leukemia virus reverse transcriptase) and 25  $\mu$ g oligo(dT)<sub>18</sub> primer. PCR amplification was carried out using 1/25 of cDNA, 0.2 mM mixed dNTPs, and 1.5 U DreamTaq DNA polymerase (Fermentas). Specific primers for rOat3, rOatp1a5, rPCFT, rMrp1, rMdr1, and rBcrp were designed using PrimerSelect software (DNASar Inc., Madison, WI). All gene-specific primer pairs and annealing temperatures are listed in Supplemental Table 1S. PCR was performed over 32 cycles with an initial denaturation step of 30 s at 95°C followed by annealing and extension at 72°C for 40 s. A final extension was carried out at 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis with regard to size of the expected fragment. Glyceraldehyde 3-phosphate dehydrogenase was used as positive control, and amplification of genomic DNA was excluded by omitting reverse transcriptase.

**Quantitative RT-PCR of Rfc1 Gene Expression.** Total RNA was prepared from  $5 \times 10^6$  Z310 cells grown to confluence using the RNeasy Mini system (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and cDNA was synthesized from total RNA (1  $\mu$ g) as delineated above. Quantitative PCR of 1  $\mu$ l of cDNA was carried out using the PCR-Master-Mix S (peqlab, Erlangen, Germany) as well as Rfc1-specific sense and antisense primers and TaqMan probe as described previously (Halwachs et al., 2007). Rfc1

mRNA expression relative to the normalized ( $\beta$ -actin) control level was calculated by the  $2^{-\Delta\Delta C_T}$  method, taking the Rfc1 PCR efficacy into account.

#### SDS-Polyacrylamide Gel Electrophoresis and Western Blot

**Analysis.** Z310 cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium desoxycholate, 2 mM sodium vanadate, and  $1\times$  protease inhibitor cocktail) and incubated for 60 min on ice. Nuclei and cell debris were pelleted at 1000g for 5 min at 4°C. Protein concentrations were measured using the bicinchoninic protein assay (Thermo Fisher Scientific, Waltham, MA). Whole-cell lysates were mixed in sample buffer [final: 2% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, and 0.1% bromophenol blue, pH 6.8] and apart from Rfc1 supplemented with  $\beta$ -mercaptoethanol (100 mM). For detection of Oat1, proteins were denatured at 37°C for 30 min. According to protein size equal protein amounts were separated on SDS/7.5% polyacrylamide gel for Oat3, Mrp1, and Bcrp or 10% SDS gels for Oat1, Rfc1, and  $\beta$ -actin. Thereafter, proteins were blotted onto nitrocellulose membrane. Unspecific binding was blocked with 5% milk powder in Tris-buffered saline-Tween [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Tween 20] for 1 h at room temperature. Membranes were probed overnight at 4°C in 3% milk powder in Tris-buffered saline-Tween 20 with the following primary antibodies: polyclonal anti-rat OAT1 (2.5  $\mu$ g/ml; kindly provided by BC Burckhardt, Georg-August University of Göttingen, Germany), polyclonal anti-OAT3 (1:200; Santa Cruz Biotechnology, Heidelberg, Germany), polyclonal anti-SLC19A1 (1:100; Abcam Inc., Cambridge, MA), anti-MRP1 MAb (1:20; generous gift from G. Fricker, Ruprecht-Karls-University, Heidelberg, Germany) anti-ABCG2 MAb (1:100; Santa Cruz Biotechnology), or anti- $\beta$ -actin MAb (1:10,000; clone AC-15). As secondary antibodies, AP-conjugated goat anti-mouse IgG (1:7000; Promega) for Bcrp and  $\beta$ -actin, AP-conjugated goat anti-rat IgG (1:5000; Santa Cruz Biotechnology) for Mrp1 or AP-conjugated goat anti-rabbit IgG (1:2000; Dako Deutschland GmbH, Hamburg, Germany) for Oat1, Oat3, and Rfc1 were used. Transporter-antibody complexes were visualized using Western Blue stabilized substrate for alkaline phosphatase (Promega). Densitometric quantification of Rfc1 protein was performed with 1D Image Analysis Software (GeneTools, Syngene, Cambridge, UK). The amount of Rfc1 in the presence of PB in consideration of the background level was expressed as the fold change compared with untreated Z310 cells.

**Immunocytochemical Analysis.** Z310 cells were cultured on Transwell-inserts as delineated above. Cells were fixed in acetone (−20°C) for 10 min and permeabilized with 0.001% (v/v) Triton X-100. Unspecific binding was blocked with 5% (v/v) bovine serum albumin in PBS. Samples were incubated overnight at 4°C with anti-SLC19A1 (1:30), BXP-21 (1:100), MRP1 (1:20), or tight junction protein-1 (1:200) for ZO-1 followed by visualization by goat anti-rabbit Alexa Fluor 488 IgG (1:400; Invitrogen, Carlsbad, CA) for Rfc1 and ZO-1, goat anti-mouse Alexa Fluor 594 IgG (1:400; Invitrogen) for Bcrp or DyLight649-conjugated donkey anti-rat IgG (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for Mrp1 over 1.5 h at room temperature. Nuclear staining was achieved by addition of 4'-6-diamidino-2-phenylindole (DAPI; 0.5  $\mu$ g/ml). Finally, the specimens were washed and mounted on slides with Fluoromount. Control incubations were generally performed by omission of primary antibodies. Confocal images of Z310 cells were taken by sequential scanning of optical sections (XY) of approximately 0.13  $\mu$ m thickness.

**Confocal Laser Scanning Microscopy.** Cell samples were analyzed using the inverted confocal laser scanning microscope Leica TCS SP5 equipped with a HCX plan apochromatic 63 $\times$ , 1.4 numerical aperture, oil immersion objective (Leica Microsystems, Wetzlar, Germany). All images were acquired in the sequential scan mode using an argon laser at 488 nm wavelength (to visualize Alexa Fluor 488), a helium-neon laser at 594 or 633 nm (to visualize Alexa Fluor 594 or DyLight649) or an UV-diode laser at 405 nm (to visualize DAPI). Image stacks were processed and analyzed with the LAS

AF1.7.0 software (Leica Microsystems) and Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

**Pretreatment of Z310 Cells.** To investigate the effect of AEDs on Rfc1 uptake activity, cells were treated with PB (43 or 430  $\mu$ M), CBZ (12.7 or 127  $\mu$ M), or gabapentin (11.7 or 117  $\mu$ M) in supplemented Dulbecco's modified Eagle's medium for 48 h. Selected concentrations of the respective drugs comply with the therapeutic plasma level in humans (Patsalos, 1999; Meyer, 1994) and the decouple of this concentration. Medium was changed every 24 h to maintain stable effective concentrations.

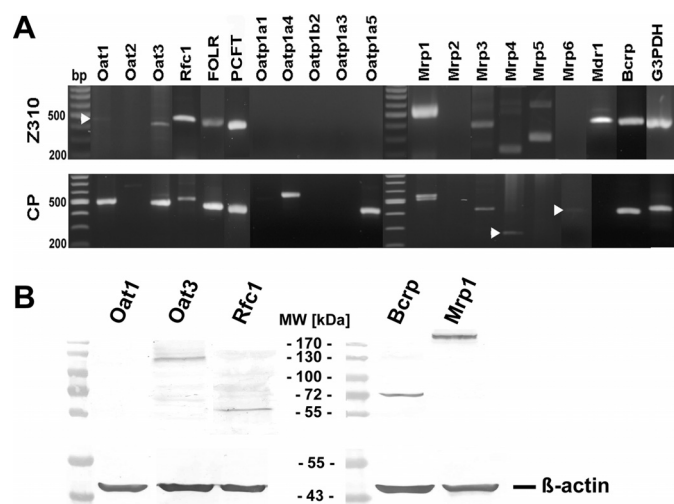
**Uptake Assays.** Measurements of [ $^3$ H]methotrexate ([ $^3$ H]MTX; specific activity,  $6.18 \times 10^8$  kBq/mmol; Moravsek Biochemicals, Brea, CA) uptake in Z310 cells were carried out by a modified method described previously (Halwachs et al., 2007). Intracellular MTX accumulation was generally examined after cells were harvested and allowed to recover in fresh culture medium for 20 min. In all cases, cell viability was  $\geq 92\%$  as assessed by trypan blue exclusion. Z310 cells were washed three times in artificial cerebrospinal fluid (aCSF: 103 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM glucose, and 1 mM sodium pyruvate, pH 7.4) (Breen et al., 2004). For sodium-free aCSF, NaCl was replaced by choline chloride and NaHCO<sub>3</sub> by choline bicarbonate as delineated previously (Breen et al., 2004). Thereafter, [ $^3$ H]MTX (5  $\mu$ M,  $2 \times 10^5$  dpm/ml) was added to 1.2 ml of cell suspension. The aliquots (100  $\mu$ l) were removed at 0.25, 0.75, 1.25, 3, 5, 10, and 20 min for determination of ABC transporter-mediated efflux activity. For investigation of MTX influx, cells were loaded with MTX as performed for the efflux studies, but samples were collected only over a short time period at 0.25, 0.75, 1.20, 1.5, 2, and 2.5 min. To obtain Michaelis-Menten uptake parameters, nonlabeled MTX at a concentration of up to 10  $\mu$ M was added to the cell suspension, and intracellular drug accumulation was measured over 3 min. Then, cells were separated by centrifugation through a silicon oil cushion. The pelleted cells were dissolved in 4 M KOH overnight, and the cell-associated radioactivity was measured by liquid scintillation counting (LS 6500; Beckman Coulter, Fullerton, CA). Protein concentration of the cell suspension was measured using the bicinchoninic protein assay.

**Kinetic and Statistical Analyses.** Statistical analysis of all data were carried out using Excel 2000 software (Microsoft, Redmond, WA).  $K_m$  and  $V_{max}$  values were obtained with Prism 4.0 (GraphPad Software Inc., San Diego, CA) by fitting to the Michaelis-Menten equation using the least-square method and confirmed by Lineweaver-Burk plot. Curve-fittings were performed by means of SigmaPlot 10 (Systat Software Inc., San Jose, CA). Student's  $t$  test was used to determine the significance of differences between respective test series. Statistical significance was assumed at  $p$  values of  $< 0.05$ .

## Results

**Expression of Organic Anion Transporters in Z310 Cells.** To obtain an overview about the constitutive expression of various organic anion transporters in the Z310 cell line, we performed RT-PCR analyses and compared the expression profile to that from rat CP that served as a positive control. As illustrated in Fig. 1A, transcripts for Oat1, Oat3, Rfc1, FOLR, PCFT, Mrp1, Mrp3, Mrp4, Mrp5, Mdr1, and Bcrp were detected in Z310 cells. This expression profile was almost identical with that found for rat CP, with the exception of Oatpla4 and Oatpla5. RNA of the latter was undetectable in the Z310 cell line. On the other hand, mRNA levels of Mdr1 were at the limit of detection in CP tissue, whereas a strong signal for Mdr1 was obtained in Z310 cells. Comparing the apparent level of gene expression for MTX transporter between the RNA sources Oat1, Oat3 and FOLR





**Fig. 1.** A, expression of organic anion transporters in Z310 cells and rat choroid plexus. Total RNA was isolated and used for RT-PCR analysis as described under *Materials and Methods*. Sizes of the expected PCR products and the specific primers that were used are listed in Supplementary Table 1S. Arrowheads indicate weak bands obtained for Oat1 in Z310 cells and Mrp4 and Mrp6 in choroid plexus tissue. RT-PCR of glyceraldehyde 3-phosphate dehydrogenase served as a positive control. B, Western blot analysis of MTX transporters in Z310 cells. Equal protein amounts (50  $\mu$ g) were separated on an SDS-polyacrylamide gel, and selective MTX carrier was detected using specific antibodies as delineated under *Materials and Methods*. Specific protein bands were obtained for Oat3 (130 kDa), Rfc1 (56 kDa), Bcrp (72 kDa), and Mrp1 (190 kDa).  $\beta$ -Actin was used as a loading control. The Western blot shown is representative of at least two independent experiments.

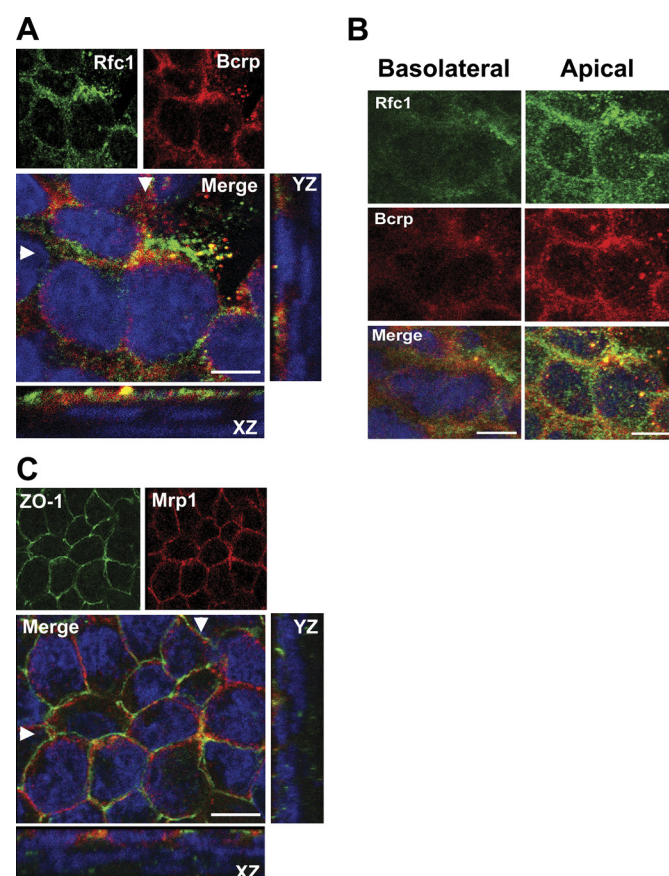
expression can be ranked higher in rat CP tissue than in Z310 cells. On the contrary, higher mRNA levels were demonstrated for Rfc1 and the detected Mrp efflux transporter in the Z310 cell line compared with rat CP.

The influx carriers Rfc1 and Oat3 as well as efflux transporters Mrp1 and Mrp4 have been suggested to be involved in MTX transport across the blood-CSF barrier in human and rodent (Nies, 2007; Spector and Johanson, 2010). Because MTX has been also identified as a substrate for Oat1 and Bcrp, we investigated protein expression of Oat1, Oat3, Rfc1, Mrp1, and Bcrp in Z310 cells. Detection of P-glycoprotein was neglected because MTX has not been identified as a substrate of this efflux carrier (Jansen et al., 2003). As shown in Fig. 1B, Oat3 protein was found at molecular masses of  $\sim$ 130 kDa as described previously (Srimaroeng et al., 2008). Oat1 protein could not be detected in Z310 cells. Because Oat1 was found only to a minor degree at the RNA level the Oat1 protein is probably below detectability, as similarly observed for rat CP tissue (Nies, 2007). Specific anti-Rfc antibody labeled a band for Rfc1 of  $\sim$ 56 kDa (Fig. 1B) corresponding to the nonglycosylated form of the carrier protein as found in rat liver (Honscha et al., 2000). This band was completely blocked by the respective peptide (Hinken et al., 2011), corroborating specificity of antibody labeling (data not shown). In regard to the expression of main MTX efflux carriers at the protein level, specific bands at 72 kDa for Bcrp and 190 kDa for Mrp1 were detected in Z310 cells (Fig. 1B).

**Subcellular Localization of Rfc1, Bcrp, and Mrp1 Protein in Polarized Z310 Monolayers.** Z310 cells were grown on Transwell filter inserts as described above. After 4 days in culture, polarized cell monolayers were obtained with transepithelial electrical resistance values ( $91.00 \pm 5.94 \Omega$

$\text{cm}^2$ ) in the same range as shown previously (Shi et al., 2008). Cell layers were fixed and examined for subcellular distribution of Rfc1, Bcrp, or Mrp1 protein via XY sectioning by confocal microscopy. Formation of monolayer barriers in the Z310 model was additionally assessed by detection of the tight junction protein ZO-1 (Fig. 2C). As shown in Fig. 2, A and B, Rfc1 is predominantly localized at the apical plasma membrane of Z310 cells. Likewise, specific staining for Bcrp was mainly observed at the apical membrane in choroidal epithelial cells (Fig. 2, A and B). Besides, Bcrp was detected by confocal microscopy in XZ views partially between cell nuclei suggesting lateral localization of Bcrp protein (Fig. 2A). In contrast, Mrp1 was predominantly found at the basolateral membrane of Z310 cells (Fig. 2C). Tight junction protein ZO-1 was detected at the lateral plasma membrane (Fig. 2C).

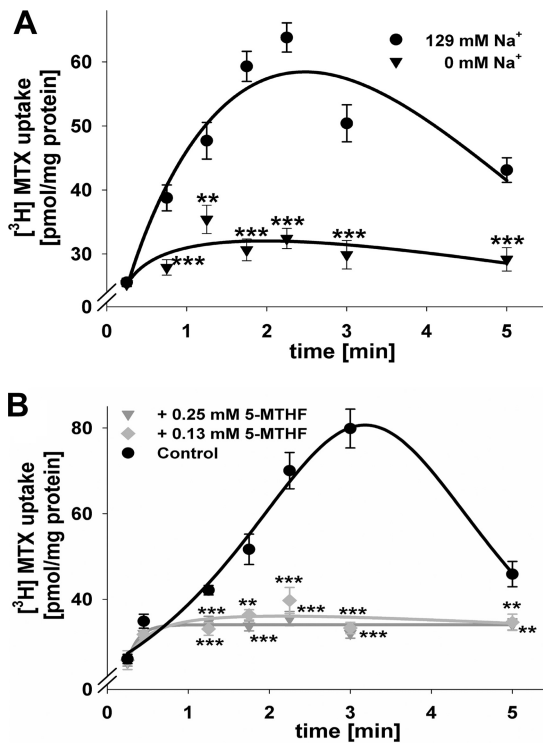
**MTX Transport in Z310 Cells.** To identify carriers involved in transepithelial transport of MTX in rat CP, we measured the impact of various substances known to interact specifically with organic anion transporters on intracellular



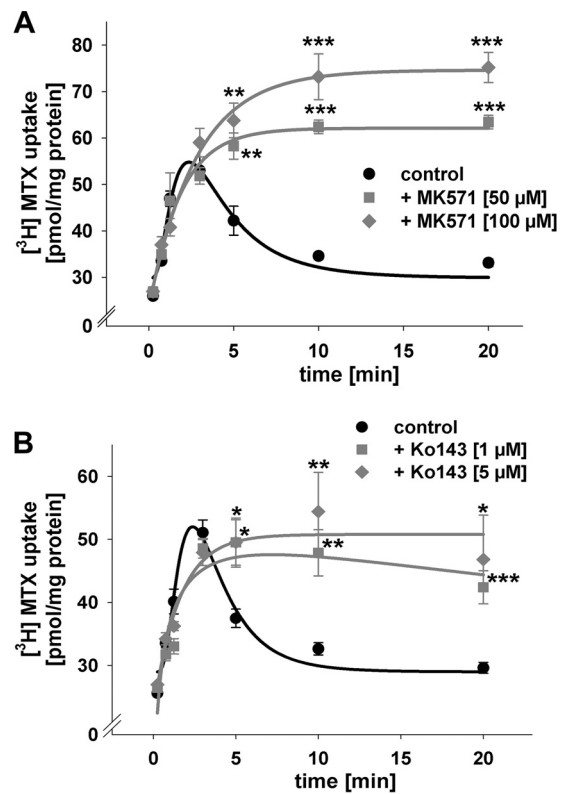
**Fig. 2.** Subcellular localization of Rfc1, BCRP, and Mrp1 and detection of ZO-1 in Z310 cells. Cells were grown on permeable Transwell filter inserts. A and B, polarized Z310 cell monolayers were fixed and stained for Rfc1 (green) and Bcrp (red). C, polarized cells were fixed and stained for Mrp1 (red) and ZO-1 (green). In general, cell nuclei (blue) were visualized using DAPI. A to C, optical XY sectioning was performed by confocal laser scanning microscopy. A and C, white arrowheads indicate positions of the corresponding XZ- or YZ-section. Rfc1 as well as Bcrp were predominantly localized to the apical surface of Z310 cells (A and B). Colocalization of Rfc1 and Bcrp results in yellow/orange. C, Mrp1 was predominantly found at the basolateral membrane and partly colocalize with ZO-1, resulting in yellow/orange staining. Control incubations included omission of primary antibody. Representative cells are shown from two independent experiments. Scale bars, 5  $\mu$ m.

MTX accumulation in Z310 cells. In untreated control cells, MTX uptake rapidly reached a maximum up to 3 min (Fig. 3). This initial uptake interval was generally followed by a rapid decrease in intracellular MTX accumulation (Figs. 3 and 4), with low steady-state levels attained after 10 min corresponding to by on average 21% of the maximum cellular MTX content at the end of the uptake phase (3 min) (Fig. 4).

Several studies indicate that Rfc1 represents the major pathway for the uptake of MTX at neutral pH (Zhao et al., 2008; Biswal and Verma, 2009). Rfc1-mediated but not PCFT- or FORL-dependent MTX influx is strictly sodium-dependent at pH 7.4 (Kneuer and Honscha, 2004; Halwachs et al., 2005). To determine the involvement of Rfc1 in MTX uptake from the CSF into CP, we therefore investigated MTX accumulation in Z310 cells over 5 min in the presence or absence of extracellular sodium. As shown in Fig. 3A, incubation of cells in sodium-depleted aCSF caused a significant reduction in MTX uptake by up to 81% within the initial uptake interval. Rfc1-mediated MTX influx was further confirmed using 5-MTHF. Incubation with both concentrations of the competitive Rfc1 inhibitor (Matherly and Goldman, 2003) almost completely abolished intracellular MTX accumulation within the initial uptake interval (Fig. 3B). MTX uptake was saturable with a  $K_m$  of  $10.1 \pm 3.3 \mu\text{M}$  and a  $V_{\text{max}}$  of  $95.8 \pm 18.3 \text{ pmol/mg protein}$  (Supplemental Fig. 1S). Contribution of Oat1 and Oat3 to MTX uptake into CP was



**Fig. 3.** Rfc1 activity in Z310 cells as measured as the sodium-dependent and MTHF-sensitive uptake of MTX. A, cells were harvested, and the culture medium was replaced by aCSF (129 mM  $\text{Na}^+$ ) or sodium-free choline-aCSF and 5  $\mu\text{M}$  MTX containing  $2 \times 10^5 \text{ dpm } ^3\text{H}$  MTX. B, cells were incubated in aCSF with MTX (5  $\mu\text{M}$ ) in the absence or presence of 0.13 or 0.25 mM MTHF. In general, uptake was stopped at the indicated time points by centrifugation of aliquots through a silicon oil cushion, and the incorporated radioactivity was measured by liquid scintillation counting. Rfc1 activity was defined as the sodium-dependent uptake of MTX (5  $\mu\text{M}$ ) over 5 min and expressed in picomoles per milligram of protein. The data represent the mean  $\pm$  S.E.M. of at least two different measurements with  $n \geq 5$ . \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



**Fig. 4.** Effect of Mrp-inhibitor MK571 or BCRP-inhibitor Ko143 on MTX accumulation in Z310 cells. A, cells were incubated with 5  $\mu\text{M}$   $^3\text{H}$ MTX in the absence or presence of 50 and 100  $\mu\text{M}$  MK571 or 1 and 5  $\mu\text{M}$  Ko143 (B) for 20 min. In general, MTX accumulation in Z310 cells was measured in aCSF at the indicated time points as delineated in the legend for Fig. 3 and expressed in picomoles per milligram of protein. The data represent the mean  $\pm$  S.E.M. of at least two different measurements with  $n \geq 5$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

assessed by incubation of Z310 cells with PAH, a known substrate of Oat1 and Oat3 (Anzai et al., 2006). PAH had no effect on MTX accumulation at 0.5 mM and only inhibited MTX uptake to a minor predominantly not significant extent at a very high concentration (1 mM) (Supplemental Fig. 2S).

The leukotriene LTD<sub>4</sub> receptor antagonist 3-((3-(2-[7-chloro-2-quinolinyl]ethenyl) phenyl)-((3-dimethylamino-3-oxopropyl)-thio-methyl]thio)propanoic acid (MK571) has been shown previously to inhibit MTX transport by the Mrp family members Mrp1, Mrp3, and Mrp4 but not Mrp 5 in various cell lines (Zeng et al., 2001; Chen et al., 2002). To investigate the involvement of these Mrp efflux carriers in MTX transport across the blood-CSF barrier, MTX accumulation was measured over 20 min in the presence or absence of MK571. After the initial uptake phase (3 min), the addition of MK571 caused a dose-dependent inhibition of the decrease in cellular MTX levels compared with untreated control cells (Fig. 4A). Furthermore, MK571-dependent MTX accumulation rapidly attained a plateau phase after 5 min with significantly increased steady-state levels within 10 min compared with the control. Contribution of Bcrp to transepithelial transport of MTX in CP was determined by incubation of cells with the specific Bcrp inhibitor 3-((3S,6S,12aS)-6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydro-pyrazino[1',2':1,6]pyrido[3,4-b]indol-3-yl)-propionic acid *tert*-butyl ester (Ko143) (Allen et al., 2002). The addition of Ko143 caused a significant



increase in steady-state MTX accumulation, achieving a significant augmented plateau level after 5 min in relation to the untreated control (Fig. 4B).

**Effect of AEDs on MTX Uptake.** To investigate the influence of the CAR-inducing AEDs PB and CBZ on Rfc1-mediated MTX uptake from the CSF into CP epithelium, Z310 cells were pretreated with 1- or 10-fold therapeutic plasma concentrations of PB or CBZ for 48 h. Incubation with the AED gabapentin lacking CAR-inducing activity (Tibussek et al., 2006) served as a negative control.

At neutral pH as existing at the blood-CSF barrier, human and rodent Rfc1 mediate uptake of MTX in a sodium-dependent manner (Kneuer and Honscha, 2004; Halwachs et al., 2005). Hence, the intracellular MTX accumulation was initially measured in aCSF containing 129 mM Na<sup>+</sup> as the overall (Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent) MTX uptake or in sodium-free aCSF representing solely the Na<sup>+</sup>-independent MTX accumulation rate. Thereafter, Rfc1 activity was defined indirectly as the difference of [<sup>3</sup>H]MTX uptake over 2.5 min in the presence or absence of extracellular sodium. Finally, sodium- and time-dependent Rfc1 activity was expressed in picomoles per milligram of protein.

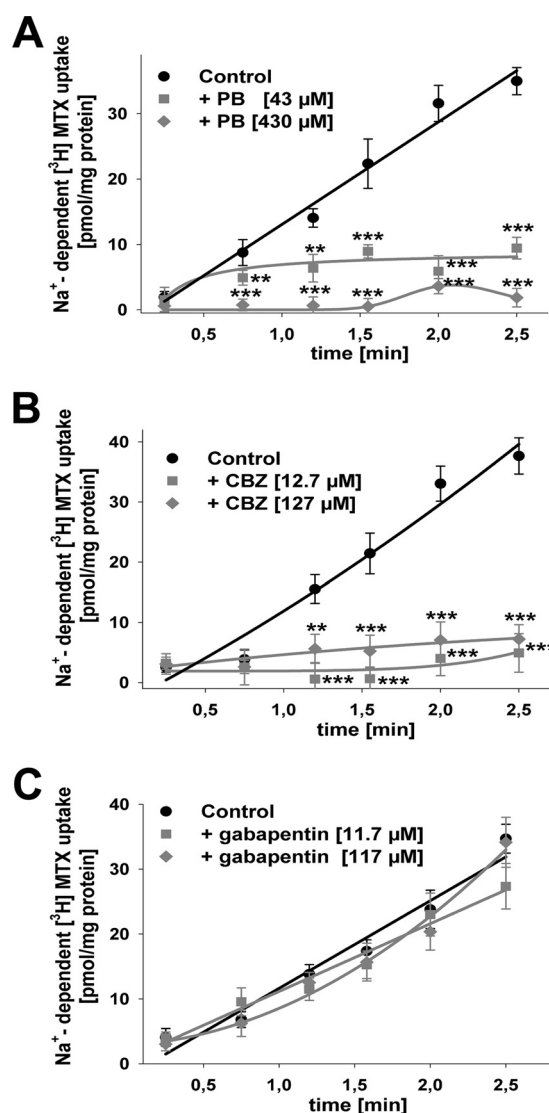
As illustrated in Fig. 5A, pretreatment with therapeutic plasma concentrations (43  $\mu$ M) of PB significantly reduced sodium-dependent Rfc1-mediated MTX uptake (by approximately 85%;  $p < 0.001$ ) within the initial uptake interval (2.5 min) compared with the untreated control. Moreover, incubation of cells with 430  $\mu$ M PB almost completely abolished Rfc1-dependent MTX influx. Similar results were obtained by pretreatment with 12.7 and 127  $\mu$ M CBZ (Fig. 5B). In contrast, incubation of cells with 11.7 or 117  $\mu$ M gabapentin was not related to a significant alteration in Rfc1-mediated MTX uptake (Fig. 5C). The sodium-independent accumulation of the drug was generally not affected significantly (data not shown).

**Impact of PB on Rfc1 Gene Expression.** To determine whether reduction of Rfc1-mediated MTX uptake by CAR-inducing AEDs is due to a decrease in Rfc1 gene expression, Rfc1 transcript levels were measured by quantitative RT-PCR. The amount of Rfc1 mRNA was normalized to the level of  $\beta$ -actin. As shown in Fig. 6A, pretreatment of Z310 cells (48 h) with 1- or 10-fold therapeutic serum levels of prototypical CAR inducer PB was not related to a significant alteration in relative Rfc1 gene expression compared with the untreated control.

**Effect of PB on the Rfc1 Protein Level.** In a further series of experiments on the regulatory mechanism of Rfc1 activity, we measured the effect of PB pretreatment on Rfc1 protein levels in Z310 cells by Western blot. Densitometric analysis of Rfc1 specific bands showed that treatment with 43  $\mu$ M PB or 430  $\mu$ M PB for 48 h resulted in a concentration-dependent significant decline in the Rfc1 protein amount to 33.14 and 18.50%, respectively, of the untreated control level (Fig. 6B).

## Discussion

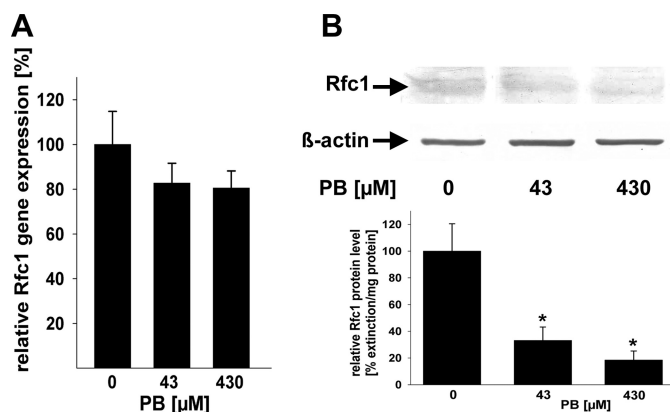
Chemotherapy with intrathecal MTX has been linked to severe neurotoxicity in patients with ALL or lymphoma. It is noteworthy that clinical studies indicated that this may be due to a decrease in the CSF clearance of the drug (Bleyer et al., 1973; Ettinger et al., 1982; Ettinger, 1982). Therefore,



**Fig. 5.** Effect of AEDs on Rfc1-mediated MTX uptake in choroid plexus epithelial cells. Z310 cells were treated with therapeutic and 10 $\times$  therapeutic concentrations of PB (A), CBZ (B), or gabapentin (C) for 48 h. In general, cellular MTX accumulation in the presence or absence of extracellular sodium was measured in aCSF at the indicated time points as delineated in the legend for Fig. 3. Rfc1 activity was defined as the sodium-dependent uptake of MTX (5  $\mu$ M) over 2.5 min and expressed in picomoles per milligram of protein. The data represent the mean  $\pm$  S.E.M. of at least two different measurements in which quadruplicates were obtained. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

patients who received intrathecal MTX suffering from neurotoxicity showed by on average 13.8-fold elevated CSF drug levels compared with patients without toxicity (Bleyer et al., 1973). MTX is known to be actively secreted from CSF into blood but the carrier proteins involved remain undefined (Spector and Johanson, 2010).

In this study, we show that the mRNA expression pattern of organic anion transporters in rat choroidal epithelial Z310 cells was almost identical with that of rat choroid plexus tissue. Endogenous transporter expression included the known MTX import carriers Oat1, Oat3, Rfc1, and PCFT (Slc46a1) and the folate-binding protein FORL (Matherly and Goldman, 2003; Nies 2007; Inoue et al., 2008). Compared with rat CP, lower expression of Oat1 and Oat3 in Z310 cells must be discussed in the context of gender and hormone-



**Fig. 6.** Effect of PB on Rfc1 expression. **A**, Z310 cells were pretreated with 43 or 430  $\mu$ M PB for 48 h. Rfc1 transcript levels were analyzed by quantitative RT-PCR using 1  $\mu$ g of total RNA and rat gene specific Rfc1 primers and probes as described under *Materials and Methods*. Relative Rfc1 mRNA levels were expressed as the percentage of the Rfc1 gene expression comparatively to the control level.  $\beta$ -Actin was used as an internal standard. The data represent the mean  $\pm$  S.E.M. of three different measurements in which quadruplicates were obtained. **B**, Z310 cells were incubated with 43 or 430  $\mu$ M PB for 48 h, and Rfc1 protein levels were assessed by Western blot. Equal protein amounts (50  $\mu$ g) were separated on a SDS/10% polyacrylamide gel and specific protein bands of  $\sim$ 56 kDa for Rfc1 ( $\rightarrow$ ) were obtained using specific anti-SLC19A1 antibody.  $\beta$ -Actin was used as a loading control and is apparent at  $\sim$ 45 kDa ( $\rightarrow$ ). The Western blot shown is representative of two independent experiments. Densitometric analysis of Rfc1 immunoreactivity in consideration of the background value is shown. Results are expressed as means  $\pm$  S.D. for duplicate determinations. \*,  $p < 0.05$ .

dependence because murine Oat1 expression was higher in male than in female kidney and rat Oat3 was only detected in male liver (Anzai et al., 2006). Because the culture of Z310 cells generated from both sexes of Sprague-Dawley rats was carried out without supplementation of male sex hormones, it is likely that expression of both transporters could be inducible by testosterone. However, high concentrations of the high-affinity Oat1 and Oat3 substrate PAH (Anzai et al., 2006) had only a minor mainly nonsignificant effect on MTX uptake. In line with previous data on the effect of PAH on MTX uptake in rat CP tissue (Breen et al., 2004), our results therefore indicate that Oat transporters do not play a significant role in MTX removal from the CSF.

Because MTX uptake was almost completely abolished in the presence of 5-MTHF and displayed to be strictly sodium-dependent, our results rather suggest that Rfc1 provides the main route for MTX uptake in Z310 cells. This is in line with previous data showing that MTX uptake in rat CP tissue was specific, concentrative, and sodium-dependent (Breen et al., 2004). Our data are further corroborated by the apical membrane localization of Rfc1 in Z310 cells supporting recent findings of subcellular distribution of Rfc1 in rat CP tissue (Hinken et al., 2011). Several publications clearly demonstrated that at neutral pH PCFT-mediated MTX transport in various cell lines is negligible (Inoue et al., 2008; Zhao et al., 2008). These data are confirmed by results derived in liver-like Huh7 cells transfected with Rfc siRNA showing that MTX transport at neutral pH is mediated largely by Rfc1 (Biswal and Verma, 2009). The FOLR is localized to the basolateral CP membrane and, in contrast to 5-MTHF, exhibits a relatively low affinity for MTX (Spector and Johanson, 2010). Thus, significant involvement of PCFT or FOLR in removal of MTX from CSF is not likely.

In line with recent data on endogenous expression of Mrp efflux transporters in isolated rat CP (Choudhuri et al., 2003), various Mrp family members were identified with predominant expression of Mrp1 in CP tissue and in Z310 cells. Our results obtained with specific Mrp inhibitor MK571 (Zeng et al., 2001; Chen et al., 2002) suggested the involvement of Mrp1, Mrp3, and Mrp4 in elimination of MTX from Z310 cells. This suggestion is corroborated by expression of Mrp1 protein at the basolateral membrane of Z310 cells. Likewise, Mrp4 also expressed in Z310 cells (Klås et al., 2010) has been localized to the basolateral CP membrane (Nies, 2007). Moreover, functional activity of both transporters has been shown recently in Z310 cells using specific subfamily member substrates (Klås et al., 2010). No information is yet available on the functional expression and subcellular distribution of Mrp3 protein. Because Mrp3 mRNA was detected in rat CP tissue and Z310 cells, contribution of Mrp3 to elimination of MTX cannot be excluded. However, with regard to the predominant expression and the strong effect of the potent Mrp1-inhibitor MK571 (Leier et al., 1996) on MTX accumulation, we suggest that Mrp1 is the prevailing Mrp family member responsible for MTX efflux in Z310 cells.

To our knowledge, this is the first report illustrating subcellular localization of Bcrp at the apical membrane of choroidal epithelial cells. Bcrp-mediated efflux of MTX in these cells was confirmed using the specific Bcrp inhibitor Ko143 (Allen et al., 2002). Because Bcrp-mediated efflux of intracellular synthesized oligoglutamate MTX derivatives has been shown previously in several tumor cell lines (Assaraf, 2006), our results suggested that apically expressed Bcrp may facilitate MTX accumulation in CSF. Although our data argue for Mrp1 as the primary active MTX efflux transporter in choroid plexus, intracellular drug accumulation by Mrp1 inhibitor MK571 was twice the level of that observed with Bcrp inhibitor Ko143. However, specific inhibitors of Bcrp activity may be useful tools to reduce neurotoxicity of intrathecally administered MTX by facilitating Mrp1-mediated drug removal from CSF into blood.

In patients with ALL or lymphoma, intrathecal MTX-induced neurotoxicity usually manifest as seizure requiring treatment with AEDs. It is noteworthy that we have shown that clinically relevant concentrations of PB or CBZ cause significant down-regulation of hepatocellular Rfc1 uptake activity through activation of the CAR signaling pathway (Halwachs et al., 2007, 2009). The nuclear receptor CAR is known to mediate regulation of carrier associated drug transport by PB or CBZ through modulation of target gene expression, including MTX efflux transporter Mrp2 (Xu et al., 2005). In fact, our results displayed that Rfc1-mediated MTX uptake in CP epithelial cells is significantly decreased by therapeutic serum concentrations of PB or CBZ. However, our data indicate that this reduction was not due to altered carrier gene expression. Likewise, long-term treatment of human ovarian carcinoma cells with CBZ significantly reduced Rfc-mediated MTX uptake but was not associated with decreased Rfc transcript levels (Toffoli et al., 2000). In addition to regulation of gene expression, PB-induced regulation of protein levels is known to affect membrane transporters such as Mrp2 (Johnson et al., 2002). Indeed, pretreatment of Z310 cells with PB resulted in a significant reduction in relative Rfc1 protein levels. Therefore, our results suggest

that regulation of Rfc1 uptake activity by CAR-inducing AEDs does not occur via CAR-dependent transcriptional regulation but by inhibition of carrier translation on the post-transcriptional level. This lack of response on the transcriptional level may be explained by absence of the CAR response element phenobarbital response enhancer module involved in PB-dependent induction of CYP450 enzymes (Xu et al., 2005) from the rfc1 gene 5'-flanking region (Honscha et al., 2000).

Altogether, this study provides data on the functional expression of MTX import and export carriers in choroidal epithelial cells. Our results further indicate that removal of intrathecally applied MTX across the blood-CSF barrier may be achieved through a two-step mechanism of transepithelial transport that includes Rfc1-mediated uptake from the CSF at the apical CP membrane followed by MTX extrusion into blood particularly via Mrp1 at the basolateral membrane. In this study, this two-step transport mechanism was illustrated by a bell-shaped transport curve reflecting a short uptake phase with intracellular MTX accumulation succeeded by avid drug efflux, resulting in a rapid decrease in cellular MTX levels. A similar curve shape has been reported for MTX accumulation in rat CP tissue (Breen et al., 2004). Because human RFC1 and rat Rfc1 have been shown to be orthologs (Kneuer and Honscha, 2004), a broader relevance of our results to other systems including human cells can be assumed. In consideration of the known dose-dependence of MTX toxicity (Shuper et al., 2002), one therefore may suggest that PB or CBZ-induced down-regulation of Rfc1 may induce intrathecal MTX neurotoxicity in patients with cancer-related seizures or potentiate drug toxicity in patients with MTX-induced seizures by a decrease in CSF clearance of the drug. Because we show that gabapentin did not lead to significant changes in Rfc1-mediated MTX uptake, new non-CAR-inducing AEDs such as gabapentin (Tibussek et al., 2006) should therefore be preferred for seizure control. Finally, consideration of drug interactions by regulation of carrier-mediated transport may help to improve antiepileptic treatment in children with ALL receiving intrathecal MTX chemotherapy.

#### Acknowledgments

We thank Wei Zheng (Purdue University, West Lafayette, IN) for donating the Z310 cell line. We also thank G. Fricker (Ruprecht-Karls-University, Heidelberg, Germany) for generously providing the anti-Mrp1 antibody and B.C. Burckhardt (Georg-August University of Göttingen, Göttingen, Germany) for kindly providing the anti-Oat1 antibody.

#### Authorship Contributions

Participated in research design: Halwachs and Honscha.

Conducted experiments: Halwachs and Lakoma.

Contributed new reagents or analytic tools: Schäfer and Seibel.

Performed data analysis: Halwachs.

Wrote or contributed to the writing of the manuscript: Halwachs and Honscha.

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