The Antiepileptic Drugs Phenobarbital and Carbamazepine Reduce Transport of Methotrexate in Rat Choroid Plexus by Down-Regulation of the Reduced Folate Carrier

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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 associated 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-

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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 xeno-biotics, 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 carriers, 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., 2003) and members of the organic anion transporter (Oat) (Anzai et al., 2006), as well as of ABC export carriers, 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).

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 μg/ml streptomycin, and 40 μg/ml gentamicin (all from PAA Laboratories). Polarized cell monolayers were obtained by culture on Polyester Transwell-Clear inserts (1.0 cm² growth area, 0.4 μm pore size; Corning Life Sciences, Wiesbaden, Germany) coated with 0.01% rat-tail collagen overnight. Z310 cells (2 x 10⁵) were suspended in 1 ml of complete growth medium supplemented with 1 μM 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 CO₂, 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 x 10⁵ Z310 cells using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Total RNA (1 μg) 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 μg oligo(dT)₁₅ 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 (DNASTar 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 x 10⁵ 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 μg) as delineated above. Quantitative PCR of 1 μl 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 (β-actin) control level was calculated by the 2−ΔΔCT 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 deoxycholate, 2 mM sodium vanadate, and 1× protease inhibitor cocktail) and incubated for 60 min on ice. Nuclei and cell debris were pelleted at 10,000 g 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 1.0% bromophenol blue, pH 6.8] and the protein concentration was calculated by 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 1.0% bromophenol blue, pH 6.8] and were denatured at 37°C for 30 min. According to protein size, equal protein amounts were separated on SDS/7.5% polyacrylamide gels for Oat3, Mrp1, and Bcrp or 10% SDS gels for Oat1, Rfc1, and β-actin. Thereafter, proteins were blotted onto nitrocellulose membrane. Unspecific binding was blocked with 5% milk powder in Tris-buffered saline-Tween 20 (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 the presence of PB in consideration of the background level was accepted. As secondary antibodies, AP-conjugated goat anti-rat IgG (1:7000; Promega) for Bcrp and β-actin, AP-conjugated goat anti-rat IgG (1:5000; 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-ABC2G2 MAb (1:100; Santa Cruz Biotechnology), or anti-β-actin MAb (1:10,000; clone AC-15). As secondary antibodies, AP-conjugated goat anti-mouse IgG (1:7000; Promega) for Bcrp and β-actin, AP-conjugated goat anti-rabbit 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 accepted 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), MRPr1 (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-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for Mrp1 and ZO-1. Cell samples were an- counter with 0.13 μm thickness.

**Confocal Laser Scanning Microscopy.** Cell samples were an- counter with 0.13 μm thickness.

**Confocal Laser Scanning Microscopy.** Cell samples were arranged using the inverted confocal laser scanning microscope Leica TCS SP5 equipped with a HCX plan apochromat 63×, 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 μM), CBZ (12.7 or 127 μM), or gabapentin (11.7 or 117 μM) 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 (Patalos, 1999; Meyer, 1994) and the decouple of this concentration. Medium was changed every 24 h to maintain stable effective concentrations.

**Uptake Assays.** Measurements of [3H]methotruxetate (3HMTX) specific activity, 6.18 × 106 Bq/mmol; Moravek 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 ≥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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 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 NaHCO3 by choline bicarbonate as delineated previously (Breen et al., 2004). Thereafter, 3HMTX (5 μM, 2 × 106 dpm/ml) was added to 1.2 ml of cell suspension. The aliquots (100 μl) 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 μM 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 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\textsubscript{m} and V\textsubscript{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, FOLR1, 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 Oatp1a4 and Oatp1a5. 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 FOLR1
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 Mrp1, and Bcrp in Z310 cells. Detection of P-glycoprotein was neglected because MTX has not been identified as a substrate for these efflux carriers (Jansen et al., 2003). As shown in Fig. 1B, Oat3 protein was found at molecular masses of ~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 ~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). 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

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**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 glycerolaldehyde 3-phosphate dehydrogenase served as a positive control. B, Western blot analysis of MTX transporters in Z310 cells. Equal protein amounts (50 μg) 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 (150 kDa), Rfc1 (56 kDa), Bcrp (72 kDa), and Mrp1 (190 kDa). β-Actin was used as a loading control. The Western blot shown is representative of at least two independent experiments.

**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 μm.
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 ± 3.3 µM and a \( V_{max} \) of 95.8 ± 18.3 pmol/mg protein (Supplemental Fig. 1S). Contribution of Oat1 and Oat3 to MTX uptake into CP was 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).

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 Na\(^+\)) or sodium-free choline-aCSF and 5 µM MTX containing 2 × 10\(^5\) dpm [\(^3\)H]MTX. B, cells were incubated in aCSF with MTX (5 µM) 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.

The leukotriene LTD\(_4\) receptor antagonist 3-((3-(2-(7-chloro-2-quinolinyl)ethenyl)-(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 Mrp5 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

**Fig. 4. Effect of Mrp-inhibitor MK571 or BCRP-inhibitor Ko143 on MTX accumulation in Z310 cells.** A, cells were incubated with 5 µM [\(^3\)H]MTX in the absence or presence of 50 and 100 µM MK571 or 1 and 5 µM 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 ± S.E.M. of at least two different measurements with \( n \geq 5 \), **, \( p < 0.01 \); ***, \( p < 0.001 \).
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⁺ as the overall (Na⁺-dependent and Na⁺-independent) MTX uptake or in sodium-free aCSF representing solely the Na⁺-independent MTX accumulation rate. Thereafter, Rfc1 activity was defined indirectly as the difference of [³H]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 μM) 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 μM PB almost completely abolished Rfc1-dependent MTX influx. Similar results were obtained by pretreatment with 12.7 and 127 μM CBZ (Fig. 5B). In contrast, incubation of cells with 11.7 or 117 μM 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 β-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 μM PB or 430 μM 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, 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-
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 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 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 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 sodium-dependent, our results rather suggest that Rfc1 provides the main route for MTX uptake in Z310 cells. 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This is in line with previous data showing that MTX uptake in rat CP tissue was sodium-dependent, our results rather suggest that Rfc1 provides the main route for MTX uptake in Z310 cells.
that regulation of Rfc1 uptake activity by CAR-inducing AEDs does not occur via CAR-dependent transcriptional regulation but by inhibition of carrier transport 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 export and import 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.

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Authorship Contributions

Participated in research design: Halwachs and Honscha.
Conducted experiments: Halwachs and Lakoma.
Contributed new reagents or analytic tools: Schafer and Seibel.
Performed data analysis: Halwachs.
Wrote or contributed to the writing of the manuscript: Halwachs and Honscha.

References


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The antiepileptic drugs Phenobarbital and Carbamazepine reduce transport of methotrexate across the rat choroid plexus by downregulation of the Reduced folate carrier

Sandra Halwachs, Cathleen Lakoma, Ingo Schäfer, Peter Seibel, and Walther Honscha

SUPPLEMENTARY FIGURE 1

Figure 2S. Concentration-dependent uptake of MTX in Z310 cells. Uptake measurement was initiated by adding $2 \times 10^5$ dpm [³H] MTX and the indicated amounts of nonlabelled MTX to Z310 cells in aCSF. After 3 min, uptake was stopped by centrifugation of aliquots through a silicon oil cushion and the incorporated radioactivity was measured by liquid scintillation counting. Insert: Corresponding Lineweaver-Burk plot. Values are mean ± SEM (n = 4).
SUPPLEMENTARY FIGURE 2

**Figure 3S.** Effect of PAH on MTX influx in Z310 choroid plexus cells. Cells were harvested and the culture medium was replaced by aCSF. Then, cells were incubated with 5 µM MTX containing $2 \times 10^5$ dpm $[^3$H] MTX in the absence or presence of 0.5 mM or 1mM PAH. Uptake of MTX 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. The data represent the mean ± SEM of three different measurements in which quadruplicates were obtained. **$p < 0.01$.**
SUPPLEMENTARY TABLE 1

Primer pairs for RT-PCR analysis and size of the expected PCR products.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Primer pairs (5′−3′)</th>
<th>Size (bp)</th>
<th>Tm (°C)</th>
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<tr>
<td>rOat1</td>
<td>ACAAGCAAGGACAAACCCGAA AGACATAGCACAATTCAAGGCTGCC</td>
<td>479</td>
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<td>Kudo et al. (2002)</td>
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<tr>
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<td>476</td>
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<td>rFOLR</td>
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<td>GAPDH</td>
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<td>441</td>
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</table>


