Pharm Res (2014) 31:1588–1604
DOI 10.1007/s11095-013-1264-4

RESEARCH PAPER

Marked Differences in the Effect of Antiepileptic and Cytostatic Drugs on the Functionality of P-Glycoprotein in Human and Rat Brain Capillary Endothelial Cell Lines

Dana Alms • Maren Fedrowitz • Kerstin Römermann • Andreas Noack • Wolfgang Löscher

Received: 4 June 2013 / Accepted: 9 December 2013 / Published online: 30 January 2014

ABSTRACT

Purpose The expression of P-glycoprotein (Pgp) is increased in brain capillary endothelial cells (BCECs) of patients with pharmacoresistant epilepsy. This may restrict the penetration of antiepileptic drugs (AEDs) into the brain. However, the mechanisms underlying increased Pgp expression in epilepsy patients are not known. One possibility is that AEDs induce the expression and functionality of Pgp in BCECs. Several older AEDs that induce human cytochrome P450 enzymes also induce Pgp in hepatocytes and enterocytes, but whether this extends to Pgp at the human BBB and to newer AEDs is not known.

Methods This prompted us to study the effects of various old and new AEDs on Pgp functionality in the human BCEC line, hCMEC/D3, using the rhodamine 123 (Rho123) efflux assay. For comparison, experiments were performed in two rat BCEC lines, RBE4 and GPNT, and primary cultures of rat and pig BCECs. Furthermore, known Pgp inducers, such as dexamethasone and several cytostatic drugs, were included in our experiments.

Results Under control conditions, GPNT cells exhibited the highest and RBE4 the lowest Pgp expression and Rho123 efflux, while intermediate values were determined in hCMEC/D3. Known Pgp inducers increased Rho123 efflux in all cell lines, but marked inter-cell line differences in effect size were observed. Of the various AEDs examined, only carbamazepine (100 μM) moderately increased Pgp functionality in hCMEC/D3, while valproate (300 μM) inhibited Pgp.

Conclusions These data do not indicate that treatment with AEDs causes a clinically relevant induction in Pgp functionality in BCECs that form the BBB.

D. Alms • M. Fedrowitz • K. Römermann • A. Noack • W. Löscher (✉)
Department of Pharmacology, Toxicology, and Pharmacy
University of Veterinary Medicine Hannover, Bünteweg 17
30559 Hannover, Germany
e-mail: wolfgang.loescher@tiho-hannover.de

D. Alms • W. Löscher
Center for Systems Neuroscience, Hannover, Germany

KEY WORDS blood–brain barrier · dexamethasone · epilepsy · nuclear receptors · pharmacoresistance

ABBREVIATIONS

AEDs	antiepileptic drugs
ANOVA	analysis of variance
BBB	blood–brain barrier
BCEC	brain capillary endothelial cell
CAR	constitutive androstane receptor
DMSO	dimethylsulfoxide
pBCEC	porcine brain capillary endothelial cell
PBS	phosphate-buffered saline
Pgp	P-glycoprotein
PXR	pregnane X receptor
rBCEC	rat brain capillary endothelial cell
Rho123	rhodamine 123
RT	room temperature

INTRODUCTION

The efflux transporter P-glycoprotein (Pgp; ABCB1) plays an important neuroprotective role at the blood–brain barrier (BBB) by extruding a variety of structurally unrelated potentially toxic compounds and preventing their accumulation within the brain (1–3). Unfortunately, Pgp impedes also the entry of various drugs that are used in the treatment of brain diseases, including anticancer drugs, antiepileptic drugs (AEDs), and HIV protease inhibitors (4). A major concern regarding the chronic administration of such drugs is the potential for induction of Pgp expression at the BBB, which may further decrease their clinical efficacy and cause drug-drug interactions (4,5). However, only relatively few studies have evaluated whether drugs used for treatment of brain diseases induce Pgp expression and functionality in brain capillary endothelial cells (BCECs) that form the BBB, and

respective data are conflicting. For instance, Lombardo *et al.* (6) reported that a 3-day exposure to various AEDs (phenobarbital, phenytoin, carbamazepine, topiramate, tiagabine and levetiracetam) induces the expression of Pgp in a rat BCEC line, GPNT. In apparent contrast, we did not determine any effect of major AEDs (phenobarbital, phenytoin and carbamazepine) on expression or functionality of Pgp in GPNT cells (7).

The known Pgp-inducing effect of older enzyme-inducing AEDs, such as phenobarbital, phenytoin, and carbamazepine, in hepatocytes and enterocytes is mediated via activation of nuclear receptors, including pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3) (8–12). However, the expression of such nuclear receptors varies widely among tissues and cell types, so that Pgp induction is tissue or cell specific (9,13). PXR expression is very low and CAR expression is below detection threshold in GPNT cells, which could explain the lack of AEDs to induce Pgp in these cells in our recent study (7). Furthermore, drug affinities for PXR and CAR vary considerably across species (14,15), so that data from rodent cell lines cannot simply be extrapolated to humans.

The purpose of this study was to compare the effects of prolonged exposure with therapeutically relevant concentrations of older and newer AEDs (phenobarbital, phenytoin, carbamazepine, topiramate, levetiracetam, valproate) on Pgp functionality in rat and human BCEC lines. Three cell lines, RBE4 (16), GPNT (17), and hCMEC/D3 (18), were used for this purpose. These cell lines markedly differ in expression of Pgp and nuclear receptors (7,19,20), and are widely used as *in vitro* models of the BBB. Our hypothesis was that RBE4 and hCMEC/D3 cells would be more susceptible to Pgp induction than GPNT cells, which, to our knowledge, has not been examined before. However, compared to our previous study (7), culturing of GPNT cells was modified to enhance their susceptibility to Pgp induction (see Methods). In addition to AEDs, we included several cytostatic drugs (doxorubicin, puromycin, vincristine, and the hPXR agonist rifampicin) with known Pgp-inducing activity in our study, because only few reports on the effects of such drugs on Pgp in BCECs are available and data are, at least in part, conflicting (7,17,20–24). Furthermore, the glucocorticoid dexamethasone, which has been shown to induce Pgp in various cell lines, including BCECs (7,9,17,25), and the Pgp inhibitor tariquidar (26) were included as reference standards, since, depending on drug concentration and cell preparation, AEDs may increase or decrease the function of Pgp (27). In addition to immortalized rat and human cell lines, some experiments were performed in primary cultures of rat and porcine brain endothelial cells (rBCECs, pBCECs). To our knowledge, this is the first study that compares the effect of various drugs on Pgp functionality in BCECs from three species, demonstrating marked quantitative differences in drug effects between the cell lines.

MATERIALS AND METHODS

BCEC Lines

The RBE4 cell line, a well established *in vitro* model of the BBB (19), was kindly provided by Prof. Françoise Roux (INSERM U26, Hôpital Fernand Widal, Paris, France). The RBE4 cell line has been obtained after transfection of a primary rat brain endothelial cell culture with the plasmid pE1A-Adenovirus encoding gene (16). RBE4 cells were cultured on collagen type I coated 100-mm tissue culture plates (Sarstedt, Nuembrecht, Germany) and maintained (according to a protocol provided by F. Roux) in alpha-MEM/Ham's F-10 (1:1 vol/vol; Gibco®/Life Technologies, Darmstadt, Germany) medium supplemented with 10% fetal calf serum (Linaris GmbH; Wertheim-Bettingen, Germany), 100 U/ml penicillin (Biochrom AG, Berlin, Germany), 100 µg/ml streptomycin (Biochrom AG), 1% L-glutamine (200 mM; Sigma-Aldrich, Taufkirchen, Germany), and 1 ng/ml basic fibroblast growth factor (bFGF; Life Technologies) in humidified 5% CO_2/95% air at 37°C. Cells were used for experiments from passage 61 to 71.

The GPNT cell line was kindly provided by Prof. Françoise Roux. The GPNT cell line has been derived from the rat brain endothelial cell line, GP8, by transfection with a selection plasmid containing the puromycin resistance gene (17). This transfection resulted in enhanced stability of the morphological phenotype of the immortalized cells and a marked increase in the expression of Pgp (17). GPNT cells were cultured on collagen type I coated 100 mm Petri-dishes and maintained (according to a protocol provided by F. Roux) in alpha-MEM/Ham's F-10 (1:1 vol/vol) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 ng/ml basic b-FGF and 5 mg/ml puromycin (only first two passages, then without puromycin) in humidified 5% CO_2/95% air at 37°C. For the drug uptake assays, GPNT cells were seeded on collagen-coated 6-well plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 1×10^4 cells/cm^2 and were grown until 100% confluence. Passages 30 to 59 were used for the uptake assays.

The initial exposure to puromycin in the GPNT cultures was reduced compared to our previous study (7) to avoid maximum induction of Pgp by puromycin already before the experiments with other known Pgp inducers and AEDs. In preliminary experiments, we compared Pgp expression by Western blot in GPNT cells that were exposed to puromycin over the first two *vs.* first four passages, showing that the longer puromycin exposure resulted in a more than twofold increase

in Pgp expression compared to the reduced exposure used for the present experiments. For uptake experiments the cells were grown for at least one passage without puromycin.

Human cerebral microvascular endothelial cells (hCMEC/D3) were kindly provided by Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France). The cell line was derived by lentiviral transfection of BCECs isolated from brain tissue obtained following surgical excision of an area from the temporal lobe of an adult patient with epilepsy (18). hCMEC/D3 cells were cultivated (according to a protocol provided by P.-O. Couraud) in endothelial cell basal medium-2 (EBM®-2, Lonza, Cologne, Germany) supplemented with 5% fetal calf serum (Linaris GmbH; Wertheim-Bettingen, Germany), 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG), 1.4 µM hydrocortisone (Sigma-Aldrich, Munich, Germany), 5 µg/ml ascorbic acid (Sigma-Aldrich), 1% lipid concentrate (Gibco®/Life Technologies), 10 mM HEPES (Gibco®/Life Technologies) and 1 ng/ml basic FGF (Life Technologies). Cells were passaged every 3–4 days on collagen type I (100 µg/ml) (Life Technologies) coated 100-mm tissue culture plates (Sarstedt) at 37°C and 5% CO_2 from passage 30 to 39. In order to determine whether the presence of hydrocortisone in the culture medium affected drug effects, an additional experiment was performed without hydrocortisone in the medium (see Results).

Primary Culture of Rat Brain Endothelial Cells (rBCECs)

Rat brain capillary endothelial cells (rBCEC) were prepared essentially as described previously (17,28) from 2 to 3 weeks-old Sprague–Dawley and Wistar rats (obtained from Charles River; Sulzfeld, Germany). For establishing the rBCEC preparation, two rat strains were used to examine possible strain differences. In short, cerebral cortices were dissected free from meninges in phosphate-buffered saline (PBS) with 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG) on ice and were mechanically dissociated with scalpels. The homogenate was suspended in enzyme solution: DMEM/F-12 (Gibco®/Life Technologies) supplemented with 1 mg/ml dispase II (Roche), 0,1 mg/ml DNAse I (Roche), 270 U/ml collagenase II (Biochrom AG), and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG) and was incubated for 1.5 h at 37°C with gentle agitation. The cell pellet was separated by centrifugation in 20% bovine serum albumin (Linaris GmbH) solution (1,000 g, 15 min, 4°C) and incubated again in the enzyme solution for 1 h at 37°C. The homogenate was filtered and the retained capillary fragments were removed from the filter with EBM-2 (Lonza) medium supplemented with 20% fetal calf serum, 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom), 1 mM HEPES (Gibco®/Life Technologies), 1% L-glutamine (200 mM, Sigma-Aldrich), and 0.5 µg/ml hydrocortisone (Sigma-Aldrich). The cells were seeded on collagen type IV (Sigma-Aldrich) coated 28 cm^2 plates and cultivated for 72 h in presence of 4 µg/ml puromycin (Sigma-Aldrich) for removal of pericytes (28) in humidified 5% CO_2/95% air at 37°C. Thereafter, puromycin was removed and replaced by 2 ng/ml basic FGF (Gibco®/Life Technologies). For uptake assays rBCEC cells from passages 1 and 2 were seeded on collagen IV coated 6-well plates. BCECs from Sprague–Dawley rats grow better and attached better to the plates than BCECs from Wistar rats, but this did not affect the uptake assays (see Results), so that cells from both rat strains were used for final analyses of data.

Primary Culture of Porcine Brain Endothelial Cells (pBCECs)

Porcine brain endothelial cells (pBCECs) were prepared as described by Patabendige *et al.* (29,30) with slight modifications. In brief, porcine brain hemispheres obtained from local abattoir were washed in PBS (Gibco®/Life Technologies). After removal of meninges and white matter, cortices were dissociated with scalpels. Brain extract was homogenized by using an overhead stirrer (Heidolph Instruments, Schwabach, Germany). Thereafter the homogenate was filtered through a 150 µm nylon mesh (SFT GmbH, Grafschaft-Gelsdorf, Germany), followed by a filtration through a 71 µm nylon mesh (SFT GmbH). Only capillaries collected on 71 µm meshes were used for functional analysis of Pgp and digested with gentle shaking at 37°C, 5% CO^2 and 95% humidity for 1 h in digest mix containing Medium199 (Gibco®/Life Technologies), 210 U/ml collagenase (Gibco®/Life Technologies), 90 U/ml trypsin (Sigma-Aldrich), 0,1 mg/ml DNase I (Roche), 20% fetal calf serum (Linaris GmbH), and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG). The capillary fragments were removed from the filters and washed three times in MEM/HEPES (Gibco®/Life Technologies) containing 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG) and 10% fetal calf serum (Linaris GmbH) by resuspendation and centrifugation (240 g, 5 min, 4°C). Thereafter the cells were seeded on collagen type I (Life Technologies)/fibronectin (Biochrom AG) coated 75 cm^2 flasks (Greiner Bio-One) in DMEM (Gibco®/Life Technologies) supplemented with 10% plasma derived bovine serum (First Link Ltd., Birmingham, United Kingdom), 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biochrom AG), 1% hour-glutamine (200 mM, Sigma-Aldrich), and 125 µg/ml heparin (Sigma-Aldrich). For removal of pericytes (30) cells were cultivated with 4 µg/ml puromycin (Sigma-Aldrich) during 72 h in humified 5% CO^2/95% air at 37°C. For uptake experiments pBCECs (passage 1) were seeded on collagen type I (Life Technologies)/fibronectin (Biochrom AG) coated 12-

well plates (Greiner Bio-One) with a density of 2×10^5 cells/cm^2.

Drug Treatment

All cell lines reached confluence after 2–3 days. In several preliminary experiments, drug exposure was started at different times (1–6 days) after cells had reached confluence, showing that starting drug exposure 3 days after reaching confluence resulted in the most consistent drug effects. Based on these data, all experiments shown in this study were performed as follows. Three days after reaching confluence, cells were washed with phosphate-buffered saline (PBS) and incubated for 3 days with or without known Pgp inducers or AEDs in different concentrations which were added to the respective cell medium. In some experiments, exposure was increased to 6 days. Shorter periods of drug exposure were not examined, because in patients, both AEDs and cytostatic drugs are typically administered over prolonged periods of time. The choice of drug concentrations was based on therapeutic plasma concentrations of AEDs in epilepsy patients (Table I) and previous Pgp studies with AEDs, dexamethasone and cytostatic drugs (7,17,31–33). Cells were closely monitored for any signs of toxicity, particularly when using cytostatic drugs. For inhibition of Pgp, tariquidar (Xenova Ltd., Slough, Berkshire, UK) was used at a concentration of 0.5 μM.

Phenytoin and carbamazepine were dissolved in ethanol (≤0.3% ethanol in final solution) and stored at −20°C. Stock solutions of other drugs, including phenobarbital and valproate (both used as sodium salt), were prepared using sterile deionised water, PBS or medium as solvent. In general, dimethylsulfoxide (DMSO) was avoided for dissolving drugs with presumed Pgp-inducing activity, because we previously found that prolonged exposure to DMSO enhances Pgp expression (7). Stock solutions of all drugs were diluted in the cell culture medium immediately before adding to the cells. Only the Pgp inhibitor tariquidar was dissolved in DMSO (<0.1% DMSO in final solution) and was added to the medium for short-term (3 h) exposure of the cells (see below). In previous experiments, determination of AED concentrations in the medium by high pressure liquid chromatography substantiated that the intended drug concentrations were obtained (34), so that lack of AED effects in the present study was not due to poor drug solubility. The medium with the respective drug was changed every 24 h. Control cells were treated either with medium, or with 0.3% ethanol. Because we did not determine any difference between cells treated with medium or 0.3% ethanol, data are shown together as controls in the Results section.

After the end of the drug treatment cells were washed with PBS and subjected to the functional assay as described below. Usually, per experimental day, at least 4 drugs (or different concentrations of one drug) and vehicle controls were tested in one cell line, at least each in triplicate. Adequate controls, also in triplicate, were included on each day. Most experiments were repeated at least once.

Functional Analysis of Pgp

Uptake assays with the cell-permeant, cationic, green-fluorescent Pgp substrate rhodamine 123 (Rho123; Sigma-Aldrich) were performed to evaluate Pgp transport function (35). The Rho123 uptake/efflux assay has previously been validated for this purpose in the three cell lines used in the present study (18,20,36–38) as well as in primary rat BCEC cultures (39). Cells were grown on collagen type I (100 μg/ml) (Gibco®/Life Technologies) coated 6-well plates (Greiner Bio-One). Following drug treatment (see above), the medium was changed to protein-reduced Opti-MEM (Gibco®/Life Technologies) for 1 h, after which cells were incubated with 5 μM (RBE4) or 10 μM (GPNT; hCMEC/D3; pBCECs) Rho123 (dissolved in ethanol; Sigma-Aldrich) in Opti-MEM by shaking for 2 h at 37°C and 5% CO_2. Protein-reduced Opti-MEM was used to minimize interactions of Rho123 with serum proteins in the medium, because Rho123 is highly bound to such proteins. The lower concentration of Rho123 used in RBE4 cells was based on the lower Pgp expression and functionality of these cells (see Results); however, for comparison some experiments in RBE4 cells were also repeated with 10 μM Rho123 (see Results). For rBCECs, which were grown on collagen type IV coated 6-well plates, 2 μM of Rho123 was used. Following incubation with Rho123, cells were washed

Table I Therapeutic Plasma Concentrations (Reference Range) of Antiepileptic Drugs in Patients With Epilepsy. Data are From Patsalos *et al.* (66)

Drug	Therapeutic plasma concentrations		Concentrations (μM) used in the present study
	μg/ml	μM	
Carbamazepine	4–12	17–51	10–100
Levetiracetam	12–46	70–270	100–300
Phenobarbital	10–40	43–172	100–300
Phenytoin	10–20	40–79	10–100
Topiramate	5–20	15–59	30–300
Valproic acid	50–100	347–693	50–600

once with ice-cold PBS and scraped in 500 µl ice cold PBS and collected in 1.5 ml tubes, which were centrifuged 8 min at 130 × *g* at 4°C. The cell pellet was resuspended in 150 µl lysis buffer (25 mM Tris, 50 mM NaCl, 0.5% (w/v) sodium deoxycholate (DOC) and 0.5% (w/v) Triton X-100). Protein determination was performed with the Pierce BCA Protein Assay kit (Thermo Scientific, Bonn, Germany) according to the manufacturer's instructions. Fluorescence was measured with the FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) and was calculated as absolute fluorescence in the cell lysate per mg of protein. Phenol red, which is usually included in the medium, did not interact with the fluorescence of Rho123, which was proven by using medium without phenol red.

To control for the extent of Pgp functionality in each experiment, the Pgp inhibitor tariquidar (0.5 µM) was added to part of the non-treated cells 1 h before Rho123 and during the 2 h of incubation with Rho123. Furthermore, a known inducer of Pgp (dexamethasone) was included as reference standard in most experiments with AEDs or cytostatic drugs.

In some experiments with RBE4 and GPNT cells, we also performed functional Pgp analysis by determining uptake of [^{3}H]-digoxin (7), but results were more variable compared to uptake assays with Rho123, so that only experiments with the latter compound were used for final analysis.

Western Blotting

Cells were scraped and lysed in buffer containing (25 mM Tris, pH 8, 50 mM NaCl, 0.5% (w/v) DOC, 0.5% (w/v) Triton X-100) supplemented with complete protease inhibitor (Roche, Mannheim, Germany). Protein concentrations in the lysates were determined by using the Pierce BCA Protein Assay kit (Thermo Scientific). Equal amounts of total protein were separated on 7.5% SDS-PAGE gels and transferred to PVDF membranes which were blocked overnight in 5% milk in phosphate buffered saline supplemented with Tween-20 (PBST: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4 mM KH_2PO4, pH 7.3, 0.05% (w/v) Tween-20) at 4°C. Membranes were incubated with primary antibodies anti-PGP (C219) 1:200 (Signet Laboratories, Dedham, MA, USA) and anti-ß-actin 1:100 (Sigma-Aldrich) for 2 h in 2% milk in PBST shaking at room temperature (RT) and washed three times for 10 min in PBS-T. Secondary antibodies anti-mouse-HRP 1:1,000 and anti-rabbit-HRP 1:1,000 (Dako, Hamburg, Germany) were incubated for 1 h in 2% milk in PBST at RT and washed three times for 10 min in PBST. Proteins were detected by enhanced chemiluminescence using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and the Chemidoc™ XRS Imager (Bio-Rad Laboratories, Munich, Germany) . Relative protein expressions were quantified densitometrically with Quantity One 1-D Analysis software (Bio-Rad Laboratories) and calculated by normalization to the reference signals of ß-actin with GraphPad Prism software (GraphPad, San Diego, CA, USA). In additional Western blot experiments, we normalized Pgp expression relative to Coomassie-stained proteins on the gel (see Results).

Statistics

Drug effects were compared with individual controls, using either Student's *t*-test or, when data were not normally distributed, the *U*-test of Mann and Whitney, using PRISM 5 software (GraphPad Software Inc., La Jolla, CA, USA). For analysis of effects of several drugs determined in the same experiment, analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's Multiple Comparison Tests were used to compare drug effects with control. Tests used were two-sided and a $P \leq 0.05$ was considered significant.

RESULTS

Pgp Expression and Functionality in RBE4, GPNT, and hCMEC/D3 Cells Under Control Conditions

As reported previously (18), monolayers of hCMEC/D3 cells were characterized by tightly packed elongated cells with a spindle-shaped morphology (Fig. 1a), similar to primary cultures of human brain endothelial cells. A similar morphology was observed for RBE4 cells (Fig. 1c), whereas GPNT cells were considerably smaller and exhibited a cobblestone shape (Fig. 1b). In Western blots from the three cell lines, Pgp was detected at the expected molecular weight (170 kDa) with different intensities between cell lines (Fig. 1d). Using equal amounts of protein (~35 µg per cell line) for blotting, the highest expression of Pgp was seen for GPNT cells and the lowest expression for RBE4 cells, with a 9-fold difference in Pgp expression between RBE4 and GPNT and a 3-fold difference between hCMEC/D3 and GPNT (Fig. 1e). hCMEC/D3 cells exhibited a 3-fold higher Pgp expression than RBE4 cells. Similar results were obtained when Coomassie staining instead of ß-actin was used for normalization (not illustrated).

As shown in Fig. 2, the differences between cell lines in functionality of Pgp (as determined by uptake of Rho123) corresponded to the relative expression of Pgp illustrated in Fig. 1. In each cell line, uptake of the Pgp substrate Rho123 was correlated with the concentration (5 or 10 µM) of Rho123 used. Uptake at 5 and 10 µM Rho123 was 74,000 *vs.* 159,000 (fluorescence in arbitrary units/mg protein) in RBE4, 9,200 *vs.* 17,400 in GPNT, and 32,400 *vs.* 64,700 in hCMEC/D3, respectively (Fig. 2). Thus, the by far highest Rho123 uptake (and thus lowest Pgp functionality) was found in RBE4 cells, followed by hCMEC/D3 and GPNT, with an 8-fold

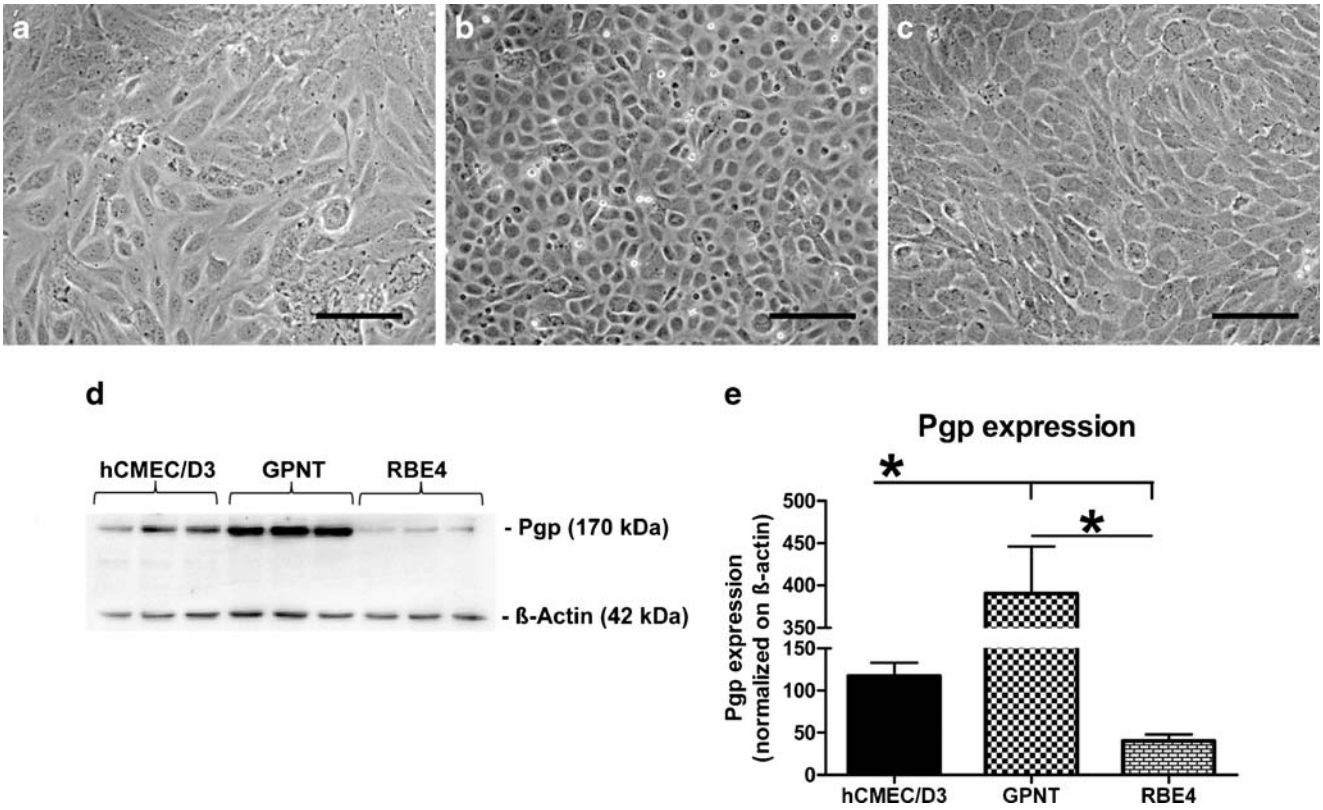

Fig. 1 Morphology and Pgp expression of the three cell lines hCMEC/D3, RBE4 and GPNT. Morphological characteristics are shown by phase contrast microscopic views of monolayers of hCMEC/D3 (**a**), GPNT (**b**) and RBE4 cells (**c**). Bars indicate 100 μm. Cells were cultivated on 6-well plates over three passages. Cell density was 236 cells/ml in A, 613 cells/ml in B and 621 cells/ml in (**d**). Pgp expression in the three cell lines is shown in (**d**) and (**e**). Sample size was 3 per cell line; all samples were compared in the same Western blot and normalized on expression of ß-actin. Relative expression (**e**) is shown as means ± SEM. Statistical difference between cell lines is indicated by asterisk ($P \leq 0.01$).

difference in uptake of Rho123 between RBE4 and GPNT. Addition of the Pgp inhibitor tariquidar (0.5 µM) significantly enhanced Rho123 uptake in all 3 cell lines (Fig. 2), but the extent of this increase depended on the basal functionality of Pgp in each cell line. Thus, in the cell line with the lowest Pgp activity (RBE4), tariquidar increased Rho123 uptake only 1.2-fold, whereas uptake was increased 12.5-fold in the cell line with the highest Pgp activity (GPNT), and hCMEC/D3 cells exhibited an intermediate (2.7-fold) increase of Rho123 uptake with tariquidar. We also compared Pgp induction by dexamethasone (1 µM) in the 3 cell lines under the same conditions, resulting in a significant decrease in Rho123 uptake only in RBE4 cells (Fig. 2). The experiment illustrated in Fig. 2 was repeated once, resulting in comparable differences between cell lines (not illustrated).

Induction of Pgp Functionality by Dexamethasone and Cytostatic Drugs

The experiment illustrated in Fig. 2 had already indicated that Pgp in the 3 cell lines differed markedly in its susceptibility to the known Pgp inducer dexamethasone. For further evaluation of such differences, the RBE4, GPNT and hCMEC/D3 cell lines were exposed over 3 days to various concentrations of dexamethasone and the cytostatic drugs doxorubicin, puromycin, rifampicin, and vincristine (Fig. 3). Furthermore, tariquidar was included in these experiments as a Pgp inhibitor. RBE4 was the most sensitive cell line to the Pgp inducing effects of dexamethasone (0.5 and 1.0 µM), doxorubicin (0.23 and 0.46 µM), and puromycin (2.65 and 5.3 µM), while rifampicin (2.5–50 µM) exerted only moderate effects (Fig. 3a). The effect of dexamethasone (1 µM) on Rho123 uptake in RBE4 was both determined with 5 µM (Fig. 3a) and 10 µM (Fig. 2a) Rho123, resulting in about the same reduction of Rho123 uptake (61% *vs.* 68%) after treatment with dexamethasone, thus indicating that the concentration of Rho123 (5 *vs.* 10 µM) did not alter the consequences of Pgp induction in this assay.

Compared to RBE4, GPNT was much less sensitive to the Pgp-inducing effect of dexamethasone (1 µM), whereas high concentrations of doxorubicin (0.92 µM) and puromycin (10.6 µM) significantly increased Pgp functionality (Fig. 3b) without inducing toxicity. In contrast, such high concentrations of doxorubicine and puromycin induced toxicity in RBE4 cells, so that Rho123 data from such concentrations were not evaluated for the latter cell type. Interestingly,

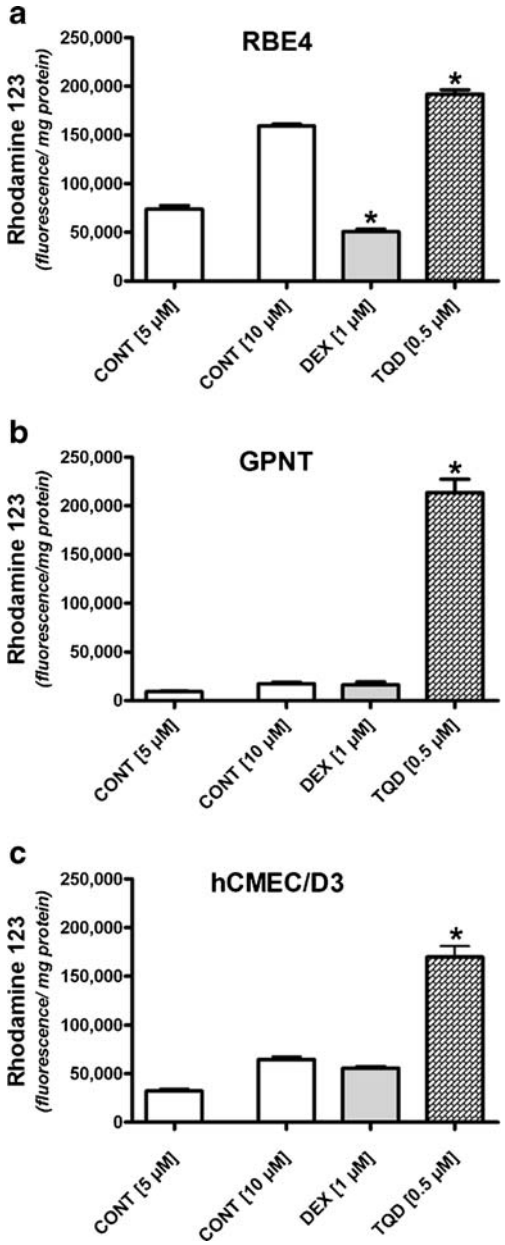

Fig. 2 Functionality of Pgp in the three cell lines hCMEC/D3, RBE4 and GPNT. Sample size was 3 per drug exposure; Rho123 uptake assays in all samples were performed in one experiment to avoid inter-day variation. Data are shown in arbitrary units as means ± SEM. In each cell line, the uptake of Rho123 was determined for 5 and 10 μM Rho123 to test the concentration-dependence of the uptake of the Pgp substrate. In addition, using 10 μM Rho123, the effects of preexposure with the known Pgp inducer dexamethasone (DEX) and the Pgp inhibitor tariquidar (TQD) were determined. Significant differences to control (10 μM Rho123) are indicated by asterisk ($P < 0.0001$).

GPNT cells were more sensitive than RBE4 cells to the Pgp inducing effect of rifampicin (Fig. 3b). hCMEC/D3 was the least sensitive cell line with respect to Pgp induction. Only high concentrations of dexamethasone (5 and 10 µM) significantly reduced Rho123 uptake, doxorubicin (0.23 µM) was not effective at all, and puromycin and rifampicin exerted only moderate effects (Fig. 3c). Interestingly, at low concentrations (5 and 10 µM), rifampicin increased the uptake of Rho123, while a significant decrease, indicating induction of Pgp, was seen at 50 µM (Fig. 3c). The vinca alkaloid vincristine, which was only tested in hCMEC/D3 cells, significantly reduced Rho123 uptake by 28% at a concentration of 20 nM (Fig. 3c). At higher concentrations of doxorubicin (0.92 µM), puromyin (2.65 µM), rifampicin (200, 500, 1,000 µM) and vincristine (40 nM) toxicity was observed in hCMEC/D3 cells, so that Rho123 data from such concentrations were not evaluated.

In view of the low sensitivity of hCMEC/D3 cells to Pgp inducers, we thought that the presence of hydrocortisone (1.4 µM) during culturing the cells (see Methods) may have had an influence, because hydrocortisone is a known Pgp inducer (40). We therefore compared the effects of dexamethasone (1 µM) and doxorubicin (0.92 µM) in cells in which we either added or removed hydrocortisone during cell culturing before the drug experiment (Fig. 4). When hydrocortisone was present in the culture medium before the experiments, uptake of Rho123 was 58.000 fluorescence units/mg protein (Fig. 4a) compared to 124,000 in the absence of preexposing to hydrocortisone (Fig. 4b), thus indicating that the preexposure to hydrocortisone markedly enhanced the functionality of Pgp. Such preexposure also significantly increased the expression of Pgp (as indicated by Western blot), in that the average Pgp expression was 1.8-fold higher in hCMEC/D3 cells cultured in the presence of hydrocortisone ($P = 0.0359$; not illustrated). Dexamethasone (1 µM) had no effect on Rho123 in cells that were preexposed to hydrocortisone (Fig. 4a), but markedly decreased Rho123 uptake in cells that were not preexposed to hydrocortisone (Fig. 4b). A similar difference was seen with doxorubicine (not illustrated), but this drug exerted toxic effects on the cells, particularly in cells that had not been preexposed to hydrocortisone. Tariquidar was slightly more active to increase Rho123 uptake in hCMEC/D3 cells in the presence (3.7-fold) *vs.* absence (2.8-fold) of hydrocortisone during cell culturing (Fig. 4). It should be noted that culturing hCMEC/D3 cells in the absence of hydrocortisone resulted in more dead cells compared to cell culturing with hydrocortisone, so that most of the experiments performed in this study with hCMEC/D3 cells used the hydrocortisone-containing culture medium originally described for these cells (18).

Induction of Pgp Functionality by Antiepileptic Drugs

In contrast to the known Pgp inducers (Figs. 3 and 4), AEDs were almost ineffective to induce Pgp in any cell line, although various drug concentrations, including concentrations that exceeded the therapeutic range (Table I), were tested (Fig. 5). In RBE4 cells, only carbamazepine (at 10 and 50 µM) and topiramate (at 100 µM) significantly decreased Rho123 uptake, but the effect size was small (Fig. 5a). In

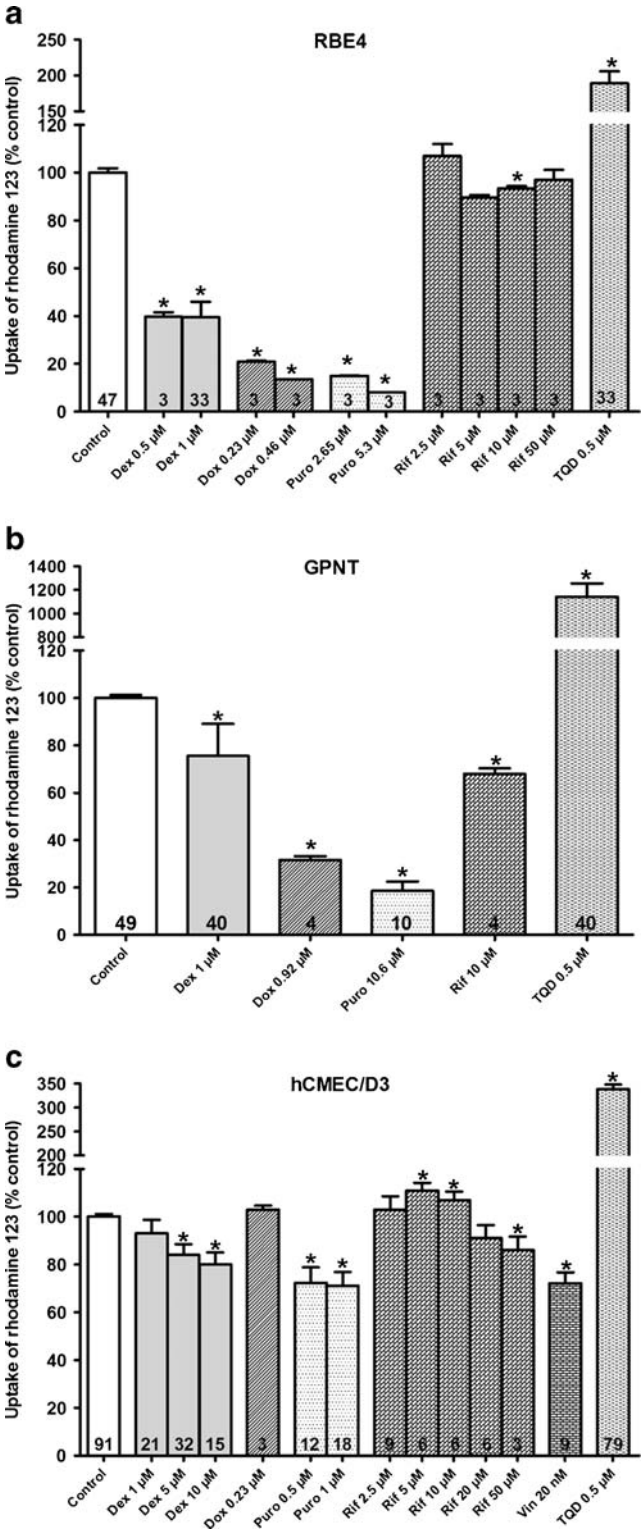

Fig. 3 Effect of known Pgp inducers and the Pgp inhibitor tariquidar on Pgp functionality as determined by the Rho123 uptake assay in the three cell lines RBE4, GPNT, and hCMEC/D3. In the figure, data from various experiments are summarized and illustrated in percent control. Usually, per experimental day, at least 4 drugs (or different concentrations of one drug) were tested in one cell line, at least each in triplicate, plus vehicle controls and dexamethasone and tariquidar as reference standards (see Fig. 4 for examples of such individual experiments). When drug effects were variable or not concentration-dependent, the experiment was repeated. Drug concentrations inducing toxic effects are not illustrated (see Results). The number of samples per drug concentration is indicated in the bars; data are shown as means ± SEM. Individual controls from the various experiments were summarized and shown as 100%. Significance of differences (as calculated by comparing with controls of the individual experiments) is indicated by asterisk ($P < 0.05$). Rho123 was used at 5 μM in experiments with RBE4 cells, but 10 μM in GPNT and hCMEC/D3 (see Methods). Data for hCMEC/D3 (**c**) are from cells preexposed to hydrocortisone during culturing of the cells. Abbreviations: Dex dexamethasone; Dox doxorubicin, Puro puromycin, Rif rifampicin, Vin vincristine, TQD tariquidar.

contrast, valproate increased the uptake of Rho123 at 300 µM, indicating inhibition of Pgp as seen with tariquidar in these cells. This effect of valproate was lost at 600 µM (Fig. 5a).

In GPNT cells, no significant effects of AEDs were seen at therapeutic concentrations or above (Fig. 5b). In hCMEC/D3, carbamazepine (at 100 µM) significantly decreased Rho123 uptake, but effect size was small (Fig. 5c). Valproate significantly increased Rho123 uptake at 300 µM but, similar to RBE4 cells, this effect was lost at 600 µM (Fig. 5c). In order to determine whether preexposure to hydrocortisone altered the efficacy of AEDs to induce Pgp, we also performed experiments in hCMEC/D3 cells without hydrocortisone preexposure (Fig. 4b). Except for a nonsignificant trend for decreased Rho123 uptake with phenytoin (50 µM), none of the AEDs induced Pgp functionality, but valproate significantly increased Rho123 uptake at 300 µM (Fig. 4b). A nonsignificant trend for such an effect was also seen in the presence of hydrocortisone (Fig. 4a; note that sample size was only 3), and this effect became significant when the experiment was repeated with a larger sample size (Fig. 5c). Such variation in the effect of an AED from experiment to experiment was also seen with some of the other AEDs in different cell lines; in such cases the experiment was repeated at least once.

The lack of any marked effects of AED exposure on Rho123 uptake prompted us to increase the length of drug exposure from 3 to 6 days for some AEDs (carbamazepine, phenytoin, topiramate in GPNT, valproate in hCMEC/D3). No alteration in the effect of AEDs was observed.

A decreased uptake of Rho123 is most likely a consequence of increased expression and/or functionality of Pgp (41). In order to determine whether drug exposure increased the expression of Pgp, we performed Western blots for some of the experiments shown in Figs. 3 and 5. As illustrated in Fig. 6, consistent with the significant decrease in Rho123 uptake, dexamethasone increased Pgp expression in RBE4 and GPNT cells. A significant increase in Pgp expression was also seen with puromycin (Fig. 6b). AEDs did not significantly alter Pgp expression with one exception: topiramate increased

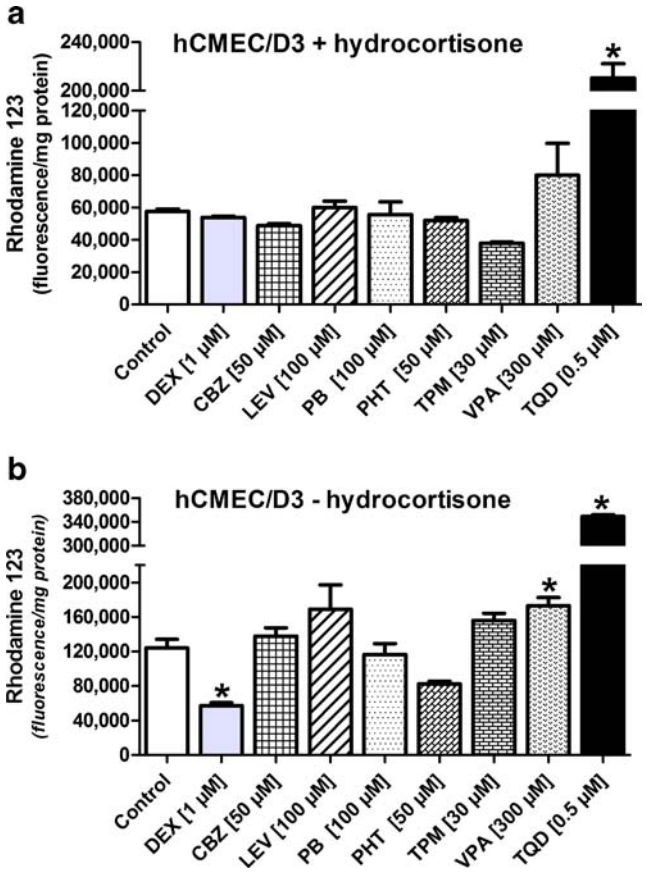

Fig. 4 Comparison of drug effects on Pgp functionality in hCMEC/D3 cells that were either preexposed to hydrocortisone (**a**) or not preexposed to hydrocortisone (**b**) during cell culturing. Data are shown in arbitrary units as means ± SEM of 3 samples per drug. All data are from the same experimental day. Significant differences to control are indicated by asterisk ($P < 0.05$). Abbreviations: *Dex* dexamethasone, *CBZ* carbamazepine, *LEV* levetiracetam, *PB* phenobarbital, *PHT* phenytoin, *TPM* topiramate, *VPA* valproate, *TQD* tariquidar.

Pgp expression in RBE4 cells (Fig. 6a) which was consistent with its significant effect on Rho123 uptake illustrated in Fig. 5a.

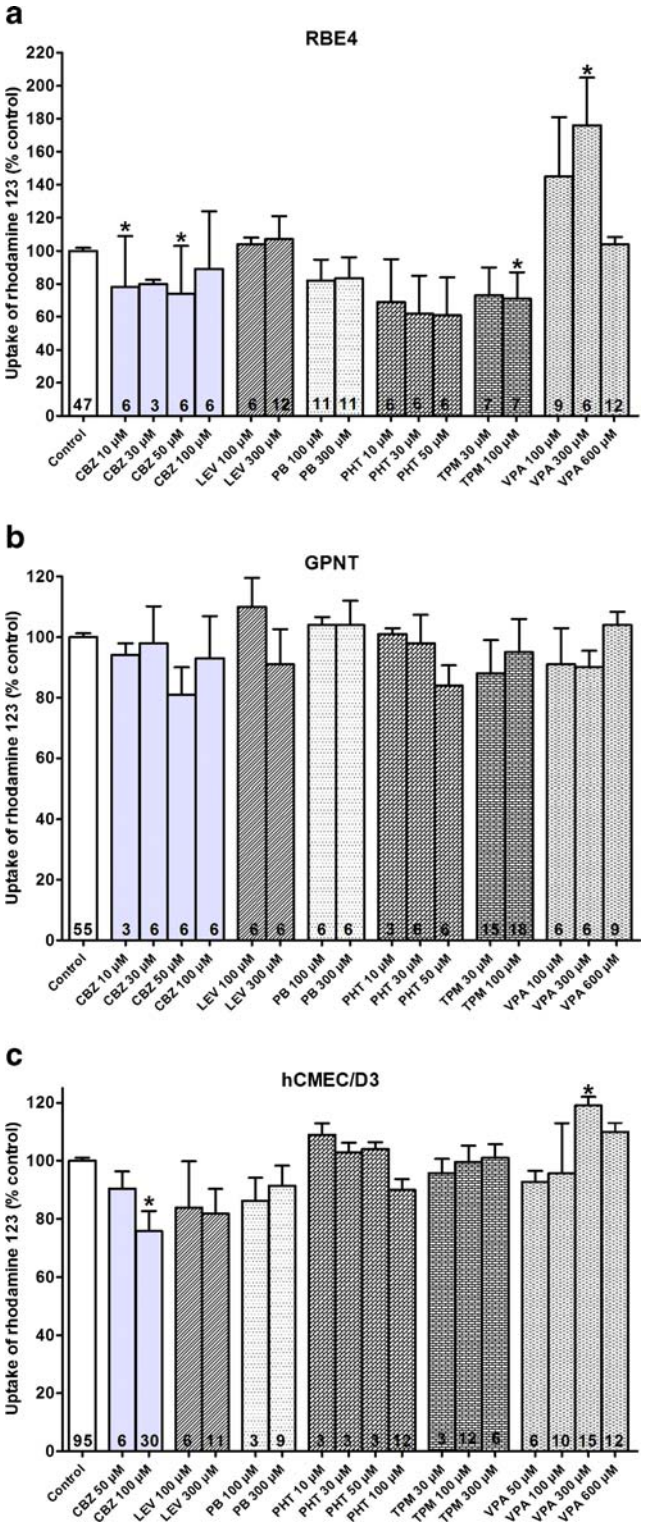

Fig. 5 Effect of AEDs on Pgp functionality as determined by the Rho123 uptake assay in the three cell lines RBE4, GPNT, and hCMEC/D3. In the figure, data from various experiments are summarized and illustrated in percent control. Usually, per experimental day, at least 4 drugs (or different concentrations of one drug) were tested in one cell line, at least each in triplicate, plus vehicle controls and dexamethasone and tariquidar as reference standards (see Fig. 4 for examples of such individual experiments). When drug effects were variable or not concentration-dependent, the experiment was repeated. The number of samples per drug concentration is indicated in the bars; data are shown as means ± SEM. Individual controls from the various experiments were summarized and shown as 100%. Significance of differences (as calculated by comparing with controls of the individual experiments) is indicated by asterisk ($P < 0.05$). Rho123 was used at 5 μM in experiments with RBE4 cells, but 10 μM in GPNT and hCMEC/D3 (see Methods). Data for hCMEC/D3 (c) are from cells preexposed to hydrocortisone during culturing of the cells. Abbreviations: CBZ carbamazepine, LEV levetiracetam, PB phenobarbital, PHT phenytoin, TPM topiramate, VPA valproate.

Comparison of Effect Size Following Drug Exposure of RBE4, GPNT and hCMEC/D3 Cells

In Table II, the maximum effect size of drug exposure on Rho123 uptake is compared across the three cell lines. With

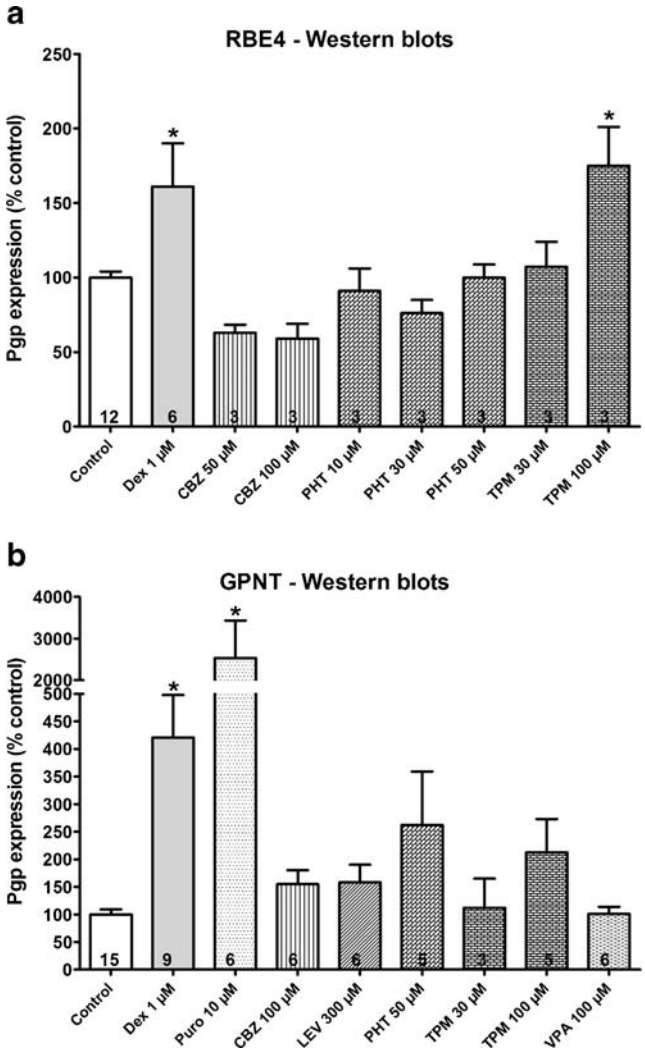

Fig. 6 For some of the experiments on Pgp functionality illustrated in Figs. 3 and 5, also Pgp expression was determined by Western blot. In the figure, data from several experiments in RBE4 and GPNT cells are summarized and illustrated in percent control. The number of samples per drug concentration is indicated in the bars; data are shown as means ± SEM. Significant differences to control are indicated by asterisk ($P<0.05$). Abbreviations: *CBZ* carbamazepine, *Dex* dexamethasone, *PHT* phenytoin, *Puro* puromycin, *TPM* topiramate, *VPA* valproate.

few exceptions, RBE4 was the most sensitive cell line, both to known Pgp inducers and AEDs. Among the 6 AEDs exanmined in this study, only carbamazepine (in RBE4 and hCMEC/D3) and topiramate (in RBE4) moderately induced Pgp functionality, while Pgp was inhibited by valproate (in RBE4 and hCMEC/D3). The inhibitory effect of valproate on Pgp in hCMEC/D3 cells was more pronounced when these cells were used without hydrocortisone preexposure (see Fig. 4b).

Drug Effects in Primary Cultures of Rat Brain Endothelial Cells (rBCECs)

For comparison with the rat RBE4 and GPNT cell lines, some experiments were performed in primary cultures of rat

Table II Effect Size Following Drug Exposure of RBE4, GPNT and hCMEC/D3 Cells in the Rhodamine 123 Uptake Assay

Treatment	Effect size (relative to control)		
	RBE4	GPNT	hCMEC/D3
Dexamethasone	**−60% (0.5 µM)**	−24% (1 µM)	−20% (10 µM)
Doxorubicin	**−87% (0.46 µM)**	−68% (0.92 µM)	N.E. (0.23 µM)[a]
Puromycin	**−92% (5.3 µM)**	−81% (10 µM)	−28% (1 µM)[a]
Rifampicin	−7% (10 µM)	**−32% (10 µM)**	−14% (50 µM)[a]
Vincristine	n.t.	n.t.	−28% (20 µM)[a]
Tariquidar	+89% (0.5 µM)	**+1042% (0.5 µM)**	+238% (0.5 µM)
Carbamazepine	**−26% (50 µM)**	N.E. (100 µM)	−24% (100 µM)
Levetiracetam	N.E. (300 µM)	N.E. (300 µM)	N.E. (300 µM)
Phenobarbital	N.E. (300 µM)	N.E. (300 µM)	N.E. (300 µM)
Phenytoin	N.E. (50 µM)	N.E. (50 µM)	N.E. (100 µM)
Topiramate	**−29% (100 µM)**	N.E. (100 µM)	N.E. (300 µM)
Valproate	**+76% (300 µM)**	N.E. (300 µM)	+19% (300 µM)[b]

For each treatment, the drug concentration with the highest effect size (in percent *vs.* control) is shown. Only significant differences to control were used for calculation of effect size. Data were calculated from the experiments shown in Figs. 3 and 5. In addition, the cell line with the most marked alteration in rhodamine 123 uptake in response to drug exposure is indicated in bold

[a] Higher concentrations were toxic

[b] Also significant increase (+ 40%) in hCMEC/D3 cells that were not preexposed with hydrocortisone (see Fig. 4)

BCECs. As shown in Fig. 7a, b, expression of Pgp in such cultures was similar to that observed in the RBE4 and hCMEC/D3 cell lines, but much lower compared to GPNT as shown in Fig. 1d, e. With increasing passages, Pgp expression significantly decreased, so that only cells of the first two passages were used for functional experiments. Functionality of Pgp in rBCECs was proven by Rho123 efflux. The average uptake of Rho123 (determined at 2 µM) was 61,514±5,161 fluorescence units/mg protein (mean ± SEM from 24 determinations) and did not differ significantly in rBCECs from Wistar or Sprague–Dawley rats (not illustrated). As shown by a representative experiment in Fig. 7c, Rho123 accumulation was not affected by exposure with dexamethasone (10 µM), but significantly reduced by puromycin (1 µM), indicating increased functionality of Pgp. When exposure with dexamethasone was increased from 3 to 6 days, also no effect on Rho123 uptake was observed (not illustrated). Tariquidar (0.5 µM) more than doubled intracellular concentrations of Rho123, indicating significant inhibition of Pgp functionality (Fig. 7c). Similar results were obtained with these drugs in rBCECs of both Wistar and Sprague–Dawley rats, so that all experiments performed in rBCECs from these two rat strains were summarized and shown together in Fig. 7d. Dexamethasone did not alter Rho123 uptake (or efflux) in any experiment, while puromycin significantly decreased

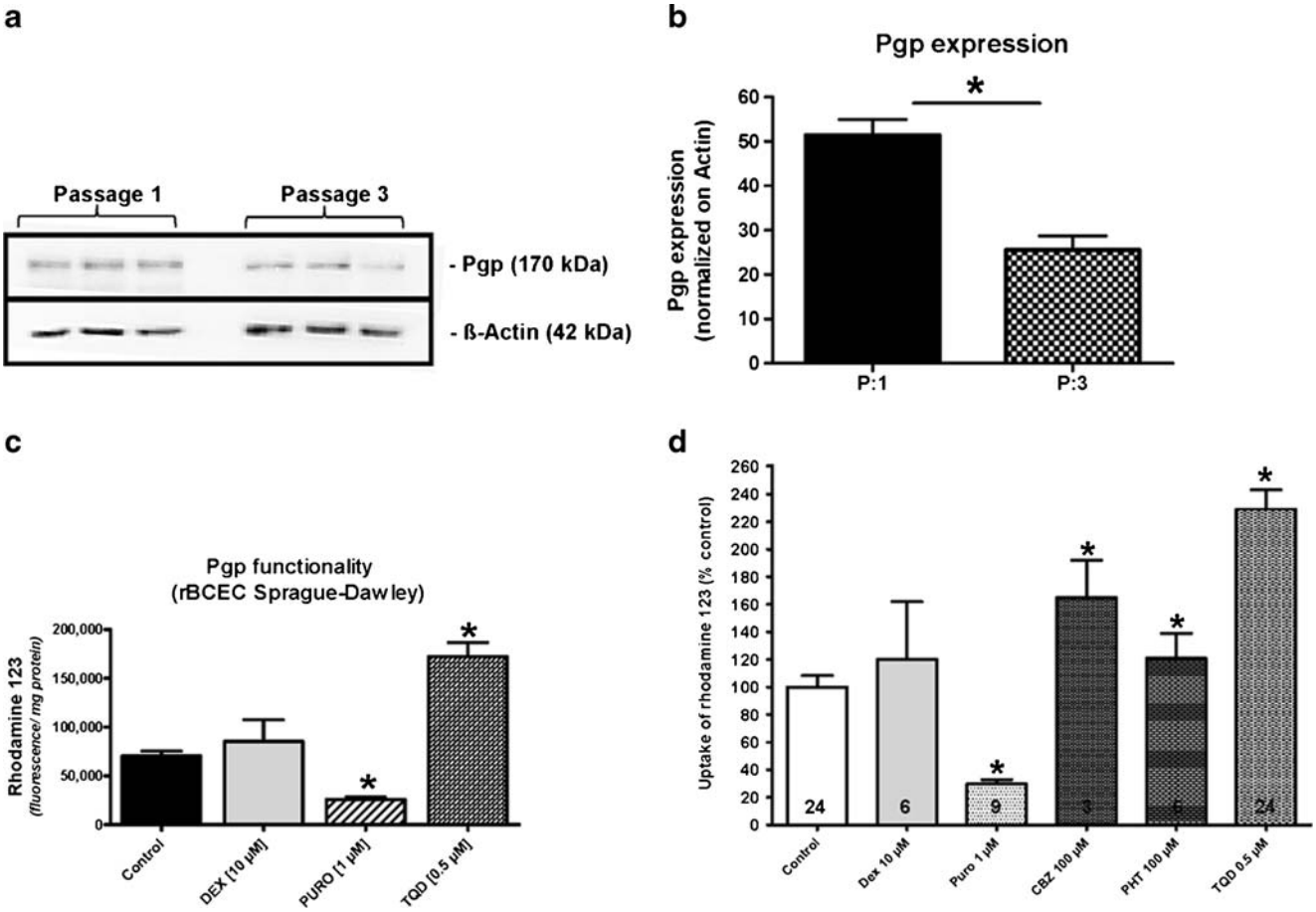

Fig. 7 Expression and functionality of Pgp in primary cultures of BCECs isolated from Wistar and Sprague–Dawley rats. Representative expression of Pgp in rBCECs isolated from Wistar rats (**a** and **b**); note the significant difference between passage 1 and 3 ($P = 0.0056$). A representative example of a Rho123 uptake experiment with rBCECs (**c**). Data are given as means + SEM of three samples per treatment; significant differences to control are indicated by asterisk ($P < 0.05$). (**d**) Data from various experiments in rBCECs from Wistar and Sprague–Dawley rats are summarized and illustrated in percent control. The number of samples per drug concentration is indicated in the bars; data are shown as means ± SEM. Individual controls from the various experiments were summarized and shown as 100%. Significance of differences (as calculated by comparing with controls of the individual experiments) is indicated by asterisk ($P < 0.05$). Abbreviations: *CBZ* carbamazepine, *Dex* dexamethasone, *PHT* phenytoin, *Puro* puromycin, *TQD* tariquidar.

uptake (by 70% on average) and tariquidar increased uptake (2.3 fold on average) in all experiments with these compounds. Similar to tariquidar, a significantly increased uptake of Rho123 was also observed with carbamazepine (100 µM), and, to a lesser extent, with phenytoin (100 µM).

Drug Effects in Primary Cultures of Porcine Brain Endothelial Cells (pBCECs)

For comparison with rat and human BCECs, some experiments were performed in primary cultures of porcine BCECs (Fig. 8). As shown in Fig. 8a, expression of Pgp in such pBCEC cultures was considerably higher than that observed in hCMEC/D3 cells. Relative Pgp protein expression (normalized to actin) of hCMEC/D3 cells was only 28% of that determined in pBCECs, which was confirmed by repeating the experiment (not shown). Functionality of Pgp in pBCECs was proven by Rho123 efflux. The average uptake of Rho123 (determined at 10 µM) was 27,404±4,384 fluorescence units/mg protein (mean ± SEM from 11 determinations). The Rho123 uptake in pBCECs was 2.4-times lower than in hCMEC/D3 cells (64,700 fluorescence units/mg protein at 10 µM; see Fig. 2c), thus reflecting the higher Pgp expression in pBCECs (Fig. 8a). Inhibition of Pgp by tariquidar (0.5 µM) caused a 3.5-fold increase in the uptake of Rho123 in pBCECs (Fig. 8b). Similar to rBCECs, dexamethasone (1 or 10 µM) did not significantly decrease the uptake of Rho123 in pBCECs, whereas a significant decrease was seen with puromycin (1 µM). Increase of the concentration of puromycin to 10 µM was associated with toxicity (not shown). Carbamazepine (100 µM) and phenytoin (µM) did not significantly alter the uptake of Rho123, although a nonsignificant trend for increased uptake was seen with carbamazepine ($P = 0.0614$).

DISCUSSION

The clinically important question whether AEDs induce Pgp at the BBB and thereby potentially reduce their own

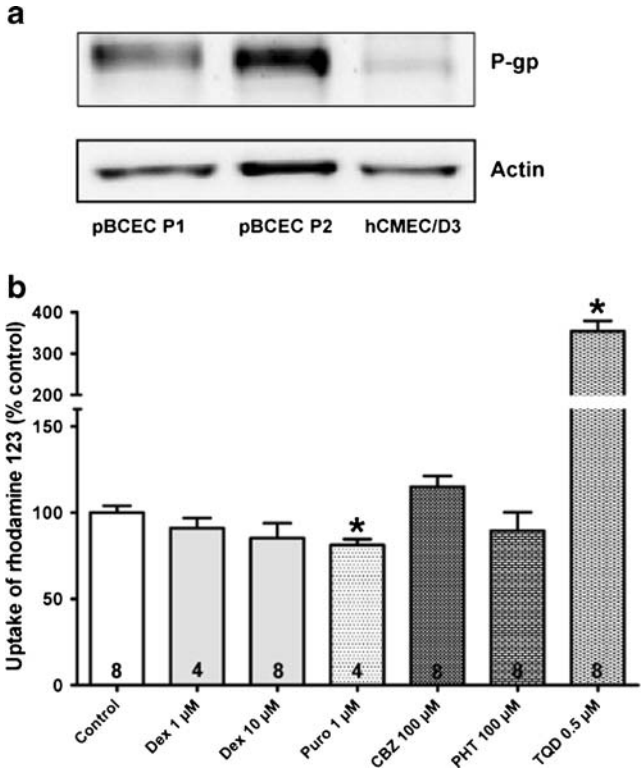

Fig. 8 Expression and functionality of Pgp in primary cultures of porcine BCECs. Representative expression of Pgp in pBCECs (**a**) for passage 1 (P1) and passage 2 (P2). For comparison, Pgp expression was determined in hCMEC/D3 cells, using equal amounts of protein (20 μg) for blotting. Note the higher Pgp expression in pBCEC *vs.* hCMEC/D3. (**b**) Data from two experiments in pBCECs are summarized and illustrated in percent control. The number of samples per drug concentration is indicated in the bars; data are shown as means ± SEM. Individual controls from the two experiments were summarized and shown as 100%. Significance of differences is indicated by asterisk ($P < 0.05$). Abbreviations: *CBZ* carbamazepine, *Dex* dexamethasone, *PHT* phenytoin, *Puro* puromycin, *TQD* tariquidar.

penetration into the brain is unresolved and previous studies on this question are equivocal (42,43). Rather than studying AED effects on Pgp expression as in most previous studies, we decided to study alterations in Pgp functionality in response to AED exposure, because only increased Pgp functionality at the BBB would reduce brain penetration of AEDs. We and others have previously shown that various major AEDs, including phenobarbital, phenytoin, topiramate, lamotrigine, levetiracetam, and oxcarbazepine, are substrates of rodent and human Pgp, whereas this is equivocal for some other AEDs, including carbamazepine and valproate (42,43). In previous experiments, we had used uptake of digoxin and vinblastine for determining AED effects on Pgp functionality in cell lines, including GPNT, but none of the AEDs tested (phenobarbital, phenytoin, carbamazepine) exerted any significant effect (7). For the present study, we used uptake of Rho123 as a functional assay of Pgp activity for studying effects of various AEDs and, for comparison, known Pgp inducers in three BCEC lines, including human hCMEC/D3 cells. A preliminary comparison of drug effects on digoxin *vs.* Rho123 uptake showed that Rho123 uptake was less variable, thus providing more reliable results on Pgp functionality in cell lines (see Methods). Rho123 is a widely used substrate of Pgp for uptake/efflux assays (18,35,37,41). Some reports have suggested that Rho123 efflux occurs also in BCRP(ABCG2)-expressing cell lines, but Robey *et al.* (44) reported that Rho123 was not able to detect BCRP activity, and also our own experiments in BCRP-transfected kidney cell lines did not detect any BCRP-mediated uptake of Rho123 (K. Römermann, unpublished data).

In the three BCEC lines used in the present study, the uptake of Rho123 markedly differed, with highest uptake in RBE4 and lowest uptake in GPNT, reflecting the significant differences in Pgp expression and functionality between these cell lines. GPNT cells are known to express much more Pgp than RBE4 cells (19), which was also seen in our experiments. In hCMEC/D3 cells, Pgp expression was higher than in RBE4 cells, but clearly below the expression seen in GPNT cells, resulting in a Rho123 uptake in between RBE4 and GPNT. As shown by directly comparing the effects of dexamethasone and tariquidar on Rho123 uptake in the three cell lines, the effect size of Pgp inducers and inhibitors depended on the magnitude of Pgp functionality. With tariquidar, the increase in Rho123 uptake was negatively correlated with the magnitude of control uptake (the higher the basal uptake of Rho123, the lower the effect of tariquidar), whereas with dexamethasone the opposite was found (the higher the basal uptake of Rho123, the higher the effect of dexamethasone). In other words, cells with high basal Pgp functionality, such as GPNT and hCMEC/D3 cells, were not very sensitive to the Pgp inducer dexamethasone but highly sensitive to the Pgp inhibitor tariquidar, whereas the opposite was true for cells with low basal Pgp functionality, such as RBE4.

The rat BCEC line RBE4 is a widely used cell line that expresses many properties also expressed by brain endothelial cells *in vivo* (19,45). However, it has only rarely been used to study effects of Pgp inducers and inhibitors in uptake/efflux assays, so that no previously published data are available on the drugs used in the present study. Most likely as a result of low Pgp expression and functionality, RBE4 was the most sensitive cell line to known Pgp inducers, such as dexamethasone, doxorubicin, and puromycin, but RBE4 cells were rather insensitive to the anti-tuberculosis drug rifampicin. The latter drug is a high-affinity ligand for human PXR (hPXR) but not for rodent PXR (46), which could explain its low efficacy in inducing Pgp in RBE4 cells; however, the more potent effect of rifampicin observed in GPNT cells may indicate that hPXR is not the only mediator of rifampicin's effect on Pgp functionality. Among the AEDs, both carbamazepine and topiramate significantly decreased uptake of Rho123 in

RBE4, indicating Pgp induction, while valproate seemed to inhibit Pgp at 300 μM, which is within the therapeutic range of this drug. Interestingly, this effect was lost at 600 μM.

In comparison to RBE4, GPNT cells are more often used to study effects of drugs on Pgp expression and functionality (6,7,17,21). The high Pgp expression of these cells is a consequence of transfection with a selection plasmid containing the puromycin resistance gene and exposure to puromycin during culturing of the cells (17,21). In a previous study of our group, effects of exposure to dexamethasone (1 μM) and doxorubicin (1 μM) on Pgp functionality and expression were quite variable (7), so that we reduced the preexposure to puromycin in the present experiments to avoid maximum induction of Pgp by puromycin already before the experiments with other known Pgp inducers and AEDs. This resulted in lower expression of Pgp (see Methods) and higher sensitivity to dexamethasone and doxorubicine, but not AEDs. Indeed, none of the AEDs used in the present study exerted any effect on Rho123 uptake, thus substantiating our previous experiments in which uptake of either digoxin or vinblastine were used to determine Pgp functionality (7). In addition, we did not observe any effect of AED exposure on Pgp expression in GPNT cells [7; present study], which is in sharp contrast to the study of Lombardo *et al.* (6), in which six AEDs (phenobarbital [300 μM], phenytoin [100 μM], carbamazepine [100 μM], topiramate [100 μM], tiagabine [30 μM] and levetiracetam [300 μM]) were reported to increase Pgp protein expression about 3-fold after 3 days exposure of GPNT cells. As discussed recently (7), the uniform increase of Pgp in the experiments of Lombardo *et al.* (6) in response to six AEDs (each tested at only one concentration) may be an artifact, at least in part, as a result of the solvent (DMSO) used in these experiments, which was avoided in our experiments with AEDs and cytostatic drugs.

The hCMEC/D3 cells are a line of human cerebral microvascular endothelial cells that was developed and first described by Weksler *et al.* in 2005 (18) and has been widely used as a model of the human BBB since then. Like other immortalized BCEC lines, hCMEC/D3 cells show insufficient tightness when grown as a cell monolayer, not developing the low paracellular permeability observed with primary BCEC cultures or kidney cells lines (e.g., LLC, MDCK; (42)), so that these cells have only limited use for bidirectional transport studies, but can be used for a variety of other applications, including efflux assays with Pgp substrates such as Rho123 (18,20,47). To our knowledge, the effects of AEDs on uptake of a Pgp substrate have not been examined previously in hCMEC/D3, but 3 days exposure to the PXR agonist rifampicin was reported to increase Pgp expression (23). In our experiments, rifampicin significantly decreased the uptake of Rho123 in hCMEC/D3 cells and similar effects were also determined for the known Pgp inducers dexamethasone, puromycin and vincristine. In contrast, most AEDs did not affect Pgp functionality in this cell line except for moderate Pgp-inducing effect of carbamazepine. Because the medium recommended by P.-O. Couraud's group for culturing the cells contains 1.4 μM hydrocortisone, we thought that this preexposure to a known Pgp-inducing agent could probably affect the ability of other drugs to induce Pgp. For instance, Martin *et al.* (11) have shown that pretreatment of cell lines with dexamethasone as a media supplement masked the induction of Pgp by other Pgp inducers. In hCMEC/D3 cells, hydrocortisone is added to the medium to enhance tight junction proteins (48), which is not needed for uptake assays such as the Rho123 assay. Nevertheless, even when these cells are used for Pgp efflux assays, medium with hydrocortisone is generally used (20) and we observed more dead cells when culturing hCMEC/D3 cells without hydrocortisone. However, because we wanted to know how preexposure to hydrocortisone affects drug effects in the Rho123 assay, we performed experiments in which hydrocortisone was completely removed from the culture medium. As expected, the hCMEC/D3 cells exhibited an increased uptake of Rho123 and became more sensitive to the Pgp-inducing effect of dexamethasone, but the effects of AEDs did not significantly change.

Overall, none of the six AEDs tested caused an induction of Pgp functionality that was comparable to the effects of dexamethasone or cytostatic drugs in the three examined BCEC lines. This could either mean that most AEDs do not induce Pgp at the BBB at therapeutically relevant concentrations or that immortalized BCEC lines are only a relatively insensitive tool to investigate potential effects of AEDs on Pgp expression and functionality. A similar lack of any robust Pgp-inducing effect of AEDs was recently also found for kidney (MDCK) cells (7) and for intestinal Caco-2 cells (D. Alms, unpublished data). Three of the AEDs examined, i.e., phenobarbital, phenytoin, and carbamazepine, have been reported to induce Pgp in hepatocytes and intestinal cells, most likely via activation of the nuclear receptors CAR or PXR (43), but whether such an effect also occurs in other cell types, such as BCECs, is less well established. In this respect, it is also important to note that induction of Pgp is tissue-specific, which may relate, at least in part, to tissue differences in the expression of nuclear receptors (9,13,25,49). In general, the brain or BCECs isolated from the brain are less sensitive to the Pgp inducing effects of several prototypical inducing agents, including dexamethasone, than intestine or liver (25,49). CAR and PXR are expressed in brain capillaries or BCECs from mouse, rat, pig and human (3) and were shown to mediate drug effects on Pgp. For instance, the PXR ligand dexamethasone (0.1–0.5 μM) increased Pgp expression and functionality in rat brain capillaries (50) and the hPXR ligand rifampicin (10 μM) increased Pgp expression in brain capillaries from hPXR transgenic mice (51). Both PXR ligands were also found to increase Pgp functionality in the present study in

immortalized BCEC lines. Exposing rat brain capillaries to the CAR activator phenobarbital increased the transport activity and protein expression of Pgp; induction of transport was abolished by a protein phosphatase 2A inhibitor (52). However, phenobarbital was used at a concentration (1 mM) that is >5-times higher than the upper limit of the therapeutic range of this AED in patients with epilepsy (170 μM; Table I). This may explain why we did not observe any significant effect of phenobarbital when using therapeutically relevant concentrations in the present experiments in BCEC lines from rats and humans.

Similarly, we did not observe any meaningful induction of Pgp in brain capillaries following treatment of rats with anticonvulsant doses of phenobarbital (30 mg/kg/day) or phenytoin (50 mg/kg/day) for 11 days (53). In apparent contrast, treating rats over 4 days with 80 mg/kg/day phenobarbital increased Pgp-mediated transport and protein expression in brain capillaries assayed *ex vivo* (52). Such high dose of phenobarbital is in the neurotoxic range of this compound and thus of no relevance for effects occurring at the much lower anticonvulsant doses (54). Such differences in drug concentrations or doses between studies may explain many if not all of the equivocal data reported on AEDs and brain Pgp induction in rodents (43). In addition to the role of drug concentration or dose used in experiments on induction of Pgp, there are substantial species differences in ligand affinities for rodent *versus* human PXR, so that data on Pgp induction in rodents cannot be simply extrapolated to humans. To our knowledge, this is the first study that compares the effect of various AEDs and known Pgp inducers on Pgp functionality in rodent and human BCEC lines at therapeutically relevant concentrations.

The only AED that was found to significantly increase Pgp functionality in hCMEC/D3 cells was carbamazepine. In other tissues, inducing effects of carbamazepine and some other AEDs, including valproate, are primarily mediated by CAR and/or PXR (8–12,55,56). With respect to the relatively small effects of carbamazepine (and the lack of other AEDs to induce Pgp at all) observed in the present study in hCMEC/D3 cells, it is important to note that both PXR and CAR are downregulated in these cells (20), which we have previously reported also for GPNT and RBE4 cells (7). However, CAR and PXR are not the only transcription factors that regulate Pgp and mediate drug effects on this transporter, but other nuclear receptors such as the aryl hydrocarbon receptor (AhR), which is highly expressed in hCMEC/D3 (20), the farnesyl-X receptor, the glucocorticoid receptor, and class I histone deacetylases (HDAC1) may also be important in this respect (3,57,58).

With respect to the induction of Pgp functionality by carbamazepine in hCMEC/D3 cells seen in our experiments, it is important to note that this drug has been shown to induce intestinal Pgp expression and functionality in humans *in vivo* (57). Whether a similar *in vivo* alteration of Pgp occurs at the BBB of humans is not known, but the fact that the *in vitro* effect of carbamazepine in a human BCEC line is in line with the *in vivo* effect of this drug on intestinal Pgp in humans may indicate that data from human cell lines reflect the *in vivo* situation.

Interestingly, the HDAC inhibitor valproate significantly inhibited Pgp function in both RBE4 and hCMEC/D3 cells at therapeutic concentrations (300 μM), but in both cell lines this effect was lost at higher concentrations. In a previous study by Weiss *et al.* (27) in a *MDR1*-transfected kidney cell line (LLC), valproate was the only AED that inhibited Pgp in therapeutic concentrations (250–500 μM), but the mechanism of this effect is not known. In human tumor cell lines, valproate (0.5–1.5 mM) was shown to induce Pgp, which was related to its HDAC-inhibitory activity (59). Thus, depending on cell line and drug concentration, valproate can both induce and inhibit Pgp functionality, which deserves further studies on the mechanisms of these effects. Biphasic effects of drugs on Pgp functionality have also been described for other drugs and are most likely a consequence of concentration-dependent interactions with different drug binding sites of Pgp (42).

In order to exclude that the lack of any marked induction of Pgp functionality by AEDs was due to the use of immortalized BCEC lines, some experiments were also performed in primary cultures of rat and porcine BCECs. Similar to the findings in rat and human BCEC lines, the known Pgp inducer puromycin induced Pgp functionality in primary BCEC cultures from both species, whereas tariquidar inhibited Pgp function. The effect size of tariquidar on Rho123 uptake was 2.3-fold, 2.7-fold, and 3.5-fold in rBCECs, hCMEC/D3, and pBCECs, respectively, reflecting the higher Pgp expression in pBCECs compared to rBCECs and hCMEC/D3 cells. However, in apparent contrast to rat and human BCEC lines, dexamethasone did not induce Pgp in primary cultures of rat and porcine BCECs. In rBCECs this could have been due, at least in part, to the preexposure with hydrocortisone and puromycin in the culture medium (see Methods). Indeed, Narang *et al.* (60) previously reported that dexamethasone increased Pgp expression and functionality in rBCECs when cells were cultured without hydrocortisone or puromycin. pBCECs were cultured without hydrocortisone, but puromycine was used to eliminate pericytes, as commonly done during preparation of primary cultures of BCECs (28). Thus, the different extent of Pgp inhibition or induction that has been observed for some of the drugs among the different BCEC preparations used in the present study may not only result from the immortalization process or the selected species, but also from the degree of Pgp induction by hydrocortisone, puromycine or none, which may strongly determine the regulatory responsiveness and sensitivity of the transporter

towards other drugs. This does, however, not explain the different sensitivity of rBCECs and pBCECs to exposure with puromycin, because this drug (at 1 μM) decreased Rho123 uptake by 70% in rBCECs but only 20% in pBCECs. Another factor that may affect the effect size of exposure of BCECs with Pgp-inducing drugs is the duration of exposure. For instance, Lemmen *et al.* (61) recently reported that induction of Pgp in pBCECs by rifampicin was maximal after 6 and 12 h of exposure, but decreased at 24 and 48 h. In the present study, all drugs were compared at the same exposure duration (3 days; in some experiments 6 days), so that transient drug effects on Pgp occurring with shorter exposure might have been missed. Furthermore, the choice of Pgp substrate used for functional analysis of Pgp may affect results obtained with Pgp inducers. For the present experiments, we used the Rho123 assay, because this assay proved to be less variable (and more sensitive to known Pgp inducers) than other assays previously used by us in this respect (see Methods).

In our experiments with primary cultures of rat and porcine BCECs, a 3 day exposure with carbamazepine and phenytoin did not induce but rather inhibited Pgp functionality, resembling the findings of Weiss *et al.* (27) with these AEDs in the calcein uptake assay in primary cultures of porcine BCECs and the bodipy-verapamil accumulation assay in LLC-MDR1 cells. To our knowledge, only one previous study used primary cultures of rat BCECs to study effects of AED exposure (62). The authors reported that all AEDs (phenobarbital, phenytoin, carbamazepine, valproic acid) increased Pgp expression about 3-fold, which was associated with a moderate (10–30%), but statistically significant decrease in the accumulation of Rho123 by phenobarbital, phenytoin and valproic acid, but not carbamazepine. However, cells were drug-exposed for 60 days at increasing drug concentrations, and the authors mentioned that a 30-day exposure to AEDs failed to induce Pgp function and level (62). This finding is difficult to interpret, because drug-provoked induction of Pgp expression and functionality is known to occur rapidly in order to allow cells to protect themselves from potential intoxication by drugs and other xenobiotics (63).

CONCLUSIONS

Our data do not support the notion that treatment with therapeutic concentrations of AEDs causes a clinically relevant induction in the functionality of Pgp in BCECs that form the BBB. Although some Pgp induction was seen with carbamazepine in both RBE4 and hCMEC/D3 cells, this was not confirmed in primary BCEC cultures. Unless there is a general difference in the sensitivity of Pgp at the BBB between *in vitro* and *in vivo* drug exposure, it thus seems unlikely that AEDs reduce their own penetration into the brain via Pgp induction, which then critically contributes to development of AED resistance in patients with epilepsy. Rather, the increased Pgp expression at the BBB that has been determined in patients with AED-resistant epilepsy may be a consequence of genetic polymorphisms in the *ABCB1* gene or, more likely, of excessive neuronal release of the Pgp inducer glutamate in response to seizures (4,46). The situation is different with cytostatic drugs, most of which induced Pgp functionality in all BCEC lines and primary BCEC cultures, although interesting differences were observed among cell lines. Further study is needed to uncover the molecular mechanisms behind these inter-cell line differences in Pgp induction. Our data demonstrate that studies on the Pgp induction potential of xenobiotics in BCECs should not rely on a single cell line, but rather a battery of cell lines should be used to avoid false negative or positive results. As shown by the present data, any Pgp-inducing compounds in the culture medium, which are often added to increase the viability or barrier function of BCECs (hydrocortisone) or to eliminate pericytes (puromycin) in *in vitro* models of the BBB, may strongly determine the regulatory responsiveness and sensitivity of Pgp towards other drugs. It remains to be determined how closely such *in vitro* experiments on Pgp induction reflect the *in vivo* situation, but, as discussed above, the study of Giessmann *et al.* (64) with carbamazepine indicates that data from human cell lines such as hCMEC/D3 provide a clue to what would happen *in vivo*. We plan to use ^{11}C-labeled Pgp ligands and positron emission tomography (PET) to further study the effect of AEDs on Pgp expression and functionality at the BBB *in vivo* (65).

ACKNOWLEDGMENTS AND DISCLOSURES

We thank Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France) for providing hCMEC/D3 cells and Prof. Francoise Roux (INSERM U26, Paris, France) for providing GPNT and RBE4 cells. The study was supported by a grant (Lo 274/10) from the Deutsche Forschungsgemeinschaft (Bonn, Germany). Dana Alms received a PhD scholarship from the FAZIT-Stiftung (Frankfurt am Main, Germany).

REFERENCES

1. Schinkel AH. P-Glycoprotein, a gatekeeper in the blood–brain barrier. Adv Drug Deliv Rev. 1999;36:179–94.
2. Fromm MF. Importance of P-glycoprotein at blood-tissue barriers. Trends Pharmacol Sci. 2004;25:423–9.
3. Miller DS. Regulation of P-glycoprotein and other ABC drug transporters at the blood–brain barrier. Trends Pharmacol Sci. 2010;31:246–54.
4. Löscher W, Potschka H. Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci. 2005;6:591–602.
5. Marquez B, Van Bambeke F. ABC multidrug transporters: target for modulation of drug pharmacokinetics and drug-drug interactions. Curr Drug Targets. 2011;12:600–20.

6. Lombardo L, Pellitteri R, Balazy M, Cardile V. Induction of nuclear receptors and drug resistance in the brain microvascular endothelial cells treated with antiepileptic drugs. Curr Neurovasc Res. 2008;5: 82–92.
7. Ambroziak K, Kuteykin-Teplyakov K, Luna-Tortós C, Al Falah M, Fedrowitz M, Löscher W. Exposure to antiepileptic drugs does not alter the functionality of P-glycoprotein in brain capillary endothelial and kidney cell lines. Eur J Pharmacol. 2010;628:57–66.
8. Kullak-Ublick GA, Becker MB. Regulation of drug and bile salt transporters in liver and intestine. Drug Metab Rev. 2003;35:305–17.
9. Xu C, Li CY, Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res. 2005;28: 249–68.
10. Urquhart BL, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. J Clin Pharmacol. 2007;47:566–78.
11. Martin P, Riley R, Back DJ, Owen A. Comparison of the induction profile for drug disposition proteins by typical nuclear receptor activators in human hepatic and intestinal cells. Br J Pharmacol. 2008;153:805–19.
12. Köhle C, Bock KW. Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. Biochem Pharmacol. 2009;77:689–99.
13. Chen T. Overcoming drug resistance by regulating nuclear receptors. Adv Drug Deliv Rev. 2010;62:1257–64.
14. Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. Steroids. 2007;72:231–46.
15. di Masi A, Marinis ED, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: molecular, functional, and biomedical aspects. Mol Aspects Med. 2009;30:297–343.
16. Roux F, Durieu-Trautmann O, Chaverot N, Claire M, Mailly P, Bourre JM, *et al.* Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. J Cell Physiol. 1994;159:101–13.
17. Régina A, Romero IA, Greenwood J, Adamson P, Bourre JM, Couraud PO, *et al.* Dexamethasone regulation of P-glycoprotein activity in an immortalized rat brain endothelial cell line, GPNT. J Neurochem. 1999;73:1954–63.
18. Weksler BB, Subileau EA, Perrière N, Charneau P, Holloway K, Leveque M, *et al.* Blood–brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J. 2005;19:1872–4.
19. Roux F, Couraud PO. Rat brain endothelial cell lines for the study of blood–brain barrier permeability and transport functions. Cell Mol Neurobiol. 2005;25:41–58.
20. Dauchy S, Miller F, Couraud PO, Weaver RJ, Weksler B, Romero IA, *et al.* Expression and transcriptional regulation of ABC transporters and cytochromes P450 in hCMEC/D3 human cerebral microvascular endothelial cells. Biochem Pharmacol. 2009;77:897–909.
21. Demeuse P, Fragner P, Leroy-Noury C, Mercier C, Payen L, Fardel O, *et al.* Puromycin selectively increases mdr1a expression in immortalized rat brain endothelial cell lines. J Neurochem. 2004;88:23–31.
22. Male DK. Expression and induction of p-glycoprotein-1 on cultured human brain endothelium. J Cereb Blood Flow Metab. 2009;29: 1760–3.
23. Zastre JA, Chan GN, Ronaldson PT, Ramaswamy M, Couraud PO, Romero IA, *et al.* Up-regulation of P-glycoprotein by HIV protease inhibitors in a human brain microvessel endothelial cell line. J Neurosci Res. 2009;87:1023–36.
24. Bachmeier CJ, Beaulieu-Abdelahad D, Ganey NJ, Mullan MJ, Levin GM. Induction of drug efflux protein expression by venlafaxine but not desvenlafaxine. Biopharm Drug Dispos. 2011;32:233–44.
25. Perloff MD, von Moltke LL, Greenblatt DJ. Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats. Xenobiotica. 2004;34:133–50.
26. Fox E, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. Expert Rev Anticancer Ther. 2007;7:447–59.
27. Weiss J, Kerpen CJ, Lindenmaier H, Dormann SM, Haefeli WE. Interaction of antiepileptic drugs with human P-glycoprotein in vitro. J Pharmacol Exp Ther. 2003;307:262–7.
28. Perrière N, Demeuse P, Garcia E, Regina A, Debray M, Andreux JP, *et al.* Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood–brain barrier-specific properties. J Neurochem. 2005;93:279–89.
29. Patabendige A, Skinner RA, Abbott NJ. Establishment of a simplified in vitro porcine blood–brain barrier model with high transendothelial electrical resistance. Brain Res. 2013;1521:1–15.
30. Patabendige A, Skinner RA, Morgan L, Abbott NJ. A detailed method for preparation of a functional and flexible blood–brain barrier model using porcine brain endothelial cells. Brain Res. 2013;1521:16–30.
31. Smith AJ, Mayer U, Schinkel AH, Borst P. Availability of PSC833, a substrate and inhibitor of P-glycoproteins, in various concentrations of serum. J Natl Cancer Inst. 1998;90:1161–6.
32. Baltes S, Gastens AM, Fedrowitz M, Potschka H, Kaever V, Löscher W. Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. Neuropharmacology. 2007;52:333–46.
33. Baltes S, Fedrowitz M, Luna-Tortós C, Potschka H, Löscher W. Valproic acid is not a substrate for P-glycoprotein or multidrug resistance proteins 1 and 2 in a number of in vitro and in vivo transport assays. J Pharmacol Exp Ther. 2007;320:331–43.
34. Luna-Tortós C, Fedrowitz M, Löscher W. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology. 2008;55:1364–75.
35. Efferth T, Lohrke H, Volm M. Reciprocal correlation between expression of P-glycoprotein and accumulation of rhodamine 123 in human tumors. Anticancer Res. 1989;9:1633–7.
36. Vautier S, Milane A, Fernandez C, Buyse M, Chacun H, Farinotti R. Interactions between antiparkinsonian drugs and ABCB1/P-glycoprotein at the blood–brain barrier in a rat brain endothelial cell model. Neurosci Lett. 2008;442:19–23.
37. Tai LM, Reddy PS, Lopez-Ramirez MA, Davies HA, Male DK, Loughlin AJ, *et al.* Polarized P-glycoprotein expression by the immortalised human brain endothelial cell line, hCMEC/D3, restricts apical-to-basolateral permeability to rhodamine 123. Brain Res. 2009;1292:14–24.
38. Yang L, Fawcett JP, Ostergaard J, Zhang H, Tucker IG. Mechanistic studies of the effect of bile salts on rhodamine 123 uptake into RBE4 cells. Mol Pharm. 2012;9:29–36.
39. Perrière N, Yousif S, Cazaubon S, Chaverot N, Bourasset F, Cisternino S, *et al.* A functional in vitro model of rat blood–brain barrier for molecular analysis of efflux transporters. Brain Res. 2007;1150:1–13.
40. Maines LW, Antonetti DA, Wolpert EB, Smith CD. Evaluation of the role of P-glycoprotein in the uptake of paroxetine, clozapine, phenytoin and carbamazapine by bovine retinal endothelial cells. Neuropharmacology. 2005;49:610–7.
41. Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, *et al.* Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol Pharmacol. 1994;46: 627–38.
42. Löscher W, Luna-Tortos C, Römermann K, Fedrowitz M. Do ATP-binding cassette transporters cause pharmacoresistance in epilepsy? Problems and approaches in determining which antiepileptic drugs are affected. Curr Pharm Des. 2011;17:2808–28.
43. Zhang C, Kwan P, Zuo Z, Baum L. The transport of antiepileptic drugs by P-glycoprotein. Adv Drug Deliv Rev. 2012;64:930–42.
44. Robey RW, Honjo Y, van de Laar A, Miyake K, Regis JT, Litman T, *et al.* A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). Biochim Biophys Acta. 2001;1512:171–82.

45. Aschner M, Fitsanakis VA, dos Santos AP, Olivi L, Bressler JP. Blood–brain barrier and cell-cell interactions: methods for establishing in vitro models of the blood–brain barrier and transport measurements. Methods Mol Biol. 2006;341:1–15.
46. Miller DS, Bauer B, Hartz AMS. Modulation of P-glycoprotein at the blood–brain barrier: opportunities to improve CNS pharmacotherapy. Pharmacol Rev. 2008;60:196–209.
47. Poller B, Drewe J, Krahenbuhl S, Huwyler J, Gutmann H. Regulation of BCRP (ABCG2) and P-glycoprotein (ABCB1) by cytokines in a model of the human blood–brain barrier. Cell Mol Neurobiol. 2010;30:63–70.
48. Forster C, Burek M, Romero IA, Weksler B, Couraud PO, Drenckhahn D. Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood–brain barrier. J Physiol. 2008;586:1937–49.
49. Matheny CJ, Ali RY, Yang X, Pollack GM. Effect of prototypical inducing agents on P-glycoprotein and CYP3A expression in mouse tissues. Drug Metab Dispos. 2004;32:1008–14.
50. Bauer B, Hartz AM, Fricker G, Miller DS. Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood–brain barrier. Mol Pharmacol. 2004;66:413–9.
51. Bauer B, Yang X, Hartz AM, Olson ER, Zhao R, Kalvass JC, *et al.* In vivo activation of human pregnane X receptor tightens the blood–brain barrier to methadone through P-glycoprotein up-regulation. Mol Pharmacol. 2006;70:1212–9.
52. Wang X, Sykes DB, Miller DS. Constitutive androstane receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood–brain barrier. Mol Pharmacol. 2010;78:376–83.
53. Seegers U, Potschka H, Löscher W. Lack of effects of prolonged treatment with phenobarbital or phenytoin on the expression of P-glycoprotein in various rat brain regions. Eur J Pharmacol. 2002;451:149–55.
54. Gladding GD, Kupferberg HJ, Swinyard EA. Antiepileptic drug development program. In: Frey H-H, Janz D, editors. Antiepileptic drugs. Berlin: Springer; 1985. p. 341–50.
55. Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, *et al.* Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. Drug Metab Dispos. 2007;35:1032–41.
56. Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, *et al.* Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. J Pharmacol Exp Ther. 2007;320:72–80.
57. Wang X, Hawkins BT, Miller DS. Aryl hydrocarbon receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood–brain barrier. FASEB J. 2011;25:644–52.
58. Xu Y, Jiang Z, Yin P, Li Q, Liu J. Role for class I histone deacetylases in multidrug resistance. Exp Cell Res. 2012;318:177–86.
59. Eyal S, Lamb JG, Smith-Yockman M, Yagen B, Fibach E, Altschuler Y, *et al.* The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver. Br J Pharmacol. 2006;149:250–60.
60. Narang VS, Fraga C, Kumar N, Shen J, Throm S, Stewart CF, *et al.* Dexamethasone increases expression and activity of multidrug resistance transporters at the rat blood–brain barrier. Am J Physiol Cell Physiol. 2008;295(2):C440–50.
61. Lemmen J, Tozakidis IE, Galla HJ. Pregnane X receptor upregulates ABC-transporter Abcg2 and Abcb1 at the blood–brain barrier. Brain Res. 2013;1491:1–13.
62. Yang HW, Liu HY, Liu X, Zhang DM, Liu YC, Liu XD, *et al.* Increased P-glycoprotein function and level after long-term exposure of four antiepileptic drugs to rat brain microvascular endothelial cells in vitro. Neurosci Lett. 2008;434:299–303.
63. Bauer B, Hartz AM, Fricker G, Miller DS. Modulation of P-glycoprotein transport function at the blood–brain barrier. Exp Biol Med (Maywood). 2005;230:118–27.
64. Giessmann T, May K, Modess C, Wegner D, Hecker U, Zschiesche M, *et al.* Carbamazepine regulates intestinal P-glycoprotein and multidrug resistance protein MRP2 and influences disposition of talinolol in humans. Clin Pharmacol Ther. 2004;76:192–200.
65. Löscher W, Langer O. Imaging of P-glycoprotein function and expression to elucidate mechanisms of pharmacoresistance in epilepsy. Curr Top Med Chem. 2010;10:1785–91.
66. Patsalos PN, Berry DJ, Bourgeois BF, Cloyd JC, Glauser TA, Johannessen SI, *et al.* Antiepileptic drugs–best practice guidelines for therapeutic drug monitoring: a position paper by the subcommission on therapeutic drug monitoring, ILAE Commission on Therapeutic Strategies. Epilepsia. 2008;49:1239–76.