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Research paper

A fluorescence-based in vitro assay for drug interactions with breast cancer resistance protein (BCRP, ABCG2)

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ABSTRACT

Purpose: To establish a fluorescence-based assay for drug interactions with the ABC-export-protein BCRP (ABCG2). *Methods:* BCRP expression was verified by immunostaining and Western blots in intact porcine brain capillaries, isolated endothelial cells (PBCECs) and in MDCKII-cells over-expressing human wild-type BCRP (MDCKII-hBCRP). Transport of fluorescent mitoxantrone across cells was determined to assess a preferred transport direction. Sensitivity of cultured cells versus mitoxantrone in the absence and in the presence of transport modulators was examined at increasing concentrations of the cytostatic using the AlamarBlue™ assay. In addition, cells were incubated with mitoxantrone in the absence and presence of increasing concentrations of different compounds with the potential to interact with BCRP. Intracellular fluorescence accumulation was measured using a flow cytometer. *Results:* Isolated capillaries as well as 7-day old PBCECs showed expression of BCRP. Cell sensitivity to mitoxantrone significantly increased in the presence of the BCRP inhibitors KO143 and GF120918. Transport of mitoxantrone across PBCEC monolayers was directed with P_{app} (apical to basolateral) 5.6×10^{-6} cm s^{-1} and with P_{app} (basolateral to apical) 2.8×10^{-5} cm s^{-1}. FACS analysis revealed a different extent of fluorescence accumulation dependent on the kind and concentration of BCRP modulating compounds. *Conclusions:* The mitoxantrone-based assay can be used as a rapid FACS screening system to assess drug interactions with BCRP at the blood–brain barrier and therefore represents a useful tool in drug profiling.

1. Introduction

Drug transporters expressed in the gastrointestinal tract and in brain microvessels may directly affect the oral bioavailability of drugs and their penetration into the central nervous system [1,2]. Particularly, transporters belonging to the ATP-binding cassette (ABC) transporter family are gaining attention for their involvement in drug absorption and disposition. Breast cancer resistance protein BCRP (ABCG2) belongs to this superfamily of proteins [3,4]. Human BCRP was originally discovered independently from three different groups: in doxorubicin-resistant breast cancer cells [5], in a human coloncarcinoma cell line selected with mitoxantrone (“MXR”) [6] and in human placenta (“ABCP”) [7]. BCRP extrudes xenobiotics and certain drugs from cells, thereby mediating drug resistance and affecting the pharmacological behaviour of many compounds. Besides, BCRP is involved in the physiologic transport of folate derivatives [8], food carcinogens (PhiP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) [9] and vitamins [10]. The BCRP gene is located in chromosome 4q22, spans over 66 kb, and consists of 16 exons ranging from 60 to 532 bp [11]. Compared to the structures of other pharmacologically relevant ABC-transporters (e.g., ABCB1 and ABCC1), BCRP is a “half-transporter,” consisting of six transmembrane domains and one ATP-binding cassette domain. Thus, the hypothesis was set up that BCRP functions as a homodimer [12,13]. Recently, it was shown that human BCRP exists as a homodimer bound through disulfide-bonded cysteine residues [14].

Besides its expression in tumor cells [15], BCRP is expressed in barrier tissues and mediates protection of the body against the toxic action of xenobiotics as well as of metabolites. For example, it can be found in the bile canalicular membrane of hepatocytes, the brush border membrane of renal proximal tubules, the apical membrane of enterocytes, the ducts and in lobules of the breast, the placenta, the choroid plexus and in others [16,17]. Apart from its function in intestinal absorption, hepatobiliary and renal excretion or placental passage, this ATP-dependent efflux-transporter contributes multidrug-resistance to a variety of functionally and structurally unrelated drugs such as camptothecin-derived topoisomerase I inhibitors, anthracyclines or flavopiridols, and shares an overlapping substrate specificity with P-glycoprotein (P-gp) [18,19]. Besides P-glycoprotein (MDR1, ABCB1), it also appears to

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limit the penetration of many therapeutic drugs across the blood–brain barrier [20–28]. Thus, the identification of BCRP-reversing compounds and the manipulation of expression mechanisms could contribute to the improvement of CNS pharmacotherapy including treatment of brain tumors [29,30].

Depending on the objective in BCRP pharmacokinetic transport studies, several in vitro assay designs have already been established: accumulation/efflux assays determining drug efflux activity with FACS (fluorescence-associated cell sorting) and mitoxantrone, BODIPY-Prazosin, Hoechst 33342, methotrexate, pheophorbide A, topotecan or estrone-sulfate as selective BCRP substrates [31–39], transport assays measuring the net flux across the confluent monolayer [37,38] and ATPase assays examining the stimulation or inhibition of ATP-dependent drug transport in membrane vesicles [39]. Besides, [40] presented a computational model to analyse structure–activity relationships between potential therapeutic drugs and BCRP efflux activity. It was demonstrated that the drug binding to the plasma membrane forms a basic precondition for drug-BCRP interplay.

One common feature of all these assay designs is the fact that they are predominantly using over-expressing cell lines and thus are focussing exclusively on increased BCRP transport. In this regard, we have tried to establish and to validate an assay using the anthracenedione mitoxantrone as selective substrate, which is sensitive enough to determine physiologic drug–BCRP interactions in the porcine blood–brain barrier model. MDCKII-hBCRP over-expressing cells have been used as positive control.

2. Materials and methods

2.1. Materials

Ascorbic acid, cyclosporin A, daidzein, 17-β-estradiol, estrone-sulfate, genistein, leukotriene C_4, MK571, naringenin, nicardipine, novobiocin, probenecid and verapamil were purchased from Sigma–Aldrich (Taufkirchen, Germany). Saquinavir was provided by Roche (Basel, Switzerland) and lopinavir by Abbott (Ludwigshafen, Germany). Valspodar (PSC-833) was obtained from Novartis (Basel, Switzerland), elacridar (GF120918) was obtained from GlaxoSmithKline (Research Triangle Park, NC, USA), and tariquidar (XR9576) was a kind gift from Prof. A. Buschauer, University of Regensburg, Germany. KO143 was kindly provided by Dr. A.H. Schinkel, the Netherlands. FTC was supplied by Alexis Biochemicals (Lörrach, Germany) and Pheophorbide A was purchased from Frontier Scientific (Carnforth, UK). BODIPY-Prazosin was obtained from Invitrogen (Karlsruhe, Germany). Culture media, fetal calf serum (FCS), horse serum (HS) and supplements were obtained from Biochrom (Berlin, Germany).

2.2. Animals and tissue

Brains from female pigs were obtained from the local slaughterhouse (Mannheim). Immediately after isolation, the brains were kept in ice-cold aCSF (artificial cerebrospinal fluid, consisting of 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, and 10 mM glucose equilibrated with carbogen to pH 7.4) buffer for approximately 1 h until start of cell isolation.

2.3. Isolation of porcine brain capillary endothelial cells (PBCECs) and cell culture

Porcine brain capillary endothelial cells were isolated as previously described [41] with some minor modifications. Briefly, brains were cleaned of meninges, choroid plexus and superficial blood vessels. Cortical gray matter was removed, minced to 1–2 mm^3 cubes and incubated in Medium 199, supplemented with 0.8 mM L-glutamine, penicillin/streptomycin (100 U/ml; 100 μg/ml), 100 μg/ml kanamycin and 10 mM Hepes, pH 7.4. Dispase II (Hoffmann LaRoche, Mannheim, Germany) was added to a final concentration of 0.5%, and the brain tissue was incubated for 2 h at 37 °C. Then the homogenate was centrifuged at 1000*g* for 10 min. The supernatant was discarded and the pellet was re-suspended in a medium containing 15% dextran (Sigma–Aldrich, Taufkirchen, Germany). Microvessels were separated from other brain tissues by centrifugation at 5800*g* for 15 min and subsequently incubated in a 20 ml medium containing 1 mg/ml collagenase-dispase (Hoffmann LaRoche, Mannheim, Germany) for 1.5–2 h. The cell suspension was filtered through a 150 μm Polymon® mesh (NeoLab, Heidelberg, Germany) and centrifuged for 10 min at 130*g*. The cell pellet was re-suspended in a medium containing 10% horse serum. This suspension was added to a discontinuous Percoll® (Amersham Pharmacia Biotech, Freiburg, Germany) gradient consisting of Percoll® 1.03 g/ml (20 ml) and 1.07 g/ml (15 ml). The loaded Percoll® gradients were centrifuged at 1000*g* for 10 min. Endothelial cells were enriched at the interface between the two Percoll® solutions. Cells were collected, washed in a medium containing 10% horse serum and filtered through a 150 μm Polymon® mesh. The final cell suspension was kept in a medium containing 20% horse serum and 10% DMSO and stored in liquid nitrogen until use.

2.4. Cell culture

Porcine endothelial cells were seeded onto rat tail-collagen (Roche, Mannheim, Germany) coated Transwell® membranes (12-well, polycarbonate filters, 0.4 μM pore size) or onto 96-well plates from Corning (Kaiserslautern, Germany) and were grown to confluency for 7 days. The medium (Medium 199 supplemented with 0.8 mM L-glutamine, penicillin/streptomycin (100 U/ml; 100 μg/ml), 100 μg/ml kanamycin and 10 mM Hepes, pH 7.4, and 10% horse serum) was changed every other day. Twenty-four hours before the experiment, the cells were set on serum-free medium (DMEM/HAM's F12 supplemented with 0.8 mM L-glutamine, penicillin/streptomycin (100 U/ml; 100 μg/ml), 100 μg/ml kanamycin and 10 mM Hepes, pH 7.4) to increase intracellular resistance.

Wild-type human BCRP over-expressing MDCKII cell line and its corresponding native cells were a kind gift from Dr. A.H. Schinkel, The Netherlands [32]. Cells were seeded onto rat tail-collagen-coated Transwell® membranes or onto 96-well plates (Corning, Kaiserslautern, Germany) at a density of 250,000 cells/cm^2 using Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Experiments were conducted on day 3.

2.5. Cytotoxicity assay

The sensitivity of PBCECs to mitoxantrone was determined using the AlamarBlue™ assay. The assay is based on the ability of mitochondrial dehydrogenase to cleave the tetrazolium rings of an MTT derivative [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] and to form formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the pink-coloured formazan product created. The colour of the AlamarBlue™ redox system can be quantified by fluorescence measurement (λ (excitation) = 530 nm, λ (emission) = 590 nm) (company data sheet, AlamarBlue™ Assay, Serotec, Düsseldorf, Germany). Briefly, cells were incubated up to 3 days in the absence or presence of transport modulators. Afterwards cells were washed twice with Krebs–Ringer buffer (KRB consisting of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM

NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) at room temperature. Porcine endothelial cells were incubated with AlamarBlue™ reagent (1:40 in KRB), and metabolic cell activity was measured for the following 6–8 h of monitoring, which increased sensitivity towards cytotoxic mitoxantrone.

2.6. Immunostaining

Isolated capillaries, porcine endothelial cells and MDCKII cells were fixed on slides for 20 min with 3% paraformaldehyde, 0.1% glutaraldehyde and 3.4% sucrose in PBS (phosphate-buffered saline consisting of 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4), washed, permeabilized for 15 min with 1% (v/v) Triton X-100 in PBS and washed again. The capillaries were incubated for 1 h with the primary antibodies BXP53 for the detection of porcine BCRP and BXP21 for the detection of hBCRP (Axxora, Grünberg, Germany). After washing, the capillaries were incubated for 45 min with the corresponding fluorochrome-conjugated secondary antibody, FITC-conjugated rabbit anti mouse/rat IgG, at a 1:20 dilution (Molecular Probes, Göttingen, Germany) in a humid chamber in the dark. Nuclei were counterstained with DAPI (1 μg/ml). Stained capillaries were viewed using confocal microscopy (Nikon C1Si-CLEM spectral imaging confocal laser scanning system, Nikon Imaging Center, Heidelberg).

2.7. Western blot

Protein samples were homogenized in lysis buffer (CelLytic™, Sigma–Aldrich, Germany) containing protease inhibitors (Complete, EDTA-free Protease Inhibitor Cocktail Tablets®, Roche Diagnostics, Mannheim, Germany). In order to detect the half-transporter BCRP as a single monomer band, the lysis buffer contained 50 mM DTT (Sigma–Aldrich, Germany). Lysates were spun down at 1000*g* and supernatants were centrifuged at 10,000*g* for 90 min. Membrane protein content was determined by using the BCA™ Protein Assay Kit (Pierce, Germany), which is based on the Biuret reaction. Membrane proteins were subjected to electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred electrophoretically on polyvinylidene difluoride membranes. The blots were then blocked overnight at 4 °C with PBS containing 1% nonfat dry milk powder and 1% bovine serum albumin (Sigma–Aldrich, Germany). After three washing steps with PBS, the proteins were hybridized for 75 min at room temperature with the monoclonal rat antibody BXP53 against BCRP in porcine endothelial cells and with monoclonal mouse antibody BXP21 against human BCRP in MDCKII cells (both from Axxora, Grünberg, Germany) (dilution 1:100 in blocking buffer). Subsequently, the blots were incubated with a secondary anti-mouse-/rat-horseradish peroxidase-conjugated antibody (KPL, Wedel, Germany) for 1 h at room temperature, followed by enhanced chemoluminescence detection (Western Lightning® Western blot Chemiluminescence Reagent Plus, Perkin-Elmer, Wiesbaden, Germany, ChemiDocXRS software).

2.8. Uptake assays

Uptake assays were performed at 37 °C using confluent monolayers of porcine brain capillary endothelial cells at day 7. Cells were grown in 96-well cell-culture plates. Cells were washed using transport buffer (Krebs-Ringer buffer). The uptake experiment was initiated by the addition of 200 μl transport buffer containing 5–25 μM mitoxantrone. Incubations were terminated after 3, 5, 10, 15, 20, 30, 60, 90 and 120 min by rapidly removing the incubation medium followed by washing the cells using ice-cold transport buffer. Finally, cells were lysed with 10% Triton X-100. Intracellular mitoxantrone fluorescence was detected as described in "transendothelial transport" measurements.

2.9. Transendothelial transport

For the study of transendothelial transport, cells were cultured in Corning Transwell® filter inserts for 7 days until confluency had been reached. Then, both compartments were equilibrated with pre-warmed transport buffer (Krebs–Ringer buffer). The whole system was kept at constant temperature (37 °C) and was supplied with 5% CO_2/95% oxygen. At time $t = 0$, mitoxantrone was added to the donor compartment and after 30, 60 and 90 min, respectively, samples were drawn from the acceptor chamber and were analysed in a fluorescence microplate reader (Tecan Safire^2, Tecan Group, Switzerland) with an excitation wavelength $\lambda = 614$ nm and emission wavelength $\lambda = 689$ nm. The acceptor chamber volume was readjusted with assay buffer after each sampling, and fluorescence values from acceptor side samples were corrected for the amount of compound removed by previous sampling. The initial rate of transport was calculated from a linear regression. Permeability coefficients (P_{app}) were calculated according to: $P_{app} = dQ/dt \cdot 1/A/C_0$ (cm s^{-1}), where dQ/dt is the rate of translocation, A is the surface of the filter membrane and C_0 is the initial concentration of the drug.

2.10. Fluorescence-activated cell sorting (FACS) uptake assay

Freshly isolated cells or porcine endothelial cells re-frosted from liquid nitrogen were incubated with 15 μM mitoxantrone alone (control) or with testing substances at increasing concentrations for 1 h. After the cells had been washed twice with ice-cold KRB, fluorescence was measured using a FACSCalibur™ flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) equipped with a 635 nm red diode laser and 670 nm band-pass filter. Stock solutions of inhibitors which were poorly soluble in buffer were prepared using dimethyl sulfoxide (DMSO) or ethanol. In this case the final concentration of DMSO or ethanol in the assay did not exceed 1% (v/v) or 0.5% (v/v), respectively. Control experiments were performed in the absence and presence of the respective solvent. DMSO or ethanol in the concentrations used had no detectable effect on the measured cell parameters.

The cell density of suspensions in DMEM/Ham's F12 1:1 was 2.5×10^7 cells/ml. By gating on forward and sideward scatter, endothelial cells were separated from the debris, and dead cells were excluded likewise using propidium iodide staining (1 μg/ml). Twenty thousand cells were sorted in one run. Data were processed and analysed with CellQuest™ Pro (Franklin Lakes, NJ, USA).

All fluorescence values were corrected by subtracting the background fluorescence. The increase in intracellular fluorescence caused by a test compound was referred to control fluorescence levels (100%) and is given as percentage of control.

To compare the findings of BCRP-related interactions with other substrates, cells were incubated likewise with 0.5 μM BODIPY-Prazosin and 10 μM Pheophorbide A for 1 h. Intracellular fluorescence was detected with λ (excitation) = 488 nm (argon laser) and a 530/30 band-pass filter to collect emitted fluorescence (BODIPY-Prazosin) and with λ (excitation) = 635 nm and a 670 band-pass filter for Pheophorbide A detection.

2.11. Statistics

All values are presented as means ± SEM. Control and treatment groups were compared by either Student's *t*-test or one-way analysis of variance, followed by a Dunnett's post hoc test. Differences were considered significant at $p^* < 0.01$, $p^{**} < 0.001$ and $p^{***} < 0.0001$. Concentration/effect plots were made by the use of the graphic software Prism (GraphPad Software, San Diego, USA).

3. Results

BCRP an export protein with an apparent molecular weight of 72 kDa significantly contributes to the barrier function of the blood–brain barrier. Next to P-glycoprotein (ABCB1), it plays an important role in maintaining brain homeostasis by limiting entry of xenobiotics including a large number of drugs into the CNS [24–27,42]. Therefore, we aimed to develop an assay, which can easily be used to study the effects of BCRP at the blood–brain barrier. Isolated endothelial cells from porcine brains have been cultured, since porcine brain tissue is easily available from local slaughterhouses. One preparation of 10 brains yields approximately 300×10^6 endothelial cells. Mitoxantrone transport, its applicability as BCRP substrate as well as assay conditions were evaluated and MDCKII cells over-expressing human wild-type BCRP (MDCKII-hBCRP cells) were used in addition to characterise transport results.

Fig. 1 shows the luminal localisation of BCRP in porcine brain microvessels (a) as well as the membrane localisation in freshly isolated endothelial cells (b), cultured brain capillary endothelial cells (c) and MDCKII-hBCRP (d) and corresponding native cells (e) by immunostaining. In addition, BCRP expression was analysed by Western blot (f). BCRP was clearly expressed in the membrane fraction of porcine endothelial cells compared to the brain homogenate and the capillary fraction as well as in the membrane of MDCKII-hBCRP cells, which is consistent with [43,44]. Looking in more detail at the expression in isolated endothelial cells a semi-quantitative analysis revealed clear differences: compared to freshly isolated cells, cells that had been stored in liquid nitrogen as well as cells which had been kept in monolayer cultures for 3–7 days showed a lower expression of BCRP (Fig. 2). Since no significant changes in the extent of BCRP expression was seen between thawed cells and cells being kept in culture for up to 7 days, the 7-day monolayers were used for permeation studies, since one-week culture seemed to be most appropriate to obtain confluent and tight cell monolayers.

Next we studied the uptake of the drug into PBCECs as a function of concentration and time. Fig. 3 shows the concentration-dependent uptake of mitoxantrone into PBCECs. Drug uptake reached steady state after approximately 60 min of incubation.

Moreover, the permeation of mitoxantrone across 7-day-old monolayer cultures of PBCECs was determined (Fig. 4). Permeation from the apical to the basolateral (a → b) compartment ("blood to brain") was rather low, with an apparent permeability coefficient of 5.6×10^{-6} cm s^{-1}, which was only very slightly influenced by addition of 10 μM fumitremorgin C (FTC) (P_{app} 6.0×10^{-6} cm s^{-1}), a potent inhibitor of BCRP-mediated transport [45]. In contrast, basolateral to apical (b → a) permeation ("brain to blood") was considerably higher (P_{app} 2.8×10^{-5} cm s^{-1}) and was significantly inhibited by 10 μM FTC (P_{app} 1.8×10^{-5} cm s^{-1}). This vectorial transport combined with the inhibition by FTC clearly indicates BCRP-mediated transport of mitoxantrone across the apical cell membrane.

In a comparative control experiment, permeation of mitoxantrone (20 μM) across MDCKII-hBCRP was determined. Whereas (a → b) permeability was negligible, (b → a) permeation of mitoxantrone was again considerably higher (P_{app} 7.3×10^{-6} cm s^{-1}) and could also be inhibited by 10 μM FTC (P_{app} 2.4×10^{-7} cm s^{-1}). Comparison of mitoxantrone permeation across native MDCKII cells and MDCKII-hBCRP cells revealed ratios of (b → a)/(a → b) transport coefficients of 9.1 and 73.0, respectively (porcine endothelial cells: 5.0). However, it had to be noted that in absolute values the (b → a) permeability reached only one-third of the value observed with PBCECs. This is in accordance with the different transcellular resistance values of both cell lines: porcine endothelial cells showed permeability coefficient values of the transcellular marker carboxyfluorescein of about 2.6×10^{-5} cm s^{-1} (a → b) and thus seemed to be leakier than the MDCKII cell line (P_{app} 2.1×10^{-6} cm s^{-1} in over-expressing MDCKII-hBCRP cells and P_{app} 9.1×10^{-7} cm s^{-1} in native MDCKII cells). The observed lower barrier tightness in porcine endothelial cells might have an impact on an increased apical to basolateral and basolateral to apical transport of mitoxantrone, and therefore might increase the absolute permeability values in comparison to those obtained from transport studies with MDCKII cells. In addition, [37] already determined permeability coefficients for mitoxantrone in the very MDCKII-derived hBCRP cell line yet presenting lower absolute

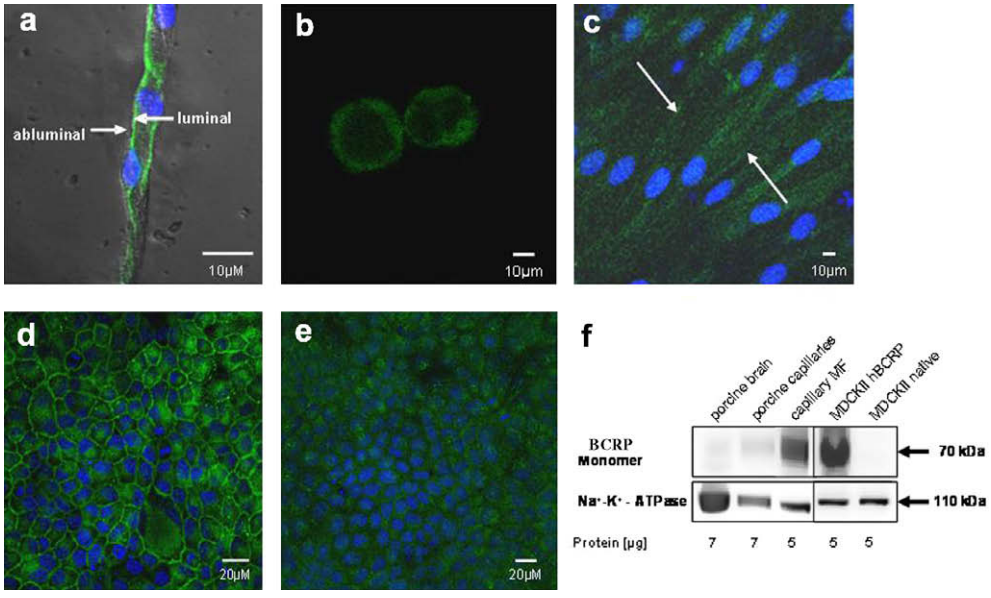

Fig. 1. Immunostaining of BCRP in an isolated porcine brain capillary (a), immunostaining of BCRP in freshly isolated porcine brain capillary endothelial cells (b), in a 7-day old PBCEC monolayer (c), in MDCKII-hBCRP cells (d) and in native MDCKII cells (e). Western blot analysis for BCRP in whole porcine brain homogenate, isolated porcine brain capillaries, the capillary membrane fraction, in MDCKII-hBCRP cells and in native MDCKII cells. Na^+–K^+–ATPase was used as internal standard (f).

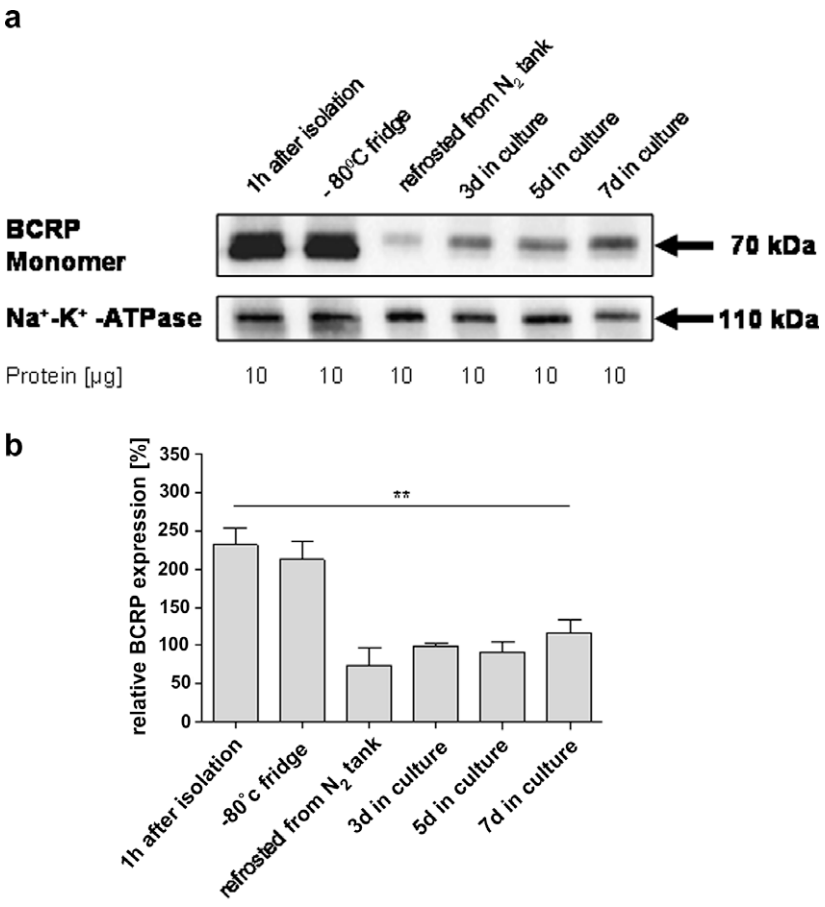

Fig. 2. Expression of BCRP in isolated PBCECs at different culture conditions as detected by Western blot (a). Densitometric analysis of Western blot: values are means ± SEM of four different experiments. BCRP monomer was normalized to the amount of internal standard Na^+–K^+–ATPase and is shown as a ratio [%] of the amount of BCRP protein expressed at day 7 after culture.

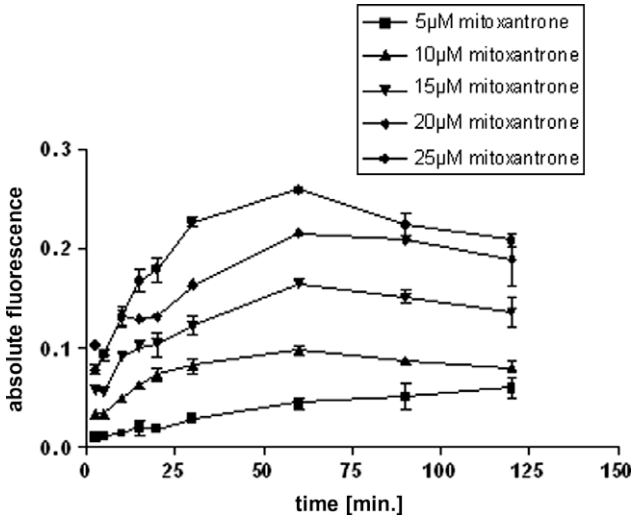

Fig. 3. Time – and concentration – dependent uptake of mitoxantrone into PBCECs (7-day old cultures).

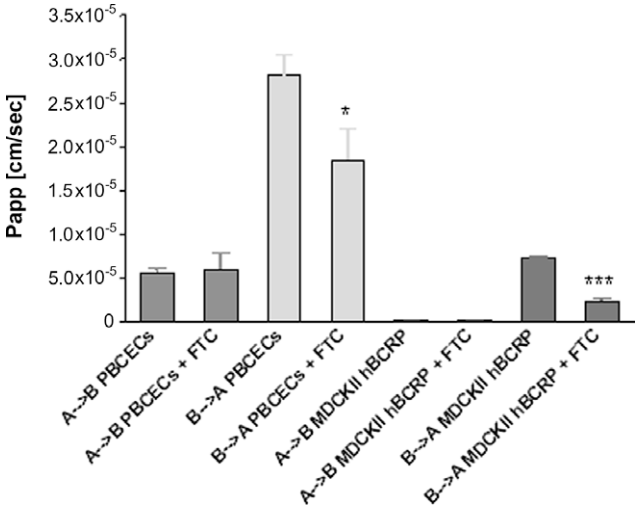

Fig. 4. Permeation of mitoxantrone (20 µM) across PBCECs (7-day old cultures) and MDCKII-hBCRP cells in the absence and presence of the BCRP inhibitor fumitremorgin C (10 µM).

transport values (P_{app} 2.0×10^{-6} cm s^{-1} (10 µM mitoxantrone) vs. 7.3×10^{-6} cm s^{-1} (20 µM mitoxantrone)). This could be due to different cell densities that were seeded onto the transwell membranes since a higher amount of cells (here about 250,000 cells/cm^2) might show a more significant vectored transport. In summary, the net ratios of efflux transport were comparable (MDCKII-hBCRP (b → a/a → b)/MDCKII (b → a/a → b) 6.1 at 10 µM mitoxantrone [37] and 8.0 at 20 µM mitoxantrone (present study). Finally, active mitoxantrone influx must not be disregarded at concentrations lower than 15 µM as was shown by Pan and Elmquist [46].

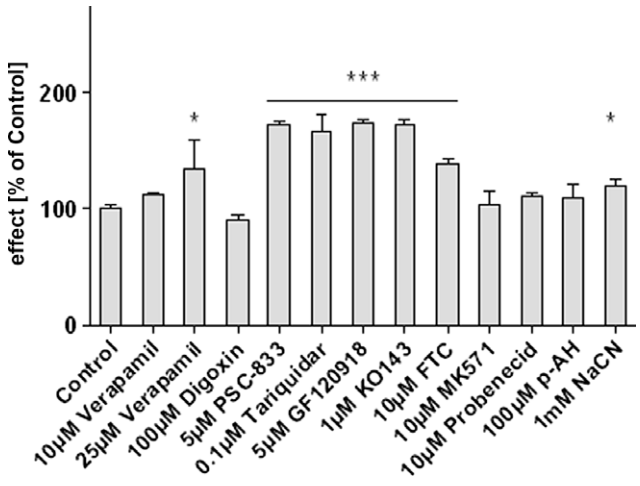

Fig. 5. Impact of transport protein inhibitors and substrates on mitoxantrone uptake (15 µM in medium) into 7-day old PBCECs.

To assess effects of other ABC-transporters such as P-gp, Mrp1 and Mrp2 on mitoxantrone transport, uptake of the drug into 7-day old PBCECs in the presence and absence of transport modulators was determined. The ability of digoxin (inhibitor of P-gp; [47]), MK571, (inhibitor of Mrp1 and Mrp2; [48]), probenecid and para-amino hippuric acid (p-AH) (inhibition of organic anion transporters; [49]), GF 120918 and tariquidar (inhibitors of P-gp and BCRP; [50,35]) and FTC and KO143 (inhibitor of BCRP; [45,51]) to affect uptake of mitoxantrone was evaluated. Cells were incubated for 60 min with 15 µM mitoxantrone in the presence of the mentioned modulators, and intracellular fluorescence was determined (Fig. 5). P-gp, Mrps and organic anion transporters had been shown to be expressed at the luminal side of brain capillaries [52–54]. However, neither digoxin nor MK571, probenecid or p-AH had a significant effect on the uptake of mitoxantrone, suggesting that these transporters have only a minor impact on cellular accumulation of the drug. In contrast, GF120918, KO143, tariquidar and PSC-833 significantly increased intracellular fluorescence intensity, presumably by inhibition of mitoxantrone efflux. Whereas FTC, GF120918 and KO143 had been described to be selective inhibitors of BCRP, the affinity of tariquidar and PSC-833 (both P-gp inhibitors) has not been really clarified. Also both appear to interact with BCRP, as the lack of effect of digoxin on mitoxantrone uptake suggests that P-glycoprotein is not involved. Mitoxantrone has been discussed to be a substrate not only for BCRP but also for P-glycoprotein [55,56] and Mrp1 [57,58], similar to BODIPY-Prazosin and Hoechst 3342 [59]. Our transport studies in porcine endothelial cells showed that higher concentrations of verapamil (25 µM) and PSC-833 (5 µM) significantly increased intracellular mitoxantrone accumulation. In comparison, the ratio of accumulated mitoxantrone fluorescence in MDCKII-hBCRP and native cells, which is to determine single BCRP-mediated drug transport and to exclude endogenous P-glycoprotein expression, only showed a significant increase after incubation with PSC-833, but not with verapamil (data not shown). This fact indicates that – based on raising intracellular mitoxantrone levels in a BCRP over-expressing cell line inhibited with PSC-833 – valspodar might also inhibit BCRP-mediated drug transport like its related substances elacridar (GF120918) and tariquidar (XR9576). Additionally, [60] discovered an inhibitory effect of verapamil on BCRP ATPase activity in Ehrlich ascites tumor tissue at concentrations higher than 50 µM. This supports our observation that verapamil inhibits mitoxantrone efflux at 25 µM but not at 10 µM. In a separate series of experiments, BCRP-mediated efflux of Pheophorbide A, which was analysed to be not a P-glycoprotein substrate [35], was also inhibited by verapamil in porcine endothelial cells (data not shown). Last, mitoxantrone did not increase intracellular calcein accumulation in PBCECs (data not shown) and thus appears to be not a high affinity substrate for porcine P-glycoprotein.

In a further set of studies, the sensitivity of PBCECs versus mitoxantrone was tested in a time- and concentration-dependent manner (Fig. 6a) by applying the AlamarBlue™ assay as viability criterium. The cells exhibited a relatively high viability even at longer incubation times for up to 24 h when the concentration did not exceed 1 µM. However, survival rates decreased at higher concentrations and at incubation times >90 min.

When the survival rates were studied after 3 days incubation, significant differences in the presence of ABC-export pump inhibitors could be observed (Fig. 6b–e). Cell survival gradually decreased in control experiments with increasing concentrations of

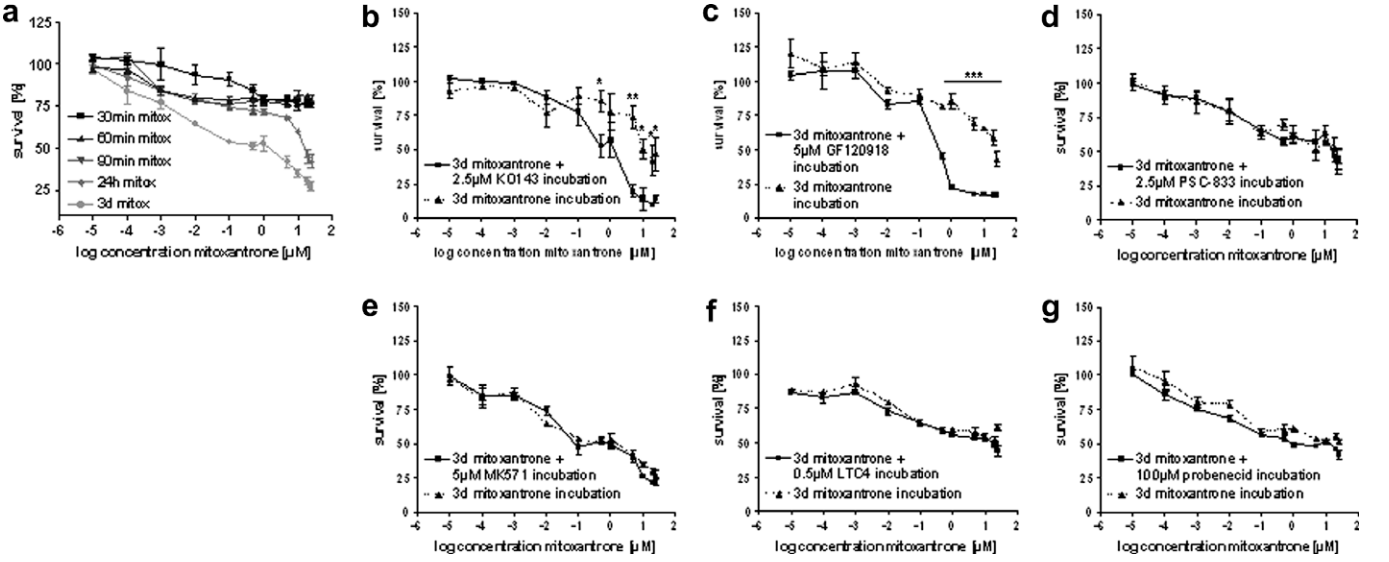

Fig. 6. (a) Sensitivity of PBCECs versus mitoxantrone as a function of time and concentration. Cell survival was tested by applying the AlamarBlue™ assay as viability criterium. (b–g) Cell survival after 3 days incubation with mitoxantrone in the absence and in presence of 2.5 µM KO143 (inhibition of BCRP), 5 µM GF120918 (inhibition of BCRP and P-gp), 2.5 µM PSC-833 (inhibition of P-gp), 5 µM MK571, 0.5 µM LTC_4 and 100 µM probenecid (inhibition of Mrps), respectively.

mitoxantrone in the absence and also in the presence of 5 μM MK571, 0.5 μM LTC_4, 100 μM probenecid (inhibition of Mrps) or 2.5 μM PSC-833 (inhibition of P-gp). Only in the presence of 5 μM GF120918 and 2.5 μM KO143 (Fig. 6f and g), a dramatic drop of viability could be observed at concentrations $\geqslant$0.5 μM, suggesting an increased sensitivity of the cells towards mitoxantrone by inhibition of BCRP, a subsequent increase in intracellular drug concentration followed by a decreased cell viability.

Since all experiments point to a BCRP-mediated transport of mitoxantrone in PBCECs, this transport was used for a screening assay to detect BCRP interactions with novel test compounds. Several assay variants were evaluated, e.g. preincubation or simultaneous incubation with the test item and mitoxantrone. Experiments with several BCRP inhibitors showed that highest effects were seen after 1 h simultaneous incubation of PBCECs with 15 μM mitoxantrone and 10 μM fumitremorgin C (data not shown).

Another technical aspect of assay performance is the shaking speed. During incubation with test compounds, the cells were shaken and by increasing the shaking speed from 200 to 700 rpm generally raised the intracellular fluorescence signal by a factor of approximately 1.5. Since PBCEC monolayers could not be shaken at higher speeds because of cell detachment from the microplate, only suspended PBCECs were incubated at this shaking speed. The increase in mitoxantrone accumulation may be the result of a better exposure of the suspended cells to the substances. In addition, the relation of the incubation solution/air surface to cell number is more favorable in Eppendorf tubes (suspension assay) than in 96-well microplates (monolayer/plate-reader assay). A larger surface means less free hydrophobic test compound available within the solution for P-glycoprotein interaction. Therefore, a smaller surface and better mixture during the incubation interval may be a prerequisite for improved cell–compound interactions of hydrophobic substances. A very similar observation has recently been made at a calcein-AM-based assay for P-glycoprotein comparing suspended and monolayers-cultured PBCECs [61].

Finally, based on these findings, standard test conditions were set up as follows: re-frosted porcine endothelial cells were simultaneously incubated with 15 μM mitoxantrone and test substances for 1 h (700 rpm) and washed twice with ice-cold KRB. Finally cells were re-suspended and intracellular fluorescence was detected via FACS analysis.

Currently, few BCRP modulators are known: fumitremorgin C, benzopyranones [62], estrogen agonists and antagonists [63], calcium channel blockers [64], HIV-protease inhibitors [65], flavonoids [66,67], immunosuppressants [68], coumermycin antibiotics [69] and tyrosine-kinase inhibitors [24,70]. Representative samples from these groups were tested for an interaction with the export protein in the mitoxantrone-based BCRP assay measuring the intracellular fluorescence accumulation in the presence of the test compounds. In addition, EC_{50} values for all tested BCRP effectors were calculated using an E_{max} model according to [71] (Table 1). Since the extent of inhibition of mitoxantrone export (i.e. increased mitoxantrone uptake) is measured and therefore no discrimination between transported substrates and non-transported inhibitors of BCRP can be done, all compounds interacting with BCRP are named "modulators" of the export protein, following a suggestion by Polli et al. [72]. Fig. 7 shows the concentration-dependent profiles for a series of drugs and phytopharmaceuticals, which had been described to interact with different affinities with the export pump including fumitremorgin C [45], novobiocin [66,73], 17-β-estradiol [63,74], estrone-sulfate [63,74], nicardipine [64], saquinavir, lopinavir [75], and naringenin [76].

All substances described to modulate BCRP increased mitoxantrone-related intracellular fluorescence. Thus, all compounds were correctly identified with regard to their predicted effect on BCRP.

Table 1
EC_{50} and E_{max} of tested compounds in porcine brain capillary endothelial cells.

Test substance	EC_{50} (μM)	E_{max} (%)
KO143	0.19 ± 0.16	241.0 ± 33.7
Fumitremorgin C	0.02 ± 0.00	192.3 ± 0.1
GF 120918	0.02 ± 0.03	165.2 ± 18.9
Lopinavir	27.59 ± 19.57	252.4 ± 19.6
Saquinavir	27.04 ± 19.99	310.6 ± 74.4
Nicardipine	1.74 ± 1.00	153.6 ± 5.75
Novobiocin	22.41 ± 16.99	199.0 ± 33.6
17-β-Estradiol	0.85 ± 0.41	143.9 ± 3.8
Naringenin	61.63 ± 49.94	308.1 ± 106.3
Estrone-sulfate	0.08 ± 0.05	122.6 ± 1.7
Genistein	51.56 ± 40.44	262.6 ± 74.9
Daidzein	0.13 ± 0.07	149.4 ± 3.6
Cyclosporine A	0.07 ± 0.04	137.79 ± 2.94

Interestingly, all these substances exhibited IC_{50} values in the submicromolar range making the discrimination solely based on IC_{50} difficult. Looking at the maximum effect, modulators could be classified as compounds with strong, moderate and weak effects on

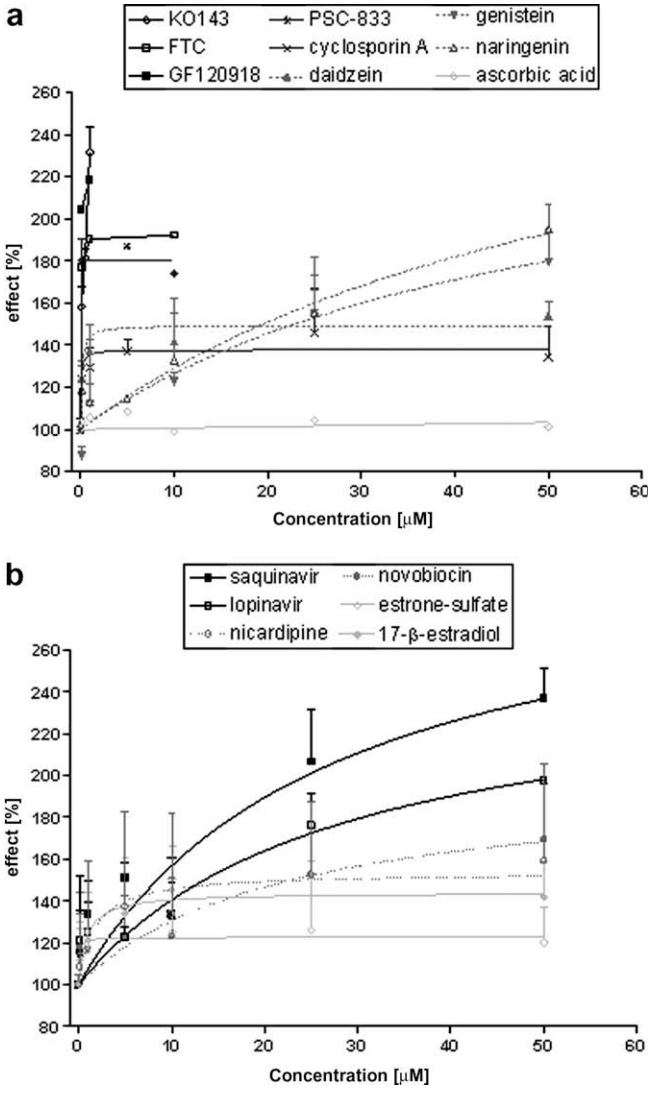

Fig. 7. Increase in intracellular fluorescence in PBCECs after simultaneous incubation with 15 μM mitoxantrone and various BCRP modulators for 1 h at 700 rpm in freshly isolated porcine brain capillary endothelial cells. Fluorescence was quantified by FACS-analysis.

BCRP. A very large increase in intracellular fluorescence greater than 160% above controls at concentrations between 1 μM and 5 μM was caused by KO143, FTC and GF120918. A somewhat weaker effect was seen by naringenin, saquinavir and lopinavir (effects >160% above control values at concentrations ⩾25 μM). 17-β-Estradiol, daidzein, nicardipine, PSC-833 and cyclosporin A showed intermediate effects with fluorescence values between 120% and 160% of controls at concentrations ⩾25 μM. Ascorbic acid and estrone-sulfate showed only little effects with fluorescence values ⩽120% above controls at 25 μM.

The low IC_{50} values correspond to a previous observation made with PBCECs in the calcein-AM assay for P-glycoprotein [77]. There, EC_{50} values were more than 70-fold lower than those determined by [78] using Caco-2VCR25 and those by [79] using MDR-CEM cells. This difference was most likely due to overexpression of the export pump in the cell lines in contrast to primary PBCECs. In over-expressing cell lines, the fluorescent indicator has to be used at higher concentrations to detect any baseline fluorescence. Additionally, effectors were also to be used at higher concentrations to detect increases in intracellular fluorescence [72]. Therefore, the sensitivity of primary PBCECs appears to be higher than the sensitivity of cell lines, over-expressing the respective export pump, consequently leading to lower EC_{50} by some orders of magnitude. This fact might be important in drug discovery and it might also play a role when information about the brain penetration of CNS-drugs is needed.

As prediction of interactions with ABC-transport proteins at the blood–brain barrier is important in screening drug candidates with CNS activity, use of simple cell-based assays may be helpful. Here we showed the use of a mitoxantrone-based assay with PBCECs. It is important to note that this assay is not able to distinguish between transported substrates of BCRP and compounds, which only inhibit BCRP function. This should be kept in mind, when drawing further conclusions in compound profiling. Nevertheless this test system proved to be a useful tool to study potential drug interactions with BCRP at the blood–brain barrier.

References

[1] M. Takano, R. Yumoto, T. Murakami, Expression and function of efflux drug transporters in the intestine, Pharmacol. Ther. 109 (2006) 137–161.
[2] F. Girardin, Membrane transporter proteins: a challenge for CNS drug development, Dial. Clin. Neurosci. 8 (2006) 311–321.
[3] H. Kusuhara, Y. Sugiyama, ATP-binding cassette, subfamily G (ABCG family), Pflugers Arch. 453 (2007) 735–744.
[4] S. Velamakanni, S.L. Wei, T. Janvilisri, H.W. van Veen, ABCG transporters: structure, substrate specificities and physiological roles: a brief overview, J. Bioenerg. Biomembr. 39 (2007) 465–471.
[5] L.A. Doyle, W. Yang, L.V. Abruzzo, T. Krogmann, Y. Gao, A.K. Rishi, D.D. Ross, A multidrug resistance transporter from human MCF-7 breast cancer cells, Proc. Natl. Acad. Sci. USA 22 (1998) 15665–15670.
[6] K. Miyake, L. Mickley, T. Litman, Z. Zhan, R. Robey, B. Cristensen, M. Brangi, L. Greenberger, M. Dean, T. Fojo, S.E. Bates, Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: Demonstration of homology to ABC transport genes, Cancer Res. 59 (1999) 8–13.
[7] R. Allikmets, L.M. Schriml, A. Hutchinson, V. Romano-Spica, M. Dean, A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance, Cancer Res. 58 (1998) 5337–5339.
[8] I. Ifergan, A. Shafran, G. Jansen, J.H. Hooijberg, G.L. Scheffer, Y.G. Assaraf, Folate deprivation results in the loss of breast cancer resistance protein (BCRP/Bcrp) expression. A role for BCRP in cellular folate homeostasis, J. Biol. Chem. 279 (2004) 25527–25534.
[9] A.E. van Herwaarden, J.W. Jonker, E. Wagenaar, R.F. Brinkhuis, J.H. Schellens, J.H. Beijnen, A.H. Schinkel, The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, Cancer Res. 63 (2003) 6447–6452.
[10] A.E. van Herwaarden, E. Wagenaar, G. Merino, J.W. Jonker, H. Rosing, J.H. Beijnen, A.H. Schinkel, Multidrug transporter Bcrp/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk, Mol. Cell Biol. 27 (2007) 1247–1253.
[11] K.J. Bailey-Dell, B. Hassel, L.A. Doyle, D.D. Ross, Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene, Biochim. Biophys. Acta 1520 (2001) 234–241.
[12] U. Henriksen, U. Gether, T. Litman, Effect of Walker A mutation (K86M) on oligomerization and surface targeting of the multidrug resistance transporter Bcrp, J. Cell Sci. 118 (2005) 1417–1426.
[13] A. Bhatia, U.H.J. Schäfer, C.A. Hrycyna, Oligomerization of the human ABC transporter ABCG2: evaluation of the native protein and chimeric dimers, Biochemistry 44 (2005) 10893–10904.
[14] K. Kage, S. Tsukahara, T. Sugiyama, S. Asada, E. Ishikawa, T. Tsuruo, Y. Sugimoto, Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization, Int. J. Cancer 97 (2002) 626–630.
[15] D.D. Ross, J.E. Karp, T.T. Chen, L.A. Doyle, Expression of breast cancer resistance protein in blast cells from patients with acute leukemia 96 (2000) 365–368.
[16] P.A. Fetsch, A. Abati, T. Litman, K. Morisaki, Y. Honjo, K. Mittal, S.E. Bates, Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues, Cancer Lett. 235 (2006) 84–92.
[17] M. Maliepaard, G.L. Scheffer, I.F. Faneyte, M.A. van Gastelen, A.C. Pijnenborg, A.H. Schinkel, M.J. van De Vijver, R.J. Scheper, J.H. Schellens, Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues, Cancer Res. 61 (2001) 3458–3464.
[18] J.D. Allen, A.H. Schinkel, Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2), Mol. Cancer Ther. 1 (2002) 427–434.
[19] O. Polgar, R.W. Robey, S.E. Bates, Bcrp: structure, function and role in drug response, Expert Opin. Drug Metab. Toxicol. 4 (2008) 1–15.
[20] T. Eisenblätter, H.J. Galla, A new multidrug resistance protein at the blood–brain barrier, Biochem. Biophys. Res. Commun. 293 (2002) 1273–1278.
[21] T. Eisenblätter, S. Hüwel, H.J. Galla, Characterisation of the brain multidrug resistance protein (BMDP/Bcrp/BCRP) expressed at the blood–brain barrier, Brain Res. 971 (2003) 221–231.
[22] S. Cisternino, C. Mercier, F. Bourasset, F. Roux, J.M. Scherrmann, Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood–brain barrier, Cancer Res. 64 (2004) 3296–3301.
[23] W. Zhang, J. Mojsilovic-Petrovic, M.F. Andrade, H. Zhang, M. Ball, D.B. Stanimirovic, The expression and functional characterization of Bcrp in brain endothelial cells and vessels, FASEB J. 17 (2003) 2085–2087.
[24] P. Breedveld, D. Pluim, G. Cipriani, P. Wielinga, O. van Tellingen, A.H. Schinkel, J.H. Schellens, The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients, Cancer Res. 65 (2005) 2577–2582.
[25] S. Bihorel, G. Camenisch, M. Lemaire, J.M. Scherrmann, Influence of breast cancer resistance protein (Abcg2) and P-glycoprotein (Abcb1a) on the transport of imatinib mesylate (Gleevec) across the mouse blood–brain barrier, J. Neurochem. 102 (2007) 1749–17457.
[26] N.A. de Vries, J. Zhao, E. Kroon, T. Buckle, J.H. Beijnen, O. van Tellingen, P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan, Clin. Cancer Res. 13 (2007) 6440–6449.
[27] J. Enokizono, H. Kusuhara, A. Ose, A.H. Schinkel, Y. Sugiyama, Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds, Drug Metab. Dispos. 36 (2008) 995–1002.
[28] J. Enokizono, H. Kusuhara, Y. Sugiyama, Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens, Mol. Pharmacol. 72 (2007) 967–975.
[29] P. Krishnamurthy, J.D. Schuetz, Role of Bcrp/BCRP in biology and medicine, Annu. Rev. Pharmacol. Toxicol. 46 (2006) 381–410.
[30] P. Breedveld, J.H. Beijnen, J.H. Schellens, Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs, Trends Pharmacol. Sci. 27 (2006) 17–24.
[31] R.W. Robey, Y. Honjo, A. van de Laar, K. Miyake, J.T. Regis, T. Litman, S.E. Bates, A functional assay for detection of the mitoxantrone resistance protein, MXR (Bcrp), Biochim. Biophys. Acta 1512 (2001) 171–182.
[32] P. Pavek, G. Merino, E. Wagenaar, E. Bolscher, M. Novotna, J.W. Jonker, A. Schinkel, Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine, and transport of cimetidine, J. Pharmacol. Exp. Ther. 312 (2005) 144–152.
[33] H. Minderman, A. Suvannasankha, K.L. O'Loughlin, G.L. Scheffer, R.J. Scheper, R.W. Robey, M.R. Baer, Flow cytometric analysis of breast cancer resistance protein expression and function, Cytometry 48 (2002) 59–65.
[34] H. Saito, H. Hirano, T. Ishikawa, High-speed screening and quantitative SAR analysis of human ABC transporter ABCG2 for molecular modeling of anticancer drugs to circumvent multidrug resistance, Mini Rev. Med. Chem. 7 (2007) 1009–1018.
[35] R.W. Robey, K. Steadman, O. Polgar, K. Morisaki, M. Blayney, P. Mistry, S.E. Bates, Pheophorbide a is a specific probe for Bcrp function and inhibition, Cancer Res. 64 (2004) 1242–1246.
[36] C.J. Henrich, H.R. Bokesch, M. Dean, S.E. Bates, R.W. Robey, E.L. Goncharova, J.A. Wilson, J.B. McMahon, A high-throughput cell-based assay for inhibitors of Bcrp activity, J. Biomol. Screen. 11 (2006) 176–183.
[37] Y. Xiao, R. Davidson, A. Smith, D. Pereira, S. Zhao, J. Soglia, D. Gebhard, S. de Morais, D.B. Duignan, A 96-well efflux assay to identify Bcrp substrates using a stably transfected MDCK II cell line, Mol. Pharm. 3 (2006) 45–54.

[38] U. Muenster, B. Grieshop, K. Ickenroth, M.J. Gnoth, Characterization of substrates and inhibitors for the in vitro assessment of Bcrp mediated drug–drug interactions, Pharm. Res. 35 (2008) 2320–2326.
[39] H. Glavinas, E. Kis, A. Pál, R. Kovács, M. Jani, E. Vági, E. Molnár, S. Bánsághi, Z. Kele, T. Janáky, G. Báthori, O. von Richter, G.J. Koomen, P. Krajcsi, Bcrp (breast cancer resistance protein/mitoxantrone resistance-associated protein) ATPase assay: a useful tool to detect drug–transporter interactions, Drug Metab. Dispos. 35 (2007) 1533–1542.
[40] P. Matsson, G. Englund, G. Ahlin, C.A. Bergström, U. Norinder, P. Artursson, A global drug inhibition pattern for the human ATP-binding cassette transporter breast cancer resistance protein (Bcrp), J. Pharmacol. Exp. Ther. 323 (2007) 19–30.
[41] J. Huwyler, G. Fricker, M. Török, M. Schneider, J. Drewe, Transport of clonidine across cultured brain microvessel endothelial cells, J. Pharmacol. Exp. Ther. 282 (1997) 81–85.
[42] D.J. Begley, ABC transporters and the blood–brain barrier, Curr. Pharm. Des. 10 (2004) 1295–1312.
[43] H.C. Cooray, C.G. Blackmore, L. Maskell, M.A. Barrand, Localisation of breast cancer resistance protein in microvessel endothelium of human brain, Neuroreport 13 (2002) 2059–2063.
[44] E. Rocchi, A. Khodjakov, E.L. Volk, C.H. Yang, T. Litman, S.E. Bates, E. Schneider, The product of the ABC half-transporter gene Bcrp (BCRP/MXR/ABCP) is expressed in the plasma membrane, Biochem. Biophys. Res. Commun. 271 (2000) 42–46.
[45] S.K. Rabindran, D.D. Ross, L.A. Doyle, W. Yang, L.M. Greenberger, Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein, Cancer Res. 60 (2000) 47–50.
[46] G. Pan, W.F. Elmquist, Mitoxantrone permeability in MDCKII cells is influenced by active influx transport, Mol. Pharm. 4 (2007) 475–483.
[47] Y. Tanigawara, N. Okamura, M. Hirai, M. Yasuhara, K. Ueda, N. Kioka, T. Komano, R. Hori, Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1), J. Pharmacol. Exp. Ther. 263 (1992) 840–845.
[48] H. Gutmann, M. Torok, G. Fricker, J. Huwyler, C. Beglinger, J. Drewe, Modulation of multidrug resistance protein expression in porcine brain capillary endothelial cells in vitro, Drug Metab. Dispos. 27 (1999) 937–941.
[49] D. Sykes, D.H. Sweet, S. Lowes, S.K. Nigam, J.B. Pritchard, D.S. Miller, Organic anion transport in choroid plexus from wild-type and organic anion transporter 3 (Slc22a8)-null mice, Am. J. Physiol. Renal Physiol. 286 (2004) F972–F978.
[50] C.M. Kruijtzer, J.H. Beijnen, H. Rosing, W.W. ten Bokkel Huinink, M. Schot, R.C. Jewell, E.M. Paul, J.H. Schellens, Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918, J. Clin. Oncol. 20 (2002) 2943–2950.
[51] J.D. Allen, A. van Loevezijn, J.M. Lakhai, M. van der Valk, O. van Tellingen, G. Reid, J.H. Schellens, G.J. Koomen, A.H. Schinkel, Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C, Mol. Cancer Ther. 1 (2002) 417–425.
[52] Y. Zhang, J.D. Schuetz, W.F. Elmquist, D.W. Miller, Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells, J. Pharmacol. Exp. Ther. 311 (2004) 449–455.
[53] H. Kusuhara, Y. Sugiyama, Active efflux across the blood–brain barrier: role of the solute carrier family, NeuroRx 2 (2005) 73–85.
[54] W. Löscher, H. Potschka, Drug resistance in brain diseases and the role of drug efflux transporters, Nat. Rev. Neurosci. 6 (2005) 591–602.
[55] S.E. Bates, R. Robey, K. Miyake, K. Rao, D.D. Ross, T. Litman, The role of half-transporters in multidrug resistance, J. Bioenerg. Biomembr. 33 (2001) 503–511.
[56] T. Litman, M. Brangi, E. Hudson, P. Fetsch, A. Abati, D.D. Ross, K. Miyake, J.H. Resau, S.E. Bates, The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2), J. Cell Sci. 113 (2000) 2011–2021.
[57] S.K. Diah, P.K. Smitherman, J. Aldridge, E.L. Volk, E. Schneider, A.J. Townsend, C.S. Morrow, Resistance to mitoxantrone in multidrug-resistant MCF7 breast cancer cells: evaluation of mitoxantrone transport and the role of multidrug resistance protein family proteins, Cancer Res. 61 (2001) 5461–5467.
[58] C.S. Morrow, C. Peklak-Scott, B. Bishwokarma, T.E. Kute, P.K. Smitherman, A.J. Townsend, Multidrug resistance protein 1 (MRP1, ABCC1) mediates resistance to mitoxantrone via glutathione-dependent drug efflux, Mol. Pharmacol. 69 (2006) 1499–1505.
[59] T. Litman, T.E. Druley, W.D. Stein, S.E. Bates, From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance, Cell Mol. Life Sci. 58 (2001) 931–959.
[60] D. Nielsen, J. Eriksen, C. Maare, T. Litman, E. Kjaersgaard, T. Plesner, et al., Characterisation of non-P-glycoprotein multidrug-resistant Ehrlich ascites tumour cells selected for resistance to mitoxantrone, Biochem. Pharmacol. 60 (2000) 363–370.
[61] M. Bubik, M. Ott, A. Mahringer, G. Fricker, Rapid assessment of P-glycoprotein–drug interactions at the blood–brain barrier, Anal. Biochem. 358 (2006) 51–58.
[62]] A. Boumendjel, E. Nicolle, T. Moraux, B. Gerby, M. Blanc, X. Ronot, J. Boutonnat, Piperazinobenzopyranones and phenalkylaminobenzopyranones: potent inhibitors of breast cancer resistance protein (ABCG2), J. Med. Chem. 48 (2005) 7275–7281.
[63] Y. Sugimoto, S. Tsukahara, Y. Imai, Y. Sugimoto, K. Ueda, T. Tsuruo, Reversal of breast cancer resistance protein-mediated drug resistance by estrogen antagonists and agonists, Mol. Cancer Ther. 2 (2003) 105–112.
[64] Y. Zhang, A. Gupta, H. Wang, L. Zhou, R.R. Vethanayagam, J.D. Unadkat, Q. Mao, BCRP transports dipyridamole and is inhibited by calcium channel blockers, Pharm. Res. 22 (2005) 2023–2034.
[65] A. Gupta, Y. Zhang, J.D. Unadkat, Q. Mao, HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/Bcrp), J. Pharmacol. Exp. Ther. 310 (2004) 334–341.
[66] Y. Imai, S. Tsukahara, S. Asada, Y. Sugimoto, Phytoestrogens/flavonoids reverse breast cancer resistance protein/Bcrp-mediated multidrug resistance, Cancer Res. 64 (2004) 4346–4352.
[67] S. Zhang, X. Yang, M.E. Morris, Flavonoids are inhibitors of breast cancer resistance protein (Bcrp)-mediated transport, Mol. Pharmacol. 65 (2004) 1208–1216.
[68] A. Gupta, Y. Dai, R.R. Vethanayagam, M.F. Hebert, K.E. Thummel, J.D. Unadkat, D.D. Ross, Q. Mao, Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (Bcrp) and reverse resistance to mitoxantrone and topotecan, Cancer Chemother. Pharmacol. 58 (2006) 374–383.
[69] K. Shiozawa, M. Oka, H. Soda, M. Yoshikawa, Y. Ikegami, J. Tsurutani, K. Nakatomi, Y. Nakamura, S. Doi, T. Kitazaki, Y. Mizuta, K. Murase, H. Yoshida, D.D. Ross, S. Kohno, Reversal of breast cancer resistance protein (BCRP/Bcrp)-mediated drug resistance by novobiocin, a coumermycin antibiotic, Int. J. Cancer 108 (2004) 146–151.
[70] Y. Nakamura, M. Oka, H. Soda, K. Shiozawa, M. Yoshikawa, et al., Gefitinib (“Iressa”, ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor, reverses breast cancer resistance protein/Bcrp-mediated drug resistance, Cancer Res. 65 (2005) 1541–1546.
[71] J. Gabrielsson, D. Weiner, Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Application, Swedish Pharmaceutical Society, 1997.
[72] J.W. Polli, S.A. Wring, J.E. Humphreys, L. Huang, J.B. Morgan, L.O. Webster, C.S. Serabjit-Singh, Rational use of in vitro P-glycoprotein assays in drug discovery, J. Pharmacol. Exp. Ther. 299 (2001) 6220–6628.
[73] C. Yang, Y.C. Chen, M.L. Kuo, Novobiocin sensitizes BCRP/MXR/ABCP over-expressing topotecan-resistant human breast carcinoma cells to topotecan and mitoxantrone, Anticancer Res. 23 (2003) 2519–2523.
[74] Y. Imai, S. Tsukahara, E. Ishikawa, T. Tsuruo, Y. Sugimoto, Estrone and 17beta-estradiol reverse breast cancer resistance protein-mediated multidrug resistance, Jpn. J. Cancer Res. 93 (2002) 231–235.
[75] J. Weiss, J. Rose, C.H. Storch, N. Ketabi-Kiyanvash, A. Sauer, W.E. Haefeli, T. Efferth, Modulation of human BCRP (Bcrp) activity by anti-HIV drugs, J. Antimicrob. Chemother. 59 (2007) 238–245.
[76] S. Zhang, X. Yang, M.E. Morris, Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport, Pharm. Res. 21 (2004) 1263–1273.
[77] B. Bauer, D.S. Miller, G. Fricker, Compound profiling for P-glycoprotein at the blood–brain barrier using a microplate screening system, Pharm. Res. 20 (2003) 1170–1176.
[78] E. Eneroth, E. Åström, J. Hoogstraate, D. Schrenk, S. Conrad, H.-M. Kauffmann, K. Gjellan, Evaluation of a vincristine resistant caco-2 cell line for use in a calcein-AM extrusion screening assay for P-glycoprotein interaction, Eur. J. Pharm. Sci. 12 (2001) 205–214.
[79] F. Tiberghien, F. Loor, Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay, Anti-Cancer Drugs 7 (1996) 568–578.