Compound Profiling for P-Glycoprotein at the Blood–Brain Barrier Using a Microplate Screening System

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Purpose. The purpose of this study was to establish a fluorescent dye (calcein–acetoxymethylester; calcein-AM)-based assay to rapidly screen compounds for interactions with p-glycoprotein (p-gp) at the blood–brain barrier and to determine whether such an assay can be useful for kinetic analysis.

Methods. Porcine brain capillary endothelial cells (PBCECs) were isolated and cultured in 96-well plates. Cells were incubated with calcein-AM in the absence and presence of substrates and inhibitors of ABC transporters and the extent of intracellularly appearing fluorescence was monitored with a fluorescence plate reader in a time- and a concentration-dependent manner.

Results. PBCECs showed stable expression of p-gp and as a result calcein-AM was extruded by the cells. In the presence of p-gp substrates and inhibitors a significant increase of intracellular fluorescence was observed (decreased calcein-AM efflux), the increase being well correlated with the p-gp affinity of the compounds used. Inhibitors of Mrp1 and Mrp2 did not influence fluorescence intensity. Time-dependent readouts and Michaelis-Menten kinetic analysis separated inhibitors into those showing competitive, mixed and non-competitive inhibition of p-glycoprotein-mediated transport.

Conclusion. The calcein-AM-assay based on PBCECs can be used as a rapid microplate screening system for interactions of drugs with p-glycoprotein at the blood-brain barrier and represents therefore a useful tool in the profiling of drugs. In addition, convenient kinetic assays can provide information about the mode of interaction.

KEY WORDS: blood–brain barrier; calcein-AM; compound profiling; microplate screening system; p-glycoprotein.

INTRODUCTION

The multidrug resistance gene product p-glycoprotein (p-gp) is a primary cellular constituent maintaining the barrier function of brain capillary endothelial cells. This ATP-driven drug efflux pump, located in the luminal membrane of endothelial cells, plays a significant role in controlling the central nervous system (CNS) exposure to many endogenous and exogenous substances by transporting them back into the blood circulation (1–3). P-gp is also present in primary brain tumors where it plays a crucial role in their clinical resistance to chemotherapy (4). Therefore, it is important to develop appropriate screening systems for compound profiling in early drug development. Several *in vitro* assays have been developed to aid drug candidate selection and optimization, for example, an ATPase activity assay (5), a monolayer efflux assay (6), a [^{3}H] colchicine accumulation assay (7), and fluorescent dye assays, such as a rhodamine 123 based test system (8) or a calcein–acetoxymethylester (calcein-AM)-based assay (9). However, each assay system has drawbacks. Cell monolayer efflux assays measuring p-gp-mediated transport directly are time- and cost-intensive, and because of low substrate concentrations, special analytics are often required. Fluorescent dye systems allow the rapid screening of a large number of compounds. However, the fluorescent marker substrates do not always exhibit exclusive specificity for p-gp but are also recognized by other ABC proteins, for example, Mrps. The direct use of a fluorescent marker, such as rhodamine 123, which is added to the test system and not formed inside the cells, requires additional washing steps, which may affect the cell monolayer integrity and result in erroneous readouts. In addition, the result may be influenced by extensive mitochondrial trapping of the fluorescent dye (10). Furthermore, when cell lines are used that overexpress p-gp, the sensitivity of the assay may be affected and data interpretation may become difficult because of the high amount of efflux protein in the plasma membrane, which requires relatively high concentrations of competitors to see inhibitory effects.

Considering the difficulties and drawbacks of available p-gp test systems, we wanted to develop an assay that could be used directly with brain capillary endothelial cells. In the present study, we decided to adapt a calcein-AM-based assay for use with primary cultured porcine brain capillary endothelial cells (PBCECs). In this assay, the fluorescent dye is formed by intracellular hydrolysis of nonfluorescent calcein-AM, a p-gp substrate (11). When p-gp is inhibited calcein-AM efflux is reduced and it enters the cells where it is hydrolyzed to calcein, a fluorescent organic anion. Calcein accumulation is measured as an indicator of p-gp inhibition. The assay was validated with a series of known p-gp-modulating agents with different affinities for the transporter. In addition to steady-state readouts, we modified the screening system to provide kinetic data, allowing the distinction of different modes of interactions of candidate drugs with p-gp.

MATERIALS AND METHODS

Isolation of PBCECs

PBCECs were isolated following recently described protocols (12,13). Brains of freshly slaughtered animals were cleaned from meninges, choroid plexus, and superficial blood vessels. Cortical gray matter was removed from brain tissue, minced to 1- to 2-mm^3 cubes and incubated in Medium 199, supplemented with 0.8 mM L-glutamine, penicillin/streptomycin (100 U mL^{-1}; 100 μg mL^{-1}), 100 μg/mL gentamicin and 10 mM HEPES, pH 7.4 (all Biochrom, Berlin, FRG). Dispase II (Hoffmann LaRoche, Mannheim, FRG) was added to a final concentration of 0.5% and the brain tissue was incubated for 2 h at 37°C. Afterwards, the homogenate was centrifuged at 1000 g for 10 min. The supernatant was discarded and the pellet was resuspended in medium now containing 15% dextran (Sigma, Taufkirchen, FRG). Microvessels were separated from other brain tissue by centrifugation at 5800 g for 15 min and subsequently incubated in 20

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mL of medium containing 1 mg mL^{-1} collagenase-dispase (Hoffmann LaRoche, Mannheim, FRG) for 1.5 to 2 h. The resulting cell suspension was filtered through a 150-μm Polymon® mesh (NeoLab Migge, Heidelberg, FRG) and centrifuged for 10 min at 130 g. Then, the cell pellet was resuspended in medium containing 9% horse serum (Biochrom, Berlin, FRG). This suspension was added to a discontinuous Percoll® (Pharmacia, Freiburg, FRG) gradient consisting of Percoll® 1.03 g mL^{-1} (20 mL) and 1.07 g mL^{-1} (15 mL). The loaded Percoll® gradients were centrifuged at 1000 g for 10 min. Endothelial cells were enriched at the interface between the 2 Percoll® solutions. Cells were collected, washed in medium with 9% horse serum and filtered through a 150-μm Polymon® mesh. The final cell suspension was kept in medium containing 20% horse serum and 10% DMSO and stored in liquid nitrogen until use.

Cell Culture

For immunostainings and calcein-AM assay experiments, porcine brain capillary endothelial cells were seeded at 250,000 cells/cm^2 on rat tail collagen-coated (Hoffmann LaRoche, Mannheim, FRG) 8-well Permanox® chamber slides (Nunc, Wiesbaden, FRG) and 96 well cell culture clusters (Costar, Wiesbaden, FRG), respectively. Cells were cultured under standard cell culture conditions for 7 days using isolation medium without gentamicin, but with 9% horse serum. Cell culture medium was changed every other day. One day prior to experiments the medium was changed to DMEM/Ham's F12 (1:1), supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 U mL^{-1}; 100 μg mL^{-1}), and 10 mM HEPES.

Immunostaining of P-gp

PBCEC monolayers grown on chamber slides were washed three times with 4°C cold Krebs–Ringer buffer (KRB), followed by fixation for 30 min with 4°C cold 3% p-formaldehyde/0.1% glutardialdehyde/3.4% sucrose. Subsequently, the cells were washed three times with 4°C KRB, permeabilized with 0.1% Triton-X-100 at room temperature, and again washed (three times, 4°C KRB). Nonspecific adsorption was prevented by treatment with 1% bovine serum albumin for 30 min at 37°C.

For immunostaining of p-gp cells were incubated for 60 min at 37°C with a 1:20 dilution of the monoclonal mouse IgG2a primary antibody C219 (Alexis, Grünberg, FRG). Cell nuclei were stained using 1.5 μM propidium iodide. The incubation was stopped by washing three times with 4°C KRB. Primary antibody binding was detected using a 1:20 dilution of a FITC-labeled secondary anti-mouse IgG antibody (Chemicon, Hofheim, FRG) for 60 min at 37°C. The cells were washed three times with 4°C KRB. Then, the chamber on top of the slide was removed and the cells were embedded with Aqua Poly Mount® (Polysciences, Heidelberg, FRG) and sealed using a coverslip and commercially available colorless nail varnish. Fluorescence microscopy was performed using a Leitz Dialux 22 microscope (Leica Microsystems, Bensheim, FRG) combined with a Visicam Visitron-1300 camera (Visitron Systems, Puchheim, FRG).

Calcein-AM Assay

For all compounds tested, stock solutions were prepared in DMSO. Dilutions were then made with KRB. The final concentration of DMSO on the cells did not exceed 1%. At this concentration, DMSO did not affect the assay. To perform calcein-AM-MDR-assay studies, cells were washed three times with 37°C KRB and subsequently incubated with increasing concentrations of the test compounds for 15 min at 37°C. Calcein-AM (MoBiTec, Göttingen, FRG) was added to a final concentration of 1 μM and incubated for 30 min at 37°C. Afterwards, the cells were immediately washed three times with ice-cold KRB and lysed with 1% Triton-X-100. Fluorescence was measured using a Fluoroskan Ascent® plate reader (Labsystems, Helsinki, FIN) with λ excitation = 485 nm and λ emission = 520 nm. Each test compound was measured with n = 12, control and background were measured with n = 12. For each tested compound at least 2 experiments were performed to prove reproducibility.

All fluorescence values were corrected by subtracting the background fluorescence. The increase in cellular fluorescence caused by a test compound was referred to the fluorescence of the control (100%). The EC_{50} of compounds tested in the calcein-AM-MDR-assay was obtained by using an E_{max} model according to References 14 and 15.

For calcein-AM-kinetic-assay studies, the cells were incubated with increasing concentrations of the test compounds for 15 min at 37°C. Immediately after adding calcein-AM to a final concentration of 1, 2, or 3 μM, respectively, the cells were placed in the fluorescence reader with a stage temperature of 37°C. Fluorescence was measured each 30 s for a total time of 60 min. Each concentration of a test compound was measured with n = 4, control and calcein-AM auto-hydrolysis were measured with n = 4, and background was measured with n = 12. For each tested compound two repetition experiments were performed to control reproducibility.

All fluorescence values were corrected by subtracting background and calcein-AM auto-hydrolysis. PSC-833 induced fluorescence was the same for all tested concentrations (0.1/0.5/1/2/5 μM) and was therefore used as reference for complete inhibition of p-gp. The difference of the PSC-833 induced fluorescence and the fluorescence values of the control as well as of the test compounds for 1, 2, and 3 μM calcein-AM, respectively, were plotted against time. The slopes of the curves between 5 and 30 min were determined in order to set up kinetics according to Michaelis-Menten. To determine the type of the interaction between a test compound and p-gp, data were plotted according to Lineweaver-Burk, apparent K_m and V_{max} were estimated and K_i was calculated.

Statistics

Data are given as mean ± SD Means were judged to be significantly different when $p < 0.05$ using an unpaired Student's t test.

RESULTS AND DISCUSSION

P-gp provides an important function in maintaining brain homeostasis by limiting the entry of xenobiotics into the central nervous system, amongst them an array of drugs (16,17). P-gp is an integral part of the blood–brain barrier and serves as a marker for cerebral capillary endothelial cells (18,19). Using the Western Blot technique, p-gp has been identified in

brain capillaries by several groups including our own (20,21). Here, we used immunostaining to demonstrate p-gp expression in primary cultures of PBCECs, which is of highest importance regarding the development of a screening system for drug interactions with p-gp (Fig. 1).

To study drug–p-gp interactions a calcein-AM-assay was used. In this assay calcein-AM serves as a high-affinity marker for p-gp activity (11). Calcein-AM is a nonfluorescent, lipophilic, and therefore highly cell-permeable ester. Once inside the cell the ester bonds are rapidly cleaved by nonspecific esterases generating highly fluorescent calcein, which is trapped inside the cell because of its hydrophilic nature and charge. Because calcein-AM is a substrate for p-gp, inhibition of the transporter decreases efflux, which results in a higher intracellular calcein fluorescence (Fig. 2).

We used two complementary models of the calcein-AM assay to investigate interactions of drugs with p-gp: 1) the calcein-AM-multidrug resistance-assay (calcein-AM–MDR assay) to determine concentration-dependent effects of drugs on p-gp at steady state; this is a high-throughput screening system (9), and 2) the calcein-AM-kinetic assay to determine the type of interaction between a drug and p-gp. To validate the assay with regard to its sensitivity and correctness, experiments were conducted with compounds well characterized for their interaction with p-gp. These standards were previously classified by Seelig (22) as p-gp substrates (codeine, cyclosporin A, digoxin, ivermectin, loperamide, morphine, nicardipine, noscapine, papaverine, PSC-833, quinidine, ritonavir, saquinavir, taxol, verapamil, and yohimbine) and nonsubstrates (adrenaline, ascorbic acid, benzylpenicillin, clonidine, caffeine, diazepam, and sucrose), respectively.

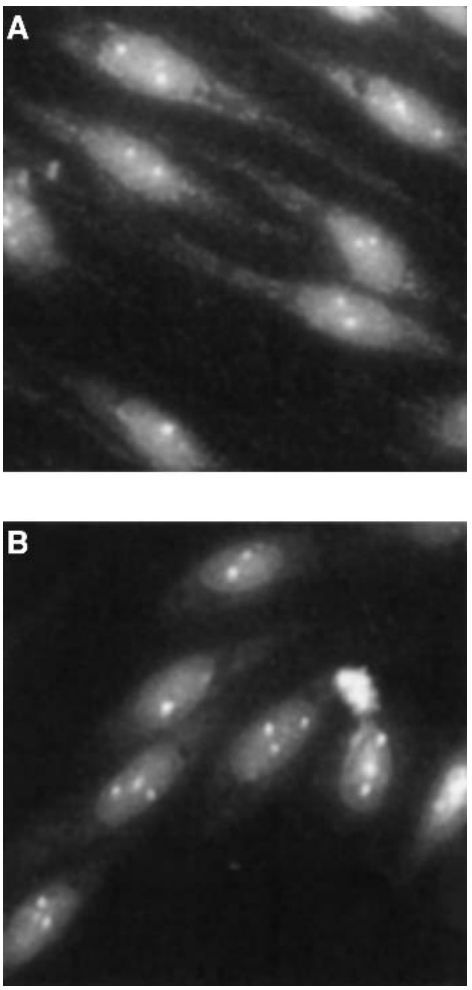

Fig. 1. Immunostaining of p-glycoprotein in primary cultures of PBCEC after 7 days in culture. Positive controls (A) were incubated with the monoclonal mouse IgG2a primary antibody C219 against p-glycoprotein and a FITC-labeled secondary anti-mouse IgG antibody. Negative controls (B) were incubated with secondary antibody only. Cell nuclei were stained with propidium iodide.

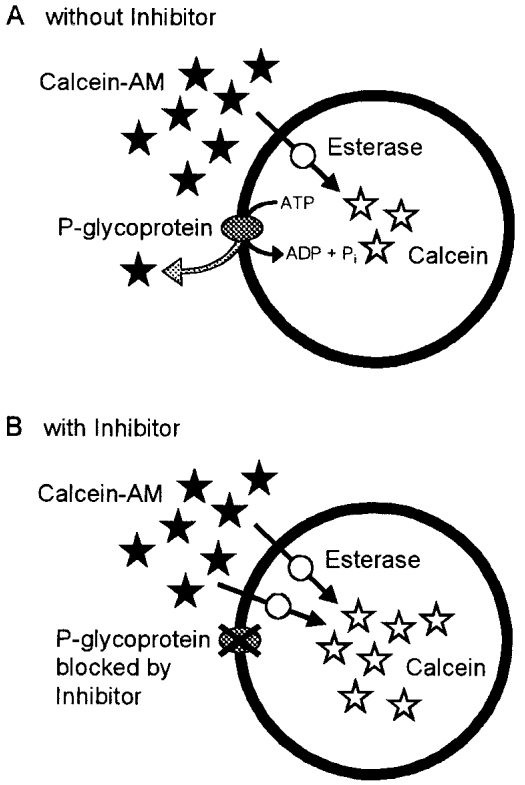

Fig. 2. Lipophilic, nonfluorescent calcein-AM readily permeates the cell membrane and enters the cell (A). Unspecific cytosolic esterases cleave the ester bond whereby highly fluorescent calcein is generated. Because of to its hydrophilicity, calcein will not cross the lipid bilayer and is therefore trapped inside the cell. Because calcein-AM is a high-affinity p-gp substrate, it is actively transported out of the plasma membrane and thereby prevented from entering the cell. Drugs interacting with p-gp, either in a competitive or noncompetitive manner, increase the amount of calcein-AM entering the cell, which results in an increase of cytosolic calcein fluorescence (B).

Figure 3 shows the concentration-dependent increase in cellular fluorescence of the tested compounds. All substances defined by Seelig (22) to be p-gp substrates increased intracellular calcein fluorescence (Fig. 3a). Thus, all compounds were correctly identified with regard to their predicted effect on p-gp. Interestingly, the group of p-gp substrates could further be classified as compounds with strong, moderate and weak effects on p-gp. A large increase in intracellular calcein fluorescence was caused by cyclosporin A, PSC-833, ivermectin, ritonavir, and nicardipine with effects >500% at concentrations up to 10 μM (Fig. 3a). Higher concentrations of these compounds could not be tested due to their low solubility in aqueous solutions. Taxol, noscapine, saquinavir, papaverine, verapamil, quinidine, morphine, and loperamide showed

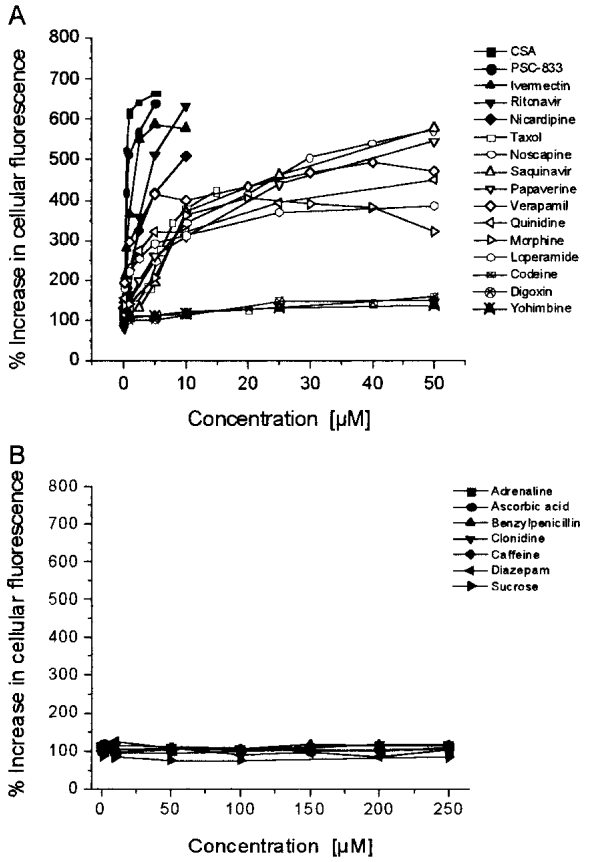

Fig. 3. Effects of p-gp modulators (A) and nonmodulators (B) tested in the calcein-AM-MDR assay (control = 100%, n = 6). P-gp modulators were classified as strong (effect > 500% at ≤ 10 μM), moderate (effect of 250–500% at 10-50 μM), or weak (effect of 125–250% at 10–50 μM), respectively. Compounds with an effect ≤ 125% at 10–250 μM were classified as non-modulators. For clarity, error bars are omitted.

moderate effects on p-gp with 250–500% increases of intracellular fluorescence intensity in the concentration range from 10 to 50 μM. Weak effects (125–250%) were observed with codeine, digoxin, and yohimbine (10–50 μM). All compounds predicted to be nonsubstrates of p-gp, namely adrenaline, ascorbic acid, benzylpenicilline, clonidine, caffeine, diazepam, and sucrose, did not increase intracellular calcein fluorescence at all in the concentration range tested (all effects < 125%, Fig. 3b). These results are in accordance with the findings of others with the tested compounds (22,23), indicating the reliability of the calcein-AM-MDR assay using PBCECs to identify p-gp substrates correctly and showing the high sensitivity of the model to distinguish between different interactions of test compounds with p-gp.

In addition, EC_{50} values for all tested p-gp effectors were calculated using an E_{max} model according to Gabrielsson and Weiner (14) and were compared to previously published EC_{50} obtained with Caco-2VCR25 cells (15) and MDR-CEM cells (24), respectively. Table I shows calculated EC_{50} values ranging from 0.6 ± 0.3 μM for cyclosporin A to 52.7 ± 8.3 μM for digoxin. The ranking of the test compounds according to their calculated EC_{50} shows a good correlation with the classification in strong, moderate and weak effectors on p-gp observed in figure 3a. However, verapamil, nicardipine, ritonavir, and

Table I. EC_{50} of Compounds Tested in the Calcein-AM-MDR Assay Using Porcine Brain Capillary Endothelial Cells (PBCECs) in Comparison to Caco-2VCR25 and MDR-CEM Cells

		Cell culture PBCECs	Caco-2VCR25	MDR-CEM
Test compound	Effect on p-gp[a]	EC_{50} [μM]	EC_{50} [μM][b]	EC_{50} [μM][c]
Cyclosporin A	S	0.6 ± 0.3	7	3.4 ± 0.6
Verapamil	M	0.8 ± 0.3	70	22.5 ± 10.6
PSC-833	S	0.8 ± 0.3		0.4 ± 0.1
Ivermectin	S	1.0 ± 0.2	9	
Loperamide	M	1.2 ± 0.4	16	
Quinidine	M	2.0 ± 0.8	157	30.5 ± 9.0
Nicardipine	S	3.4 ± 0.4		
Morphine	M	4.7 ± 2.9		
Ritonavir	S	8.1 ± 3.2		
Papaverine	M	14.3 ± 2.1		
Noscapine	M	16.8 ± 2.6		
Yohimbine	W	16.9 ± 9.8		
Saquinavir	M	21.9 ± 6.8		
Taxol	M	29.8 ± 19.3		
Codeine	W	44.3 ± 22.9		
Digoxin	W	52.7 ± 8.3		

Note: Test compounds are listed according to their EC_{50}. EC_{50} were determined using an E_{max} model according to (13).
[a] according to figure 3a.
[b] EC_{50} values obtained from (14).
[c] EC_{50} values obtained from (23).
S, strong; M, moderate; W, weak.

yohimbine show a somewhat deviating behavior. The dose-response curve of verapamil shown in Fig. 3a is characterized by a steep increase at low concentrations (< 5 μM), which is comparable to strong effectors, but is then followed by a plateau with only a slight additional effect at higher concentrations (5–50 μM). This also applies for yohimbine, though less markedly. Nicardipine and ritonavir show in comparison to the other strong effectors a less steep increase at a concentration < 5 μM, which is similar to the one of moderate effectors. Considering that calculation of EC_{50} using the E_{max} model is based on the shape of the dose-response curves, the overestimated potency of verapamil and yohimbine and the underestimated potency of nicardipine and ritonavir in affecting p-gp activity, respectively, according to their EC_{50} can be explained. Surprisingly, PSC-833 with an EC_{50} of 0.8 ± 0.3 μM was not the most potent compound of all standards tested, although PSC-833 is considered to be 5-10 fold more potent *in vivo* than, for example, cyclosporin A (25). The difference in activity on p-gp *in vivo* is caused by a lower protein binding of PSC-833 of 50%, whereas the protein binding of cyclosporin A is 97%. However, under experimental conditions *in vitro* using serum-free buffer cyclosporin A is not bound to proteins and therefore as potent as PSC-833 (7,26), which explains the similar EC_{50} values in our assay.

The ranking of the compounds according to their EC_{50} as tested in PBCECs correlates well with the data from Caco-2VCR25 cells (verapamil not taken into consideration) and with MDR-CEM cells (PSC-833 not taken into consideration). However, EC_{50} values determined using PBCECs are up to more than 70 fold lower than those determined by

Eneroth et al. (15) using Caco-2VCR25 and by Tiberghien and Loor (24) using MDR-CEM cells. This striking difference is most likely due to p-gp overexpression in the cell lines in contrast to primary cultured PBCEC. In p-gp overexpressing cell lines calcein-AM has to be used at higher concentrations to detect any baseline fluorescence. Additionally, p-gp effectors have also to be used at higher concentrations to detect increases in intracellular calcein fluorescence (27). This is in agreement with Eneroth *et al.* (15), who saw remarkable differences in Caco-2VCR25 cells compared to Caco-2VCR100 cells. Therefore, the sensitivity of primary cultured PBCEC is much higher than the sensitivity of p-gp overexpressing cell lines, consequently leading to EC_{50} lower by some orders of magnitude. This fact might be important in the drug discovery of p-gp-affecting compounds on the one hand and it might play a role when information about the brain penetration of CNS drugs is needed on the other hand.

Because calcein-AM is a substrate of Mrp1 (11) and calcein is a substrate of both, Mrp1 and Mrp2 (28), the effect of these outwardly directed ABC transporters was studied as possible confounding influences on the calcein-AM-assay for p-gp. Figure 4 shows the effect of two compounds well known to interact with Mrp-proteins, leucotriene C_4 (LTC_4), and MK571, on the assay performance. 0.5 μM LTC_4 produced no statistically significant increase of cellular fluorescence, whereas 2.5 μM MK571 and LTC_4 plus MK571 did increase fluorescence ($p < 0.05$). The following results support these data. PBCEC were incubated with PSC-833, cyclosporin A, verapamil, and ivermectin to inhibit p-gp activity, and, in addition treated with 2.5 μM MK571 to inhibit Mrp1 (Fig. 5a). Only the treatment with 5 μM ivermectin plus 2.5 μM MK571 showed a significant effect ($P < 0.05$) over 5 μM ivermectin alone. None of the other combinations of p-gp effector and MK571 showed a significant change. This indicates only a small influence of Mrp1 on the calcein-AM-assay. This is also true for Mrp2. In Fig. 5b, none of the combinations of p-gp effector and LTC_4 had any effect compared to the p-gp effector alone. These findings confirm observations of others, who show a significant downregulation of Mrp-protein expression in primary cultures of PBCECs (Török et al., University Hospital Basel, personal communication). Presumably the cells lose their Mrp expression in culture, which would explain the minor effects observed with LTC_4 and MK571 in the present assay setup. Interestingly, this is contradictory to some cultures of immortalized rat cells (29).

According to these results the calcein-AM assay seems to be specific for drugs interacting with p-gp in this cell culture model. However, Eisenblätter and Galla (30) have recently shown that in primary porcine brain capillary endothelial cell cultures a protein is expressed, which is most related to the human and mouse breast cancer resistance protein. This protein in addition to p-gp might play an important role in the exclusion of xenobiotics from the brain. Therefore, an influence of that protein on our assay cannot completely be excluded.

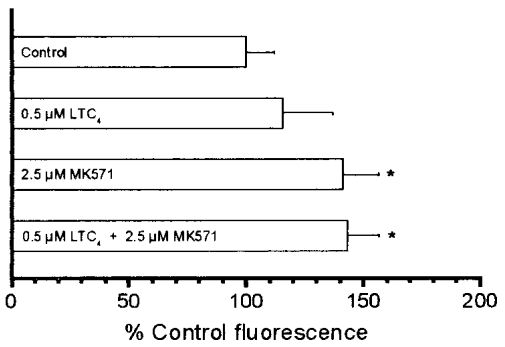

Fig. 4. Influence of Mrp1 and Mrp2 effectors on the calcein-AM assay. Data shown as % of control fluorescence. Mean ± SD, n = 6, *$p < 0.05$.

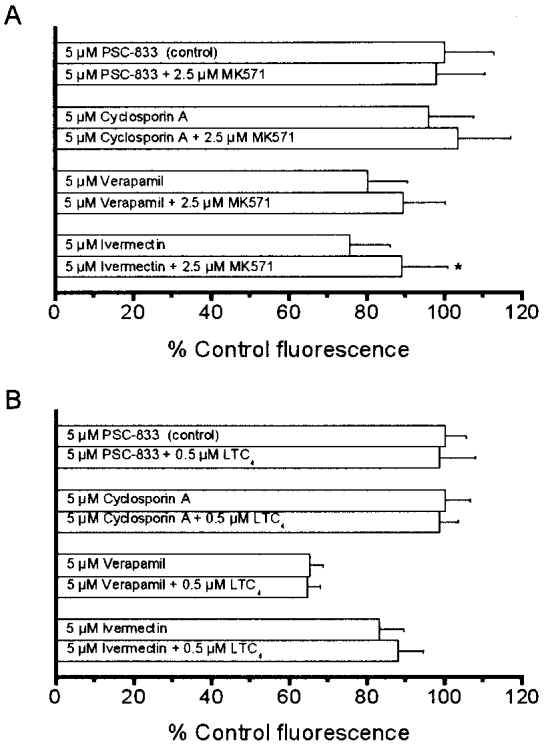

Fig. 5. Influence of Mrp1 (A) and Mrp2 (B) on the calcein-AM assay. Data shown as % of control fluorescence (5 μM PSC-833). Mean ± SD, n = 6, *$p < 0.05$ to ivermectin.

Because the calcein-AM-MDR assay does not allow us to distinguish the mechanism of inhibition, we established a calcein-AM kinetic-assay to determine the type of interaction between drugs and p-gp. Nicardipine, PSC-833, ritonavir, taxol, and vinblastine were taken as test compounds, with calcein-AM as substrate for p-gp. The transporter was assumed to turn over by an enzyme-like mechanism as described previously in the literature (11,31,32). To determine the mode of p-gp–drug interaction, curves were plotted according to Lineweaver-Burk. Apparent K_m and V_{max} were estimated from the lines and, when appropriate, K_i was calculated. Experiments showed that the activity of p-gp was completely blocked with 0.1 μM PSC-833 (not shown). Therefore, PSC-833 was taken as reference for complete inhibition of p-gp. Figure 6 depicts V vs. S and Lineweaver-Burk plots for nicardipine (a), and vinblastine (b). For nicardipine the Lineweaver-Burk plot indicates a mixed-type inhibition (Fig. 6a), suggesting more than one binding site for p-gp as already proposed by others (33–35). The calculated K_i for nicardipine was 1.37 ± 0.63 μM (Table II). The data for vinblastine (Fig. 6b) indicate competitive inhibition, with a $K_i = 0.70 \pm 0.41$ μM. For ritonavir and taxol, Lineweaver-Burk plots were similar to vinblastine in that both caused competitive inhibi-

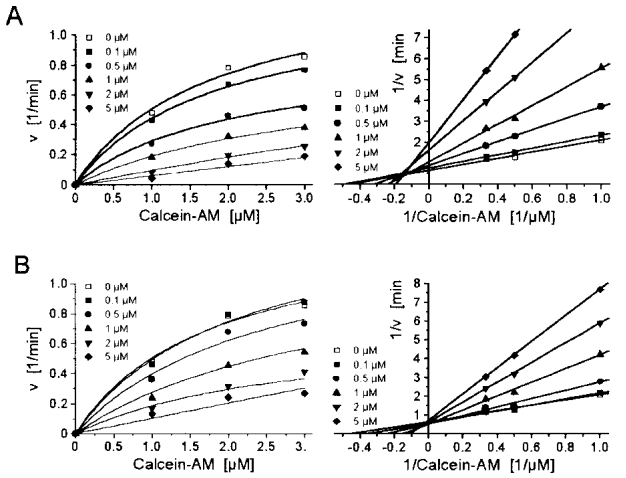

Fig. 6. V vs. S and Lineweaver-Burk plots for Nicardipine (A) and Vinblastine (B) using the calcein-AM-kinetic-assay to determine the type of interaction between drugs and p-glycoprotein (n = 4).

tion (not shown). Table II summarizes K_i and type of p-gp inhibition of the drugs tested in the calcein-AM-kinetic assay.

CONCLUSIONS

Reliable prediction of interactions with p-gp at the blood–brain barrier and characterization of the kinetics of this process may be useful in screening drug candidates with CNS activity. In the present study, we introduced a microplate screening system to determine interactions of drugs with p-gp at the blood–brain barrier *in vitro*. The system is based on a calcein-AM-assay and primary cultures of porcine brain capillary endothelial cells. Constant p-gp expression in these cells was demonstrated by immunostaining. Using the calcein-AM-MDR assay, a series of reference compounds was correctly identified with regard to their proposed interaction on p-gp (22). Thus, a mutual validation was performed, one for the prediction model and the second for the assay itself. It is important to note, that the assay is not able to distinguish between transported substrates of p-gp and compounds, which only inhibit p-gp function. This should be kept in mind, when drawing further conclusions in compound profiling.

Compounds interacting with p-gp could further be distinguished on the basis of their strong, moderate or weak effects in the calcein-AM-MDR-assay. EC_{50} of all compounds affecting p-gp activity were calculated using an E_{max} model. The ranking of the drugs according to their EC_{50} correlated well with published data. Importantly, EC_{50} determined in primary cultures of PBCECs were up to 70 fold lower than those determined in p-gp overexpressing cell lines, indicating a higher sensitivity of primary cultured brain capillary endothelial cells. Mrp1 and Mrp2 do not affect or only have a negligible influence on the calcein-AM assay, as indicated by experiments with MK571 and LTC_4. Furthermore, using the calcein-AM-kinetic assay, we could show different types of interaction between drugs and p-gp, which may become of special interest when interactions with different binding sites on p-gp are studied (35).

Table II. K_i and Mode of P-gp Inhibiton of Compounds Tested in the Calcein-AM, Kinetic Assay

Test compound	K_i [μM]	Mode of inhibition
Nicardipine	1.4 ± 0.6	Mixed
Vinblastine	0.7 ± 0.4	Competitive
Ritonavir	2.1 ± 1.1	Competitive
Taxol	10.6 ± 6.1	Competitive

Together, the data indicate that the calcein-AM assay based on primary cultures of porcine brain capillary endothelial cells can be used as a rapid microplate screening system for p-gp interactions of drugs at the blood–brain barrier and therefore is a useful tool in the compound profiling of drugs.

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