Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 396–403
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Compound profiling for ABCC2 (MRP2) using a fluorescent microplate assay system

Frank Förster [a], Astrid Volz [b], Gert Fricker [a,*]

[a] Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, Heidelberg, Germany
[b] Boehringer Ingelheim GmbH, Biberach, Germany

Received 17 July 2007; accepted in revised form 3 October 2007
Available online 9 October 2007

Abstract

Purpose: To establish a fluorescent dye (Glutathione methylfluorescein, GSMF) based assay to rapidly screen compounds for drug efflux interactions with the ABC-protein ABCC2 (MRP2). *Methods:* MDCK-cells overexpressing ABCC2 were cultured until confluency in 96-well plates. Cells were incubated with chloromethylfluorescein-diacetate (CMFDA) in the absence and presence of increasing concentrations of potential substrates and inhibitors of ABCC2. After formation of GSMF the extent of intracellular fluorescence was monitored with a fluorescence plate reader in a time- and a concentration-dependent manner. *Results:* MDCK cells showed stable expression of ABCC2 and, as a consequence, GSMF was extruded by the cells across the apical membrane in an energy-dependent manner. The incubation conditions (optimum CMFDA concentration; glutathione dependency, membrane toxicity) were elaborated. Determination of intracellular glutathione concentration indicated that under the chosen conditions glutathione is not rate limiting for the assay performance. Known inhibitors of ABCB1 (P-GP) and ABCG2 (BCRP) did not influence intracellular fluorescence intensity, but a significant increase of intracellular fluorescence was observed in the presence of MRP2-substrates and inhibitors accompanied with a concomitant decrease of GSMF efflux. *Conclusions:* The GSMF-assay based on fluorescence accumulation in MRP2-overexpressing MDCK cells can be used as a rapid microplate screening system for interactions of drugs with MRP2 and therefore represents a useful tool in drug profiling.

Keywords: MRP2; ABCC2; CMFDA; Fluorescence assay; ABC-protein

1. Introduction

Membrane transport proteins play a crucial role in absorption, distribution and elimination of many drugs, drug candidates and metabolites. Particularly the export proteins ABCB1 (P-GP; MDR1-gene product; *p*-glycoprotein), ABCC1/2 (MRP1/2) and ABCG2 (BCRP) gain interest due to their role in drug resistance of tumor cells as well as their protective function in barrier tissues like gut, placenta, liver, kidney and blood–brain barrier. The fact that these export proteins recognize drugs as substrates has led to search and development of inhibitors for, e.g., P-GP, such as Valspodar (PSC-833), Elacridar (GF120918), Zosuquidar (LY335979), Tariquidar (XR9576), and others [1].

In parallel, test systems have been set up to screen novel drug candidates with respect to potential interactions with ABC export proteins. For P-GP fluorescent based calcein-AM assays have been established, which can be used with P-GP over-expressing cell lines, Caco-2 cells [2,3] as well as brain capillary endothelial cells [4,5]. Alternatively, the uptake of Rhodamin-123 can be determined in absence or presence of P-GP inhibitors, or the consumption of ATP is used as a measure of P-GP activity [6,7].

Whereas these assay systems have been generally accepted to evaluate potential interactions with P-GP,

* Corresponding author. Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, INF 366, 69120 Heidelberg, Germany. Tel.: +49 6221 548336; fax: +49 6221 545971.
E-mail address: gert.fricker@uni-hd.de (G. Fricker).

0939-6411/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2007.10.003

relatively little has been done to establish similar assays for other ABC-proteins, e.g., members of the ABCC family, although their importance for drug disposition is comparable to that of P-GP. One study examined the effects of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its fluorescent free acid, BCECF, on ABC transporters, showing that the ester is a mixed substrate of P-GP/BCRP, whereas after hydolysis inside the cell the free acid was recognized by MRP [8].

A decade ago, ABCC2 (MRP2) was identified in rat and human liver, which was also called 'canalicular Multispecific Organic Anion Transporter' (cMOAT) [9–12]. Similar to P-GP it is also expressed in other barrier tissues, however its substrate selectivity differs from that of P-GP in recognizing predominantly organic anions, glutathione conjugates and small peptides [13–15].

The goal of the present work was the design of an assay for MRP2, which is reliable, easy to perform and allows the screening of larger numbers of compounds with respect to interaction with MRP2.

2. Materials and methods

2.1. Cells

MDCKII-MRP2 transfected with human ABCC2 were a kind gift from Prof. Dr. P. Borst (Netherland Cancer Institute, Amsterdam, NL). For transfection the retroviral vector (pCMV)-neo had been used, in which a HindIII–NcoI DNA fragment containing the complete predicted MRP2 open reading frame (GenBank Accession No. U49248) was inserted, resulting in pCMV-cMRP2 [16].

2.2. Materials

Murine antibodies against MRP2 (clone M_2III-6) and against Na^+/K^+-ATPase (clone M7-PB-E9) as well as secondary anti-mouse-IgG-FITC antibodies were obtained from Alexis, Grünberg, FRG. Cyclosporin A was from Novartis (Basle, CH), Cerivastatin was from Bayer-Schering (Leverkusen, FRG). All other materials were obtained from the usual commercial sources at the highest purity available.

2.3. Cytotoxicity assay (Alamar Blue assay)

Mitochondrial dehydrogenases play a major role in energy supply of cells. Their activity can be used as indicator of cellular vitality. Mitochondrial dehydrogenase activity was measured with Alamar blue, a MTT derivative. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay [17] is based on the ability of mitochondrial dehydrogenase to cleave the tetrazolium rings of MTT and form formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals, which are solubilised. The number of surviving cells is directly proportional to the level of the pink coloured formazan product created. The colour can then be quantified using a simple colorimetric assay.

Cell monolayers cultured in 96-well plates were washed twice with KRB at 37 °C and then incubated with test compound for 90 min at 37 °C. After the incubation monolayers were washed twice with KRB to remove turbid medium. Before removal of the buffer after the last washing step, cell monolayer integrity was checked by an inverse microscope. Buffer was replaced by 200 μl Alamar blue reagent (5 μl Alamar blue solution + 195 μl KRB) per well. Ninety-six-well plates were placed in a preheated fluorescent plate reader (Fluoroscan Ascent, Labsystems, Frankfurt, FRG, or Tecan Safire XFLUOR4, Tecan Safire, Crailsheim, FRG) at 37 °C. Increasing fluorescence of reduced Alamar blue was measured over 8 h at 539/590 nm to detect conversion of the dye by mitochondrial dehydrogenases. All experiments were performed as quadruplicate.

2.4. Determination of glutathione

Intracellular glutathione was determined with an assay (Cayman, IBL Immuno Biological Laboratories, Hamburg, FRG) based on the reaction of sulfhydryl groups with 5,5′-dithiobis-nitrobenzoic acid (DTNB, Ellman's reagens) under formation of 5-thiobenzoic acid (TNB). Eventually formed mixed disulfide GSTNB is processed to TNB and GSH by glutathione reductase. According to the recommendation of the supplier, the cells were not trypsinized prior to fluorescent measurements, since proteolytic enzymes tamper the results. Therefore, the results were referred to cm^2 monolayer area.

2.5. Immunostaining

MDCKII-MRP2 cells were cultured on 8-well object slides, which had been coated with rat tail collagen. After careful washing with KRB, the cells were incubated for 30 min with fixation solution (*p*-formaldehyde 3.0 mg, 0.4 ml 25% glutardialdehyde, saccharose 3.4 g, PBS ad 100 ml) prior to a 10 min permeabilisation with Triton X-100 (0,1%) in KRB. After a washing step the cells were incubated for 30 min with blocking solution (1% BSA in KRB). Then, the cells were incubated overnight with 100 μl primary antibody (clone M2III-6, mouse IgG2a; Alexis, Grünberg, FRG) against MRP2. Subsequently, cell monolayers were washed 3× with KRB and then incubated with secondary antibody (anti-mouse-IgG-FITC from sheep) Alexis, Grünberg, FRG) for 1 h. After a final washing step, the cells were coated with Aqua Polymount® and subjected to fluorescence microscopy (Leitz Dialux 22 mikroscope, (Leica Microsystems, Heidelberg, FRG).

2.6. Uptake studies

Cells were kept in culture in 24 and in 96-well plates, respectively. Prior to transport experiments, they

were washed twice with KRB at 37 °C. Then, the cells were incubated with 1 μM CMFDA in absence or in presence of potential MRP2 modulators at increasing concentrations. Typically the modulators had been dissolved in DMSO. In control experiments we confirmed that the cell monolayers tolerated up to 1% DMSO in the incubation medium without functional impairment. All monolayers were incubated for 90 min at 37 °C and under constant circular shaking at 50 rpm. Then, the culture plates were placed on ice, medium in the apical compartment was removed and the cells were washed twice with ice-cold KRB. Then, the cells were lysed by incubation with 200 μl of 1% Triton X-100 in KRB for 30 min under constant shaking. Finally, the culture plates were subjected to fluorescence quantification in a fluorescent plate reader (Tecan Safire XFLUOR4, Tecan Safire, Crailsheim, FRG) with filter settings of λ_{ex} of 485 nm and λ_{em} of 516 nm. The extent of transport inhibition in presence of MRP2-modulators was quantified from fluorescence values using the software GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

2.7. Permeation experiments

To determine directed permeation of MRP2-substrates MDCKII-MRP2 cells were cultured on permeable Transwell© cell culture inserts. Prior to the experiments, cell culture medium was replaced by KRB at 37 °C (0.5 ml in the apical compartment, 1.5 ml in the basolateral compartment). Cells were allowed to recover from media change for 30 min at 37 °C, then the transepithelial resistance was determined and the transport experiments were started by adding test compounds to either the apical or basolateral compartment. The culture plates were kept at 37 °C at orbital shaking (50 rpm, min) and samples of 100 μl were collected every 15 min for up to 90 min. Aliquots were replaced by buffer. Samples were pipetted onto 96-well plates and excreted fluorescent compound was quantified in a fluorescence plate reader (Fluoroscan Ascent, Labsystems, Frankfurt, FRG, or Tecan Safire XFLUOR4, Tecan Safire, (Crailsheim, FRG).

2.8. Western blot analysis

Cell monolayers were washed twice with KRB and homogenized in mammalian tissue lysis buffer (CelLytic® MT Cell Lysis Reagent for mammalian tissues, Sigma–Aldrich) supplemented with protease inhibitors (Complete, EDTA free Protease Inhibitor Cocktail Tablets®, Roche Diagnostics, Mannheim). After lysates had been spun down at 1000g supernatants were centrifuged at 10,000*g* for 90 min and pellets were resuspended in lysis buffer. Protein content was determined by using the BCA™ Protein Assay Kit (Pierce, Rockford), which is based on the Biuret reaction.

Proteins (45 μg per lane) were subjected to electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred electrophoretically on polyvinylidene difluoride membranes at 350 mA for 60 min. The blots were then blocked overnight at 4 °C with PBS containing 1% non-fat dry milk powder and 1% bovine serum albumin (Sigma–Aldrich). Then, the blotting membranes were incubated for 1 h with 10 ml primary antibody (1:50) against MRP2 (MAB M2III-6, murine IgG2a, Alexis, Grünberg, FRG). Primary antibody solution was removed and the membranes were incubated for 1 h with secondary antimouse-horseradish peroxidase-conjugated antibody (1:10,000) from sheep (KPL, Gaithersburg, MD, USA), followed by enhanced chemoluminescence detection (Immun-Star AP Chemoluminescent Kit, Bio-Rad Laboratories, Hercules, CA, ChemiDoc XRS software).

3. Results

3.1. Assay principle

CMFDA, 5-chloromethylfluorescein-diacetate (Fig. 1), is a non-fluorescent, lipophilic derivative of fluorescein, which easily permeates across the cell membrane. Inside the cells it is cleaved by unspecific esterases forming the fluorescent intermediate 5-chloromethylfluorescein CMF. CMF is hydrophilic and exhibits a very slow permeation across cell membranes. In a second step, the chloromethyl group reacts with intracellular thiol groups, e.g., glutathione. Glutathione methylfluorescein, GSMF, is produced, which is even more hydrophilic. GSMF is a substrate of MRP2 [18–20] and is actively excreted out of the cells. Interactions of test-compounds with MRP2 result in an increased intracellular fluorescence intensity.

3.2. Detection of MRP2 in MDCKII-MRP2 cells

Prior to transport experiments MRP2 was detected by Western blotting and immunostaining, respectively. Western blots revealed a strong signal in the MW range of

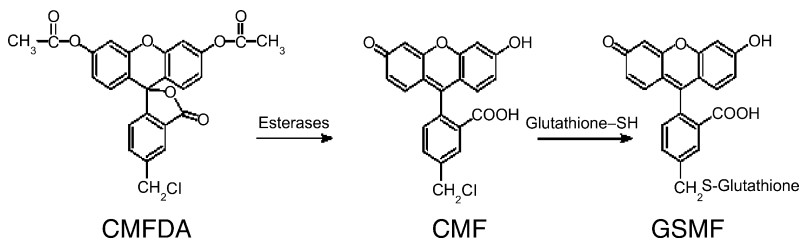

Fig. 1. Structure of chloromethylfluorescein diacetate (CMFDA), CMF (chloromethylfluorescein) and GSMF (glutathione methylfluorescein).

190 kDa. Immunostaining showed predominant localisation of MRP2 in the cell membrane (Fig. 2).

3.3. ATP-dependency of GSMF-transport

ABC-protein mediated transport is coupled to the consumption of ATP and, therefore, it is sensitive to metabolic poisoning. One possibility to identify ATP-dependent processes is incubation of cells with cyanide, which inhibits cytochrome oxidase and, thus, the formation of cellular ATP. In order to study whether GSMF excretion is ATP-dependent MDCKII-MRP2 cells were pre-incubated with 0.1 and 1.0 mM NaCN, respectively, and then the formation of extracellular GSMF was determined after 45 min incubation with 1.0 μM CMFA. Preincubation with increasing doses of cyanide led to a concentration dependent decrease of extracellular GSMF (data not shown), indicating that excretion of GSMF by MDCKII-MRP2 cells is indeed an energy-dependent process.

3.4. Directed excretion of GSMF

Functional activity of export proteins in cell monolayers can be determined by preferential transport of a substrate to the luminal (apical) or abluminal (basolateral) buffer compartment dependent on the localisation of the studied export protein in either membrane [21]. To study directed excretion of GSMF, confluent cell monolayers were incubated with 1 μM CMFDA from the apical or basolateral compartment, respectively, and time-dependent excretion of GSMF was determined. After 90 min $64.4 \pm 4.2\%$ and $3.7 \pm 1.3\%$ of applied CMFDA were recovered as GSMF from the apical and basolateral compartment, respectively (Fig. 3). When transport was studied in presence of 100 μM MK571, a potent inhibitor of rodent Mrp- and human MRP-proteins [22], a significant decrease of apical GSMF excretion could be observed, indicating that GSMF is excreted by MRP2 across the apical membrane of MDCKII-MRP2 cells.

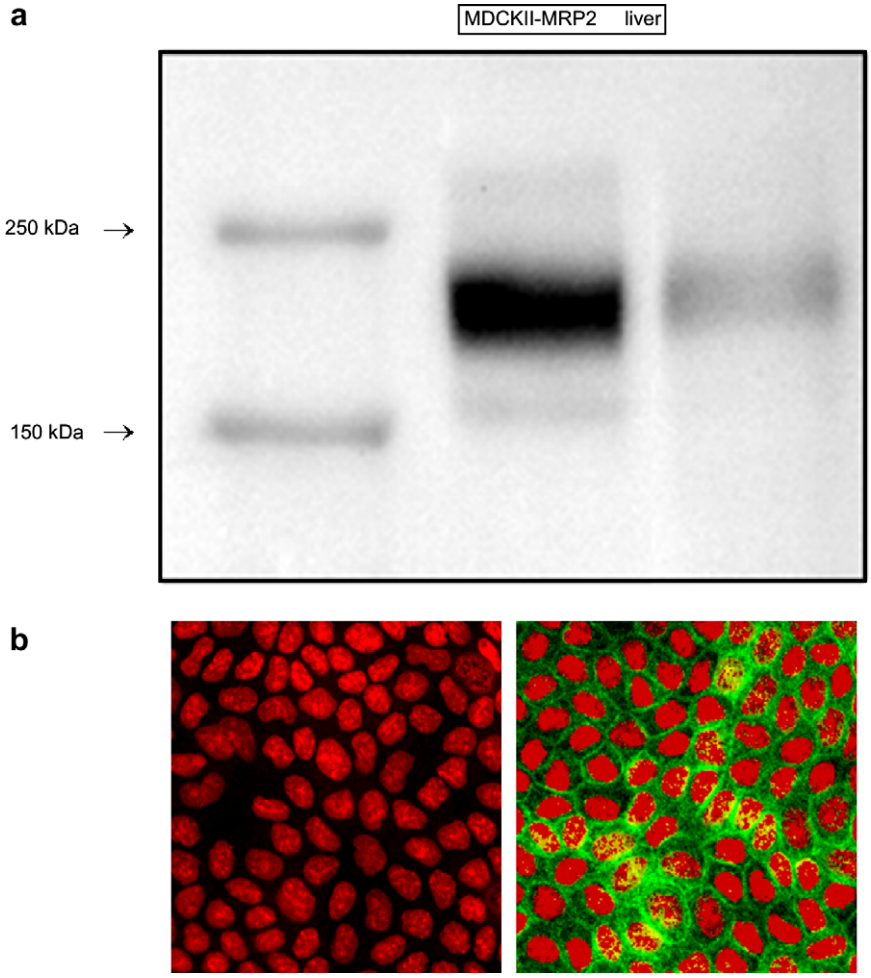

Fig. 2. (a) Western blot of ABCC2, lane 1, molecular weight standards; lane 2, MDCKII-MRP2 cells; lane 3, liver homogenate (positive control). (b) Immunolocalisation of MRP2 in the membranes of overexpressing MDCKI-MRP2 cells. Left photo, negative control; right photo, positive staining of MRP2.

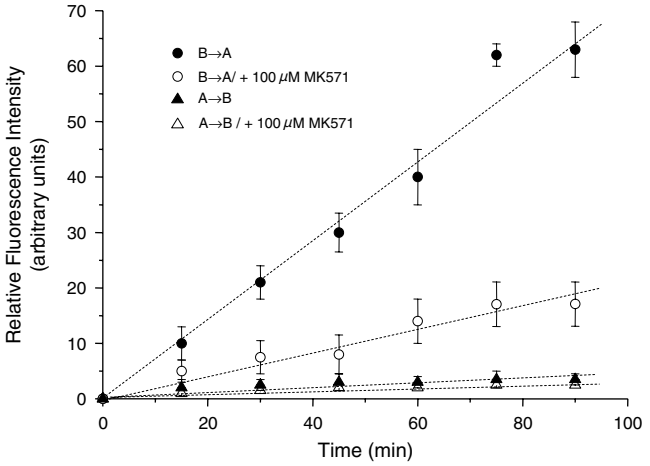

Fig. 3. Excretion of GSMF after incubation of cell monolayers with 1 μM CMFDA in absence or in presence of 100 μM MK571, an inhibitor of Mrp-proteins (■ basolateral → apical; ● basolateral → apical, + MK571; ▲ apical → basolateral, △ apical → basolateral, +MK571) (Means ± SE, $n = 6$).

3.5. Determination of intracellular glutathione concentration

An important step of the assay is the intracellular conjugation of fluorescent chloromethylfluorescein with glutathione. The product of this reaction, GSMF, is actively excreted by MRP2. In order to assess, whether the intracellular concentration of glutathione is rate limiting for the overall outcome of the assay, we determined the amount of glutathione/cm^2 confluent monolayer. It amounted to 4.9×10^{-5} μmol/cm^2 and 4.0×10^{-5} μM/cm^2 at day 3 and 4 of culture, respectively. Under the assumption that an equimolar conjugation of CMF occurs, we concluded that the intracellular amount of glutathione is not rate limiting, because at most 0.3×10^{-6} μmol/cm^2 CMF is present under standard conditions.

Recent studies showed that cellular depletion of glutathione may be accompanied by a reduced excretion of MRP2-substrates [23,24]. Therefore, we incubated MDCKII-MRP2 monolayers for 3 days with medium containing 100 μM L-buthionine–sulfoximine, an inhibitor of γ-glytamyl-cysteine synthesise [25]. Incubation of these cells with CMFDA resulted in a fourfold increased intracellular fluorescence as compared to untreated cells, indicating that the fluorescent intermediate CMF was not quantitatively conjugated and excreted.

3.6. Assay development – days of culture

In contrast to permeation assays requiring confluent monolayers tight confluency is not an indispensable premise for uptake and efflux experiments [21]. In order to assess the impact of culture time on assay performance, we studied the increase of intracellular fluorescence in absence and in presence of MK571, which is known to be a potent MRP2 inhibitor. All cells exhibited an increase in intracellular fluorescence intensity dependent on the concentration of MK571 with IC_{50} values of 9 ± 2, 14 ± 3, 18 ± 2 and 17 ± 3 μM at day 2, 3, 4 and 7 of culture. Maximum fluorescence intensity was similar in all experiments. Other studies with Caco-2 cells showed that expression of functional MRP2 has a very early onset after seeding the cells [26,27] and that in contrast to P-GP no fully differentiated monolayer is required. Based on the present result, we decided to use cells for transport experiments, which had been in culture for at least 4 days.

3.7. Assay development – assessment of appropriate CMFDA concentration

In order to determine a CMFDA concentration, which results in an intracellular fluorescence being sensitive enough to discriminate between MRP2-substrates of different affinity to the carrier protein, cells were pre-incubated with CMFDA at 0.1, 1.0, 2.5 and 5.0 μM at 4 °C for 45 min, then extracellular CMFDA was washed away and the cells were kept at 37 °C for the time intervals indicated in Fig. 4. Intracellular fluorescence at 37 °C was dependent on the initial concentration in the incubation medium, then it showed a relatively rapid decline and remained constant at a low level after approximately 30 min. The decrease can be explained by rapid transformation of CMFDA, subsequent conjugation of CMF and extrusion of GSMF out of the cells.

3.8. Assay development – exclusion of other export protein

In order to assure that neither CMFDA nor CMF and GSMF are substrates of other export proteins, e.g., ABCB1 (P-GP) or ABCG2 (BCRP, breast cancer resistance protein) specific inhibitors for both proteins were added during monolayer incubations with MK571. 2 μM LY335979 [28,29] was used to inhibit P-GP, 1 μM

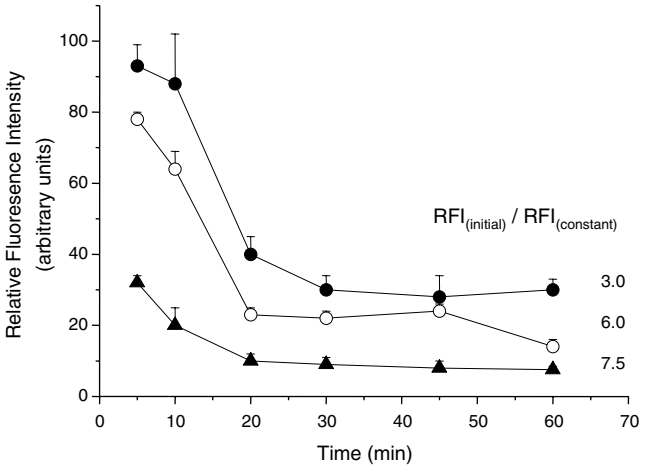

Fig. 4. Assessment of appropriate CMFDA concentration; cells were pre-incubated with CMFDA at 1.0 (▲), 2.5 (○) and 5.0 (●) μM at 4 °C for 45 min and then intracellular fluorescence was determined at 37 °C at the indicated time intervals ($n = 6$; means ± SE).

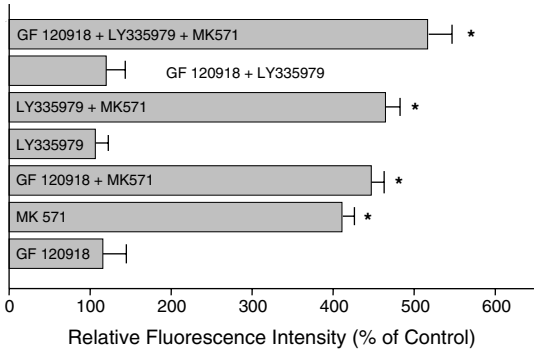

Fig. 5. Effect of inhibitors of ABC-export proteins on intracellular fluorescence accumulation. GF120918 and LY335979 (inhibitors of *p*-glycoprotein and BCRP) have no effect on the intracellular fluorescence, only in presence of MK571 (MRP2-inhibitor) an accumulation can be observed ($n = 6$; means ± SE; $*p < 0.05$).

GF120918 (Elacridar) [30] was used to inhibit P-GP and BCRP, As illustrated in Fig. 5, none of the P-GP and BCRP inhibitors had an effect on intracellular fluorescence accumulation. Although fluorescence in the presence of P-GP and BCRP inhibitors was slightly higher than in presence of MK571 alone, measured fluorescence values were not significantly different from each other in presence of any inhibitor. There was also no decrease in intracellular fluorescence, which might have been indicative for a hindered uptake of CMFDA by P-GP or BCRP. Western blots, performed as control experiments, showed negligible expression of the 2 export pumps in the used cells, which was in clear contrast to MRP2-expression.

3.9. Assay development – determination of cytotoxicity

MK571 was used as reference inhibitor for complete MRP2 inhibition. In order to exclude that observed effects are the result of cytotoxicity at higher concentrations of MK571, an Alamar Blue® assay based on mitochondrial dehydrogenase activity was performed. Fig. 6 shows that the formation of dye being indicative for cell viability was not influenced by higher concentrations of MK571, whereas 0,1% Triton X-100 totally abolished formazan formation.

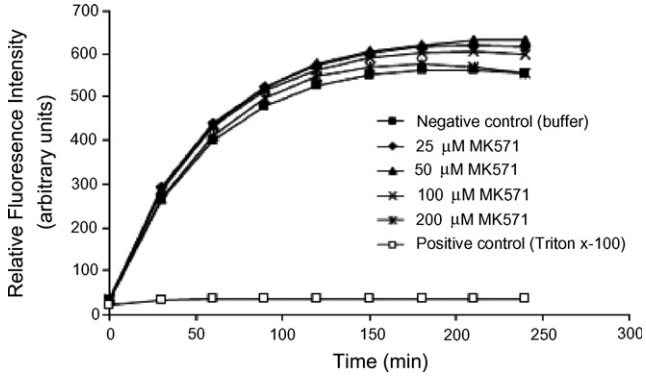

Fig. 6. Alamar Blue® assay based-effect of increasing concentrations of MK571 upon cell viability. The unaltered formation of dye indicates that cell viability is not influenced by higher concentrations of MK571. In controls, 0,1% Triton X-100 totally abolished formazan formation, suggesting complete loss of cell viability (means of $n = 6$).

3.10. Assay validation

The usefulness of the assay was studied by determining the interaction of a series of substances with potential interaction with MRP2. Since the assay does not discriminate between transported substrates and non-transported inhibitors of MRP2 all compounds interacting with MRP2 are named "modulators" of the export protein, following a suggestion by [2]. Fig. 7 shows the concentration dependent profile for a series of drugs and phytopharmaceuticals previously described to interact with different affinity with the export pump, namely MK571 [28,31], cerivastatin [32], Na^+-fusidate [33], probenecid [28], rifampicin [34], indomethacin [28], bromosulfophthalein [28], myricetin [35], sulfinpyrazone [28] and paracetamol [36].

All substances described to be MRP2 substrates increased intracellular fluorescence. Thus, all compounds were correctly identified with regard to their predicted effect on MRP2. Interestingly, the group of MRP2 substrates could further be classified as compounds with strong, moderate and weak effects on MRP2. Already at low concentrations a large increase in intracellular fluorescence was caused by MK571, cyclosporine A and myricetin (effects >500% above control values at concentrations up to 100 µM. Fusidate, indomethacin, cerivastatin and rifampicin showed an intermediate effect with fluorescence values to up to 500% above control values at concentrations up to 500 µM. Probenecid and BSP showed only little effect with fluorescence values up to 150% above controls at 500 µM and Sulfinpyrazone and Paracetamol did not show any interaction with MRP2. Table 1 lists the IC_{50}-values

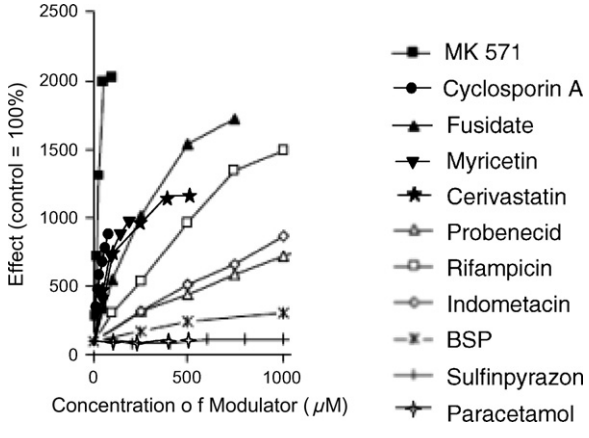

Fig. 7. Effect MRP2-modulators tested in the CMFDA-assay (control in absence of modulator = 100%, $n = 6$). MRP2 modulators can be classified as strong modulators (effects >500% above control values at concentrations ⩽100 µM, moderate modulators (effects >150% < 500% at 500 µM and weak or no modulators with effects <150 5 at 500 µM. For clarity, error bars are omitted.

Table 1
IC50-values of MRP2-modulators

Test compound	IC_{50} (μM)
MK571	21.2 ± 1.1
Cyclosporin A	5.6 ± 0.2
Myricetin	190.0 ± 35.0
Cerivastatin	198.9 ± 21.3
Na^{+}-fusidate	247.0 ± 1.0
Rifampicin	399.5 ± 3.6
Probenecid	818.6 ± 16.4
BSP	909.3 ± 123.5
Sulfinpyrazone	>1000
Paracetamol	>1000

for selected compounds. They are based on the administered concentrations and looking at theses values, it should be noted that they are best estimates obtained from a very complex cellular system comprising a uptake step, an unknown intracellular concentration and the efflux step.

These results are in accordance with the findings of others with the tested compounds, indicating the reliability of the CMFDA assay using MDCKII-MRP2 cells to identify MRP2 substrates correctly and showing the high sensitivity of the model to distinguish different extents of interaction of test compounds with MRP2.

4. Discussion

The presence of multiple efflux transporters in barrier tissues like intestinal epithelia, liver, kidney, blood–brain barrier or placenta recognizing a broad spectrum of substrates is a key determinant for the disposition of many drugs inside the body. Therefore, reliable screening procedures for the prediction of drug candidate/transport protein interactions may be useful in early drug development and for estimation of potential drug/drug interactions during clinical phases. In the present study, a rapid fluorescence based microplate screening assay has been introduced to determine drug interactions with MRP2.

The assay conditions were elaborated and a series of model compounds was identified correctly in accordance with published data. Compounds interacting with MRP2 could be distinguished on the basis of a strong, moderate or weak effect in the CMFDA-MRP2-assay. But, it should be kept in mind that is does not distinguish between transported MRP2 substrates and compounds inhibiting only MRP2-activity. Nevertheless, it provides a useful tool in early compound profiling during drug development.

Acknowledgements

The financial support of F. Förster during this project by Boehringer Ingelheim GmbH is gratefully acknowledged. Parts of this study were reported at the 32. CRS Annual Meeting 2005, Miami, FL, USA, as oral presentation.

References

[1] P. Breedveld, J.H. Beijnen, J.H.M. Schellens, Use of *P*-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs, Trends Pharmacol. Sci. 27 (2006) 7–25.
[2] J. Polli, S. Wing, J. Humphreys, L. Huang, J. Morgan, L. Webster, C. Serabjit-Singh, Rational use of in vitro *P*-glycoprotein assays in drug discovery, J. Pharmacol. Exp. Ther. 299 (2001) 620–628.
[3] A. Eneroth, E. Astrom, J. Hoogsraate, D. Schrenk, S. Conrad, H. Kaufmann, 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.
[4] 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.
[5] 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.
[6] G.A. Scarborough, Drug-stimulated ATPase activity of the human *P*-glycoprotein, J. Bioenerg. Biomembr. 27 (1995) 37–41.
[7] D. Schwab, H. Fischer, A. Tabatabaei, S. Poli, J. Huwyler, Comparison of in vitro *P*-glycoprotein screening assays: recommendations for their use in drug discovery, J. Med. Chem. 46 (2003) 1716–1725.
[8] C.J. Bachmeier, W.J. Trickler, D.W. Miller, Drug efflux transport properties of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its fluorescent free acid, BCECF, J. Pharm. Sci. 93 (2004) 932–942.
[9] R. Mayer, J. Kartenbeck, M. Büchler, G. Jedlitschky, I. Leier, D. Keppler, Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes, J. Cell Biol. 131 (1995) 137–145.
[10] M. Büchler, J. König, M. Brom, J. Kartenbeck, H. Spring, T. Horie, D. Keppler, cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats, J. Biol. Chem. 271 (1996) 15091–15098.
[11] C.C. Paulusma, P.J. Bosma, G.J.R. Zaman, C.T.M. Bakker, M. Otter, G.L. Scheffer, R.J. Scheper, P. Borst, R.P.J. Oude Elferink, Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene, Science 271 (1996) 1126–1128.
[12] D. Keppler, J. Kartenbeck, The canalicular conjugate export pump encoded by the cmrp/cmoat gene, Prog. Liver Dis. 14 (1996) 55–67.
[13] S. Dallas, D.S. Miller, R. Bendayan, Multidrug resistance-associated proteins: expression and function in the central nervous system, Pharmacol. Rev. 58 (2006) 140–161.
[14] S. Choudhuri, C.D. Klaassen, Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters, Int. J. Toxicol. 25 (2006) 231–259.
[15] R.G. Deeley, S.P. Cole, Substrate recognition and transport by multidrug resistance protein 1 (ABCC1), FEBS Lett. 580 (2006) 1103–1111.
[16] Evers M. Kool, L. van Deemter, H. Janssen, J. Calafat, L.C.J.M. Oomen, C.C. Paulusma, R.P.J. oude Eleferink, F. Baas, A.H. Schinkel, P. Borst, Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney cells expressing cMOAT (MRP2) cDNA, J. Clin. Invest. 101 (1998) 1310–1319.
[17] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[18] T. Abe, T. Mori, S. Hori, K. Koike, M. Kuwano, Multidrug resistance protein (MRP), Nippon Rinsho 55 (1997) 1077–1082.

[19] H. Gutmann, G. Fricker, M. Török, S. Michael, C. Beglinger, J. Drewe, Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake, Pharm. Res. 16 (1999) 402–407.
[20] H. Roelofsen, C.J. Soraka, D. Keppler, J.L. Boyer, Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2(cMoat) to the apical domain in hepatocytes couplets, J. Cell Sci. 111 (1998) 1137–1145.
[21] Y. Zhang, C. Bachmeier, D.W. Miller, In vitro and in vivo models for assessing drug efflux transporter activity, Adv. Drug Deliv. Rev. 55 (2003) 31–51.
[22] I. Leier, J. Hummel-Eisenbeiss, Y. Cui, D. Keppler, ATP-dependent para-amino-hippu-rate transport by apical multidrug resistance protein MRP2, Kidney Int. 57 (2000) 1636–1642.
[23] Y. Cui, J. Koenig, J.K. Buchholz, H. Spring, I. Leier, D. Keppler, Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells, Mol. Pharm. 55 (1999) 929–937.
[24] C.G. Dietrich, D.R. de Waart, R. Ottendorf, L.G. Schoots, R.P.J. Oude Elferink, Increased bioavailability of the food derived carcinogen 2-amino-1-methyl-6-phenylimidazol [4,5-b]pyridine in MRP2-deficient rats, Mol. Pharm. 59 (2001) 974–980.
[25] O.W. Griffith, A. Meister, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine), J. Biol. Chem. 254 (1979) 7558–7560.
[26] T. Hirohashi, H. Suzuki, X.Y. Chu, I. Tamai, A. Tsuji, Y. Sugiyama, Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2), J. Pharm. Exp. Ther. 292 (2000) 265–270.
[27] H. Prime-Chapman, V. Moore, B.H. Hirst, Antibiotic exposure does not influence functional expression in Caco-2 cells, J. Drug Target 1 (2005) 1–6.
[28] A. Haimeur, G. Conseil, R.G. Deeley, S.P.C. Cole, The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation, Curr. Drug Metab. 5 (2004) 21–53.
[29] C.Q. Xia, N. Liu, D. Yang, G. Miwa, L.S. Gan, Expression, localization and functional characteristics of breast cancer resistance protein in Caco-2 cells, Drug Metab. Dispos. 33 (2005) 637–643.
[30] F.G. Luo, P.V. Paranjpe, A. Guo, E. Rubin, P. Sinko, Intestinal transport of irinotecan in Caco-2 cells and MDCK II cells over-expressing efflux transporters PGP, CMOAT and MRP1, Am. Soc. Pharmacol. Exp. Ther. 30 (2002) 763–770.
[31] A.H. Schinkel, J.W. Jonker, Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview, Adv. Drug Deliv. Rev. 55 (2003) 3–29.
[32] S. Matsushima, K. Maeda, C. Kondo, M. Hirano, M. Sasaki, H. Suzuki, Y. Sugiyama, Identification of the hepatic efflux transporters of organic anions using double-transfected Madin–Darby canine kidney II cells expressing human organic anion transporting polypeptide 1B1 (OATP1B1)/multidrug resistance-associated protein 2 OATP1B1/multidrug resistance 1, and OATPaBA(breast cancer resistance protein), J. Pharmacol. Exp. Ther. 314 (2005) 1059–1067.
[33] K.A. Bode, M.G. Donner, I. Leier, D. Keppler, Inhibition of transport across the hepatocyte canalicular membrane by the antibiotic fusidate, Biochem. Pharmacol. 64 (2002) 151–158.
[34] S. Oswald, T. Giessmann, D. Luetjohann, D. Wegner, D. Rosskopf, W. Weitschies, W. Siegmund, Disposition and sterol-lowering effect of ezetimibe are influenced by single-dose coadministration of rifampin, an inhibitor of multidrug transport proteins, Clin. Pharmacol. Ther. 80 (2006) 477–485.
[35] J.J. van Zanden, A. de Mul, H.M. Wortelboer, M. Usta, P.J. van Bladeren, I.M. Rietjens, N.H. Cnubben, Reversal of in vitro cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin, Biochem. Pharmacol. 69 (2005) 1657–1665.
[36] C.I. Ghanem, P.C. Gomez, M.C. Arana, M. Perassolo, M.L. Ruiz, S.S. Villanueva, E.J. Ochoa, V.A. Catania, L.A. Bengochea, A.D. Mottino, Effect of acetaminophen on expression and activity of rat liver multidrug resistance-associated protein 2 and *P*-glycoprotein, Biochem. Pharmacol. 68 (2004) 791–798.