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Research paper

Development of a fluorescence-based assay for drug interactions with human Multidrug Resistance Related Protein (MRP2; ABCC2) in MDCKII-MRP2 membrane vesicles

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ABSTRACT

Purpose: To establish a fluorescence-based assay for drug interactions with the ABC-export-protein MRP2 (ABCC2).
Methods: Apical membrane vesicles were isolated by differential centrifugation from polarized MDCKII cells and MDCKII cells transfected with human MRP2. Vesicle fractions were characterized by electron microscopy, determination of the marker enzyme alkaline phosphatase and Western blot analysis of MRP2. Vesicle orientation was determined by measurement of 5′-nucleotidase activity in the absence and in the presence of detergents. To assess MRP2 activity, the uptake of the fluorescent MRP2-substrate 5-(6)-carboxy-2′,7′-dichlorofluorescein (CDF) was determined in the absence and in the presence of other compounds potentially interacting with MRP2.
Results: Apical membrane vesicles could be isolated from cells in considerable purity as indicated by electron microscopy, enrichment of alkaline phosphatase and high enrichment of MRP2 in vesicles of MDCKII-MRP2 cells. About half of the vesicles showed "inside-out" orientation. CDF was taken up into the membrane vesicles in a time- and concentration-dependent manner following a Michaelis–Menten type of kinetics with a K_M of 39 μM and a V_{max} of 465.3 fmol/(mg protein × min). Thereby, uptake into vesicles from transfected cells was significantly higher than uptake into vesicles from control cells. Presence of known MRP2-substrates/inhibitors in the incubation medium decreased CDF uptake into the vesicles in a concentration-dependent manner, whereas nonsubstrates/inhibitors had no effect.
Conclusions: This CDF-based uptake assay can be used as a rapid and sensitive screening system to assess drug interactions with human MRP2 and therefore represents a useful tool in compound profiling.

Beside their physiological function to transport nutrients and metabolites many membrane transport proteins also recognize drugs. Thereby, members of the ATP-binding cassette (ABC) transporter super-family have gained special attention for their involvement in drug absorption and disposition. In humans, 49 proteins of this super-family are expressed, and overall the MDR-gene product p-glycoprotein (ABCB1) has been regarded to be of high importance for drug bioavailability, as it is expressed in almost all barrier tissues including gastrointestinal tract, liver, kidney, blood–brain barrier, placental barrier, and others. Thus, it recognizes an abundance of substrates and pumps them out of the respective tissue [1–3]. Beside p-glycoprotein, also other ABC-transport proteins are of relevance for drug transport, including breast cancer resistance protein (BCRP, ABCG2) and members of the Multidrug Resistance Related Protein family (MRPs), such as MRP2 (ABCC2) [4–6]. In many tissues, the mentioned ABC transporters are expressed simultaneously and act complementary, since they exhibit a partial overlapping substrate recognition. Interestingly, in the gastrointestinal tract, there appears to be an increasing gradient of p-glycoprotein expression from proximal to distal parts of the intestine, whereas the extent of expression of MRP2 decreases from proximal to distal segments [7,8]. Both proteins recognize a huge variety of drugs including cytostatics, HIV therapeutics, antibiotics, lipid lowering agents, and many others. While p-glycoprotein mainly finds lipophilic and cationic compounds as substrates, MRP2 preferentially transports anionic compounds, including many secondary metabolites, such as glutathione conjugates, glucuronides, or sulfates. Similar to p-glycoprotein, MRP2 has two nucleotide binding sites and consists of two transmembrane domains with each having six membrane spanning regions. But, in addition, it has a third transmembrane domain with five membrane spanning sequences at its amino terminal end [9,10].

Potential interactions of drug candidates with these export proteins are of high relevance for drug development as they give hints

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with respect to bioavailability and drug–drug interactions on the level of transport proteins with consequences for pharmacokinetics and unwanted side-effects. Therefore, there is an urgent need for fast, cheap, and valid assays for an early assessment of potential interactions. Such assays may be based on cells overexpressing the respective transport protein with direct detection of substrate translocation or an indirect determination by measuring the inhibition of transport of a fluorescent or radioactively labelled marker substrate. In addition, with membrane vesicles the consumption of ATP may be measured as a marker of transport protein activity. In vesicles, also the uptake of a substrate by ABC-transport proteins may be measured. However, a certain fraction of the vesicles has to be orientated "inside-out" with the cytoplasmic, inner membrane surface of a cell being oriented towards the outer medium in the vesicles. This technique allows a direct contact of ATP and substrate without prior pre-loading, which is of special relevance for MRP2-substrates, often bearing a negative charge and therefore exhibiting a low passive membrane permeability.

The present study aims to develop a vesicle-based uptake assay for rapid and valid determination of MRP2 transport/interaction of novel drug candidates.

1. Materials and methods

1.1. Materials

Murine antibody against MRP2 (clone M2III-6) and MK571 were obtained from Alexis Biochemicals (Lörrach, FRG). Alkaline phosphatase antibody (clone 3A8) was from Abnova (Heidelberg, FRG). Secondary anti-mouse-horseradish peroxidase-conjugated antibody was from KPL (Wedel, FRG). Cyclosporine A was purchased from Novartis (Basle, CH). Sf9-MRP2-VT vesicles were from Solvo Biotechnology (Budapest, HU). All other materials were obtained from the usual commercial sources at the highest purity available.

1.2. Cells

MDCKII-MRP2 cells stably 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.

MDCKII cells and MDCKII-MRP2 cells were grown in Dulbecco's MEM supplemented with 10% fetal bovine serum, penicillin/streptomycin (200 μg/ml), and kanamycin (1 μg/ml) at 37 °C, 5% CO_2, and 95% relative humidity.

1.3. Vesicles

Vesicles from MDCKII cells and MDCKII-MRP2 cells were prepared as described previously [11]. Briefly, cells were washed with HBSS (Sigma–Aldrich, Steinheim, FRG), scraped in ice-cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) supplemented with protease inhibitors (100 μM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM E64) and gently stirred on ice for 1 h. The cell homogenate was then centrifuged at 100,000*g* for 1 h at 4 °C. The resulting pellet was resuspended in Tris–sucrose buffer (10 mM Tris–HEPES, 250 mM sucrose, pH 7.4) and homogenized with a tight-fitting glass/Teflon douncer (30 strokes). After subsequent centrifugation at 1000*g* for 15 min at 4 °C, the resulting supernatant was centrifuged at 100,000*g* at 4 °C, and the resulting pellet was resuspended in a small amount of Tris–sucrose buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle. Finally, aliquots of the vesicles were frozen in liquid nitrogen and stored at −80 °C until use.

1.4. Electron microscopy

Membrane fractions were separated from Tris–sucrose buffer by low-speed centrifugation and ice-cold cacodylate buffer (0.1 M, pH 7.0) containing 1% glutaraldehyde and 1% OsO_4 was added to the pellet. After incubating for 1 h at 4 °C and washing twice with cacodylate buffer, the pellet was incubated for 16 h at 4 °C with 2% aqueous uranyl acetate. The pellet was then washed again, dehydrated in an acetone series and embedded in Spurr's resin. After ultra-thin sections were cut, samples were stained with lead citrate and examined with a Philips CM10 transmission electron microscope at 80 kV.

1.5. Western blot

Protein content in cell homogenates and isolated vesicle fractions was determined by using the BCA™ Protein Assay Kit (Thermo Scientific, FRG), which is based on the biuret reaction. Cell homogenates and vesicle fractions were then subjected to electrophoresis on a 7.5% SDS–polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membranes. The blots were then blocked for 1 h at 4 °C with PBS-T containing 1% nonfat dry milk powder and 1% bovine serum albumin (Sigma–Aldrich, Steinheim, FRG). After three washing steps with PBS-T, proteins were hybridized overnight at 4 °C with the monoclonal mouse antibody M2III-6 against human MRP2 in MDCKII cells (Alexis Biochemicals, Lörrach, FRG) (dilution 1:50 in blocking buffer). Subsequently, the blots were incubated with a secondary anti-mouse-horseradish peroxidase-conjugated antibody (KPL, Wedel, FRG) for 1 h at room temperature, followed by enhanced chemiluminescence detection (Western Lightning® Western Blot Chemiluminescence Reagent Plus, Perkin Elmer, Wiesbaden, FRG, ChemiDocXRS software).

1.6. Enzyme determination

Alkaline phosphatase (EC 3.1.3.1) activity was determined using the QuantiChrom™ Alkaline Phosphatase Assay Kit from BioAssay Systems (Hayward, USA) according to the manufacturer's instructions. Briefly, cell homogenates and vesicle fractions were incubated with *p*-nitrophenyl phosphate which is hydrolyzed by alkaline phosphatase. Absorbance of the yellow coloured reaction product *p*-nitrophenol was then quantified in a plate reader at 405 nm. Activities were calculated with the help of a calibrator and normalized to protein contents in the samples.

1.7. Vesicle orientation

Orientation of the isolated vesicles was assessed by measuring the activity of the ectoenzyme 5′-nucleotidase (EC 3.1.3.5) using a previously described method [12]. Shortly, membrane fractions were incubated in the presence or the absence of 0.1% Triton X-100 for 15 min at 37 °C with 1.67 mM AMP as a substrate in a buffer consisting of 100 mM Tris and 3.34 mM $MgSO_4$ (pH 7.4). Enzyme activity was subsequently determined by measuring the amount of liberated phosphate using a colorimetric method [13]. Because AMP is not taken up into intact vesicles, it can only be hydrolyzed by 5′-nucleotidase in vesicles with right side-out configuration. Hence, the activities measured in the absence of Triton X-100 correspond to the right side-out vesicles, whereas those measured in the presence of Triton X-100 correspond to the total activity of right side-out and inside-out vesicles, respectively. The

difference between the two activities therefore approximates the activity of the inside-out vesicles in the sample [14].

1.8. Transport assay

Frozen vesicles were thawed for 1 min at 37 °C and samples with a protein content of 20 μg were transferred into separate wells of a 96-well plate. In the case of inhibition experiments, samples were preincubated with the respective compound for 5 min at 37 °C under gentle agitation. Samples were thereafter incubated at 37 °C in the presence of 4 mM ATP in 100 μl Tris–sucrose buffer (10 mM Tris–HEPES, 250 mM sucrose, pH 7.4) containing 10 mM $MgCl_2$ and the fluorescent substrate CDF at an appropriate concentration. For control experiments, ATP was replaced by AMP. The reaction was stopped at given times by placing the plates on ice followed by the addition of 100 μl ice-cold incubation medium. Uptake rates were determined by using a rapid filtration technique. Therefore, samples were transferred to a 96-well filterplate (MultiScreen$_{HTS}$-HV plate Durapore® PVDF, 0.45 μm, Millipore, Schwalbach, FRG) and incubation medium was removed with the help of a vacuum manifold. Following three washing steps with 200 μl Tris–sucrose buffer, vesicles were solubilized by addition of 100 μl SDS–HEPES buffer (1% SDS, 7.5 mM HEPES, pH 7.4) for at least 30 min. Finally, the plates were subjected to fluorescence quantification in a fluorescent plate reader (Fluoroskan Ascent®, Labsystems, Frankfurt, FRG) with filter settings of λ_{ex} of 485 nm and λ_{em} of 520 nm.

1.9. Statistics

All values are presented as means ± SD. Concentration/effect plots were made by use of the graphic software SigmaPlot (Systat Software, San Jose, USA).

2. Results and discussion

MRP2 (ABCC2) is an export protein with an apparent molecular weight of approximately 190 KDa, which is expressed in most barrier tissues throughout the body [15]. Besides p-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), it significantly contributes to the active extrusion of many drugs and drug metabolites [16,17]. Therefore, we aimed to develop an assay, which can easily be used to study a potential impact of MRP2 on bioavailability and disposition of novel drug candidates.

Membrane vesicles were isolated from parent MDCKII cells and MDCKII-MRP2 cells overexpressing the human isoform of the export protein. Fig. 1 shows an electron microscopic image of the final vesicle fraction from MDCKII-MRP2 cells. The majority consists of vesicles with a diameter of up to 2 μm besides some more electron-dense structures presumably originating from intracellular organelles such as mitochondria. SDS gel electrophoresis, subsequent Western blotting and immuno-detection using a specific antibody revealed a significant enrichment of MRP2 in the membrane fraction compared to the total cell homogenate (Fig. 2). In vesicles from MDCKII-MRP2 cells the staining intensity was 5-times higher than in vesicles originating from MDCKII cells. The increase of activity in the membrane fraction over activity in the total cell homogenate of alkaline phosphatase, a marker enzyme of apical membranes was 5.4-fold, which corresponds to similar values found in MDCKII cells and other cell lines [18,19]. Vesicle orientation was assessed by measuring the activity of the ectoenzyme 5′-nucleotidase. The orientation was reproducible and yielded approximately 54% of inside-out oriented vesicles, which also fits well to the literature data. van Aubel et al. [20] found approximately 65% inside-out oriented vesicles after preparation from Sf9 cells.

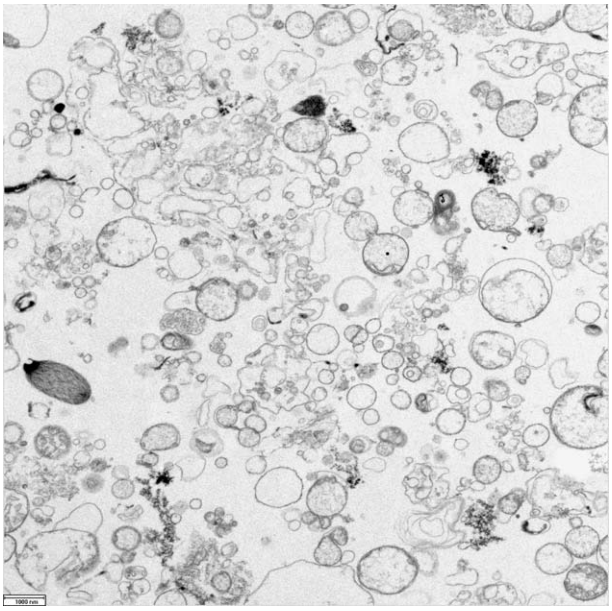

Fig. 1. Electron micrograph of a vesicle fraction from MDCKII-MRP2 cells. Magnification1500-fold.

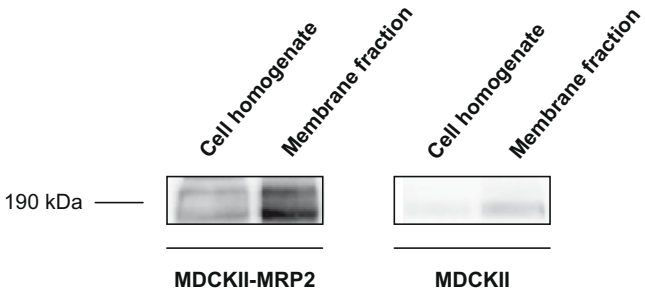

Fig. 2. Western blot analysis for MRP2 in cell lysates and vesicle suspensions from MDCKII-MRP2 cells and parent MDCKII cells. 20 μg protein were subjected to SDS gel electrophoresis, MRP2 was detected using a monoclonal primary antibody (M2III-6, mouse IgG, 1:50) and a secondary anti-mouse antibody from goat (1:10,000).

The requirements for an assay substrate require a low passive membrane permeability, which allows an easy detection of an active transport component, and a high selectivity for MRP2. Several fluorescent compounds appeared to be suitable to be used as transport substrates. However, none of the known fluorescent substrates is absolutely selective for MRP2. Most of them are recognized by other export proteins, not only by MRPs (Table 1). From these substrates, 5-(6)-carboxy-2′,7′-dichlorofluorescein (CDF) was chosen (Fig. 3), which can easily be detected in a fluorescent plate reader at 529 nm. Its transport into the MDCKII-MRP2

Table 1
Recognition of fluorescent substrates by ABC transport proteins.

Substance	Recognition	Reference
Calcein	MRP2, MRP1, P-GP	[1,2]
CDF	MRP2, MRP3, MRP5	[3,4]
Fl-MTX	MRP1, MRP1, MRP3, OATP2	[5,6]
Fluo-3	MRP2, MRP1, P-GP, OATP8	[7,9,10]
Fluo-cAMP	MRP2, Mrp1, MRP4	[11]
SG-B	MRP2, MRP4	[12]
SG-MF	MRP2, MRP1	[13]

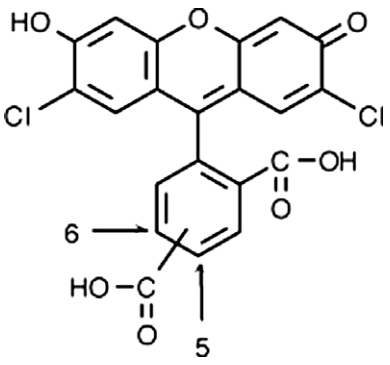

Fig. 3. Structure of 5-(6)-carboxy-2′,7′-dichlorofluorescein.

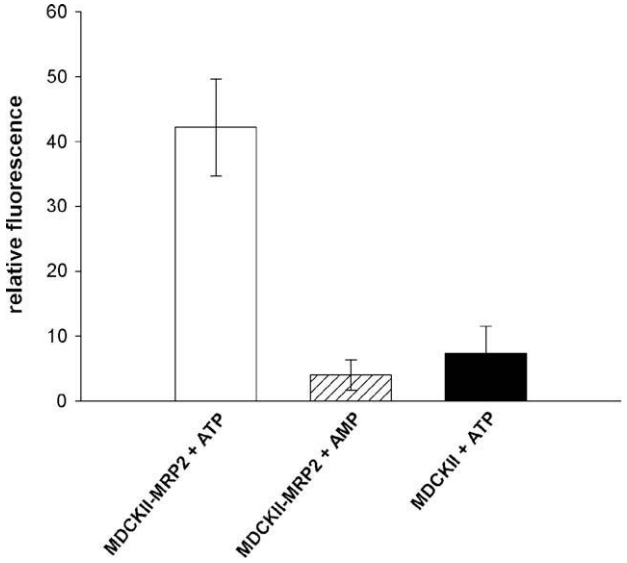

Fig. 4. Uptake of CDF into vesicles from MDCKII-MRPII cells and parent MDCKII cells in the presence of ATP and AMP, respectively.

vesicles in the presence of ATP was 11-times higher than in the absence of the nucleotide, suggesting an ATP-dependent uptake mechanism. In addition, uptake into parent MDCK cells was significantly lower, indicating that the observed uptake was indeed mediated by MRP2 (Fig. 4). Uptake of CDF was concentration dependent, linear over time for at least 60 min and temperature dependent. Kinetic analysis revealed a Michaelis–Menten type of uptake with a km of 39 μM and a V_{max} of 465.3 fmol/(mg protein × min). A Hill plot showed a Hill coefficient of $n_H = 0.9788$, suggesting a single binding site without cooperativity (Fig. 5a–c).

Based on these findings standard test conditions were set up as follows: MDCKII-MRP2 membrane vesicles were incubated with for 15 min at 37 °C with an incubation medium containing 50 μM CDF and further treated as described earlier. Finally, vesicle associated fluorescence was detected with a fluorescence plate reader. Since many compounds show a very poor solubility in aqueous solution, stock solutions of DMSO are frequently in use, which are then diluted for the respective assays. In order to check whether DMSO affects the assay performance, the transport of CDF was determined in the absence and in the presence of increasing concentrations of DMSO between 1% and 5% of the total volume. In none of all these experiments, a difference compared to the control values was observed (data not shown), indicating that DMSO in the used concentration range does not affect this assay.

Many MRP2-substrates and inhibitors have been identified in the literature, mainly organic anions, including secondary metabolites, such as glucuronides, glutathione conjugates and sulfates. Since in this assay the extent of inhibition of CDF uptake is measured, and therefore, no discrimination between transported substrates and nontransported inhibitors of MRP2 can be done, all compounds interacting with MRP2 are named "modulators" of the export protein, following a suggestion by Polli et al. [21]. An interaction with the export protein was tested by measuring the intracellular fluorescence accumulation in the presence of the potential MRP2 modulators. In addition, IC_{50}-values for all tested MRP2 modulator were calculated (Table 2). Fig. 6 shows the concentration-dependent profiles for a series of drugs and test compounds, which had been described to interact with different affinity with the export pump, including the LTD_4-receptor antagonist MK571 [22], the uricosurics benzbromarone [23] and probenecide [24], the synthetic bile acid derivative Na^+-fusidate [25], analgetic indometacin [26], cytostatic methotrexate [27], immunosuppressive cyclosporin A [22] and also analgetic antipyrine, for which no MRP2 modulation has been described so far. In addition, metabolites were tested in the assay, e.g. estradiol-17β-glucuronide [28] and its parent compound 17β-estradiol, testosterone-β-D-glucuronide and its parent compound testosterone, coumarin and coumarin-glucuronide.

All substances described to modulate MRP2 decreased CDF related vesicle associated fluorescence. Thus, all compounds were correctly identified with regard to their predicted effect on MRP2.

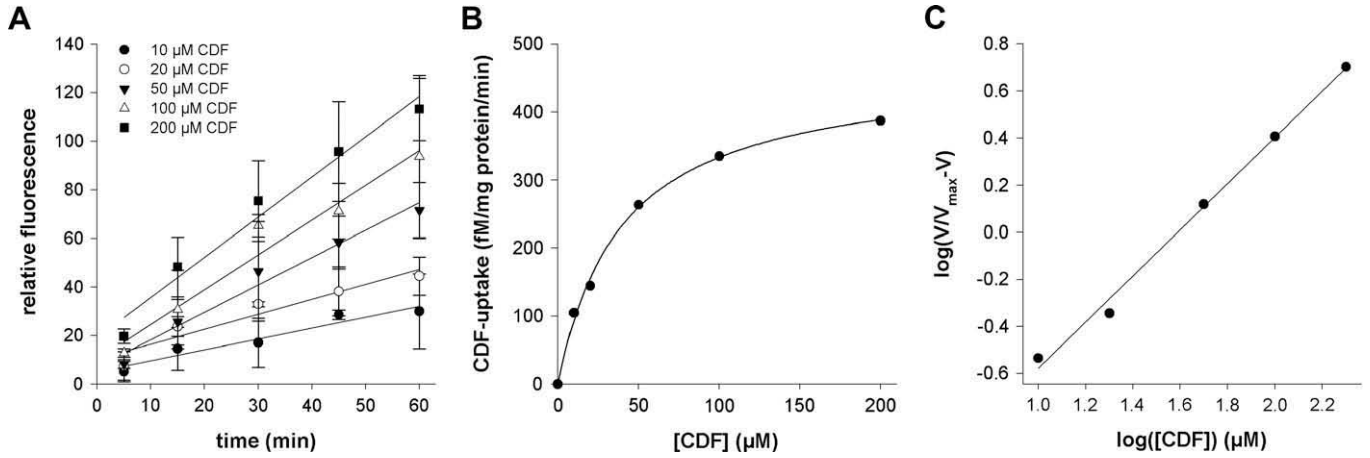

Fig. 5. Uptake of CDF into vesicles from MDCKII-MRPII cells. (a) Time-dependent uptake, (b) concentration-dependent uptake rates, and (c) Hill plot, with a Hill coefficient of 0.9788, indicating a single binding site.

Table 2
Affinity of tested MRP2 modulators to the MRP2 export pump.

Test compound	IC_{50} (μM)	Maximum inhibition (%)	CDF concentration at maximum inhibition (μM)
MK571	3.82 ± 0.18	82.22 ± 3.44	15
Benzbromarone	1.97 ± 0.26	84.47 ± 7.66	10
Cyclosporine A	5.55 ± 0.54	49.04 ± 3.58	20
Na^+-Fusidate	148.33 ± 0.18	97.21 ± 0.64	1000
Indometacin	135.12 ± 0.15	99.89 ± 1.11	2000
Probenecide	182.37 ± 0.66	92.26 ± 5.36	3000
Ivermectin	–	43.24 ± 21.51	100
Methotrexate	–	22.44 ± 18.48	100
Antipyrine	–	7.33 ± 5.421	100
Coumarin	–	0	100
Estradiol	–	16.94 ± 6.34	100
Estradiol-17β-glucuronide	19.89 ± 0.19	81.10 ± 5.24	100
Testosterone	–	0	100
Testosterone-β-glucuronide	–	38.41 ± 22.09	100

Presence of MK571 in the incubation medium resulted in a significant and dose-dependent inhibition of CDF transport into the vesicles, with an apparent K_i of 3.8 ± 0.2 μM. Based on findings in the literature, MK571 appears to be very selective for MRPs; thus, it turns out to be an ideal reference substrate in this vesicle assay. The strong inhibitory effect is supported by data created by others, e.g. low IC_{50}-values of 4 μM for para-aminohippuric acid transport [29] and 2.6 μM for estradiol$_2$-17β-glucuronide transport [30].

Another strong inhibitor of CDF uptake into the vesicles was uricosuric benzbromarone with an IC_{50} of 1.97 ± 0.26 μM. This finding is paralleled by the observation of a very strong inhibition of N-ethylmaleimide-S-glutathione transport in membrane vesicles expressing MRP6 with a maximum inhibition of transport by benzbromarone at a concentration below <30 μM [23].

Cyclosporine A inhibited CDF transport also with a low IC_{50} of 5.55 μM. However, compared to MK571 and benzbromarone the maximum effect (40% inhibition at 20 μM) was significantly lower than for the former two compounds. Previous data showed an IC_{50} of approximately 10 μM for para-aminohippuric acid transport by MRP2 [29] and 5.5 μM for CDF in similar experiments [31].

Looking at the maximum effect modulators could be classified as compounds with strong, moderate and weak effects on MRP2. A very large decrease in vesicle associated fluorescence at relatively low concentrations (⩽20 μM) was caused by MK541, benzbromarone and cyclosporine A. Fusidate, indometacin, and probenecide caused good inhibition of fluorescence accumulation, but only at very high concentrations. Recently, we made a similar observation in MRP2-expressing cells [32], suggesting that the

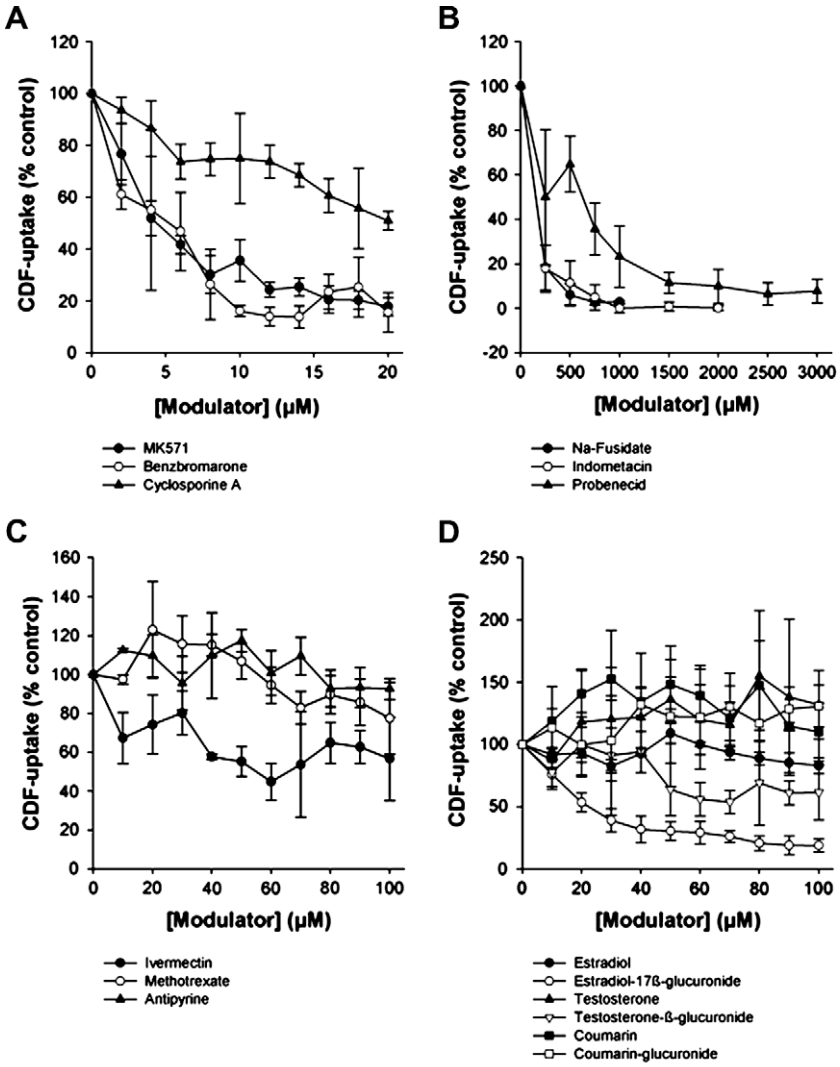

Fig. 6. Uptake of 50 μM CDF into vesicles from MDCKII-MRPII cells in the absence and in the presence of potential modulators of MRP2-mediated transport.

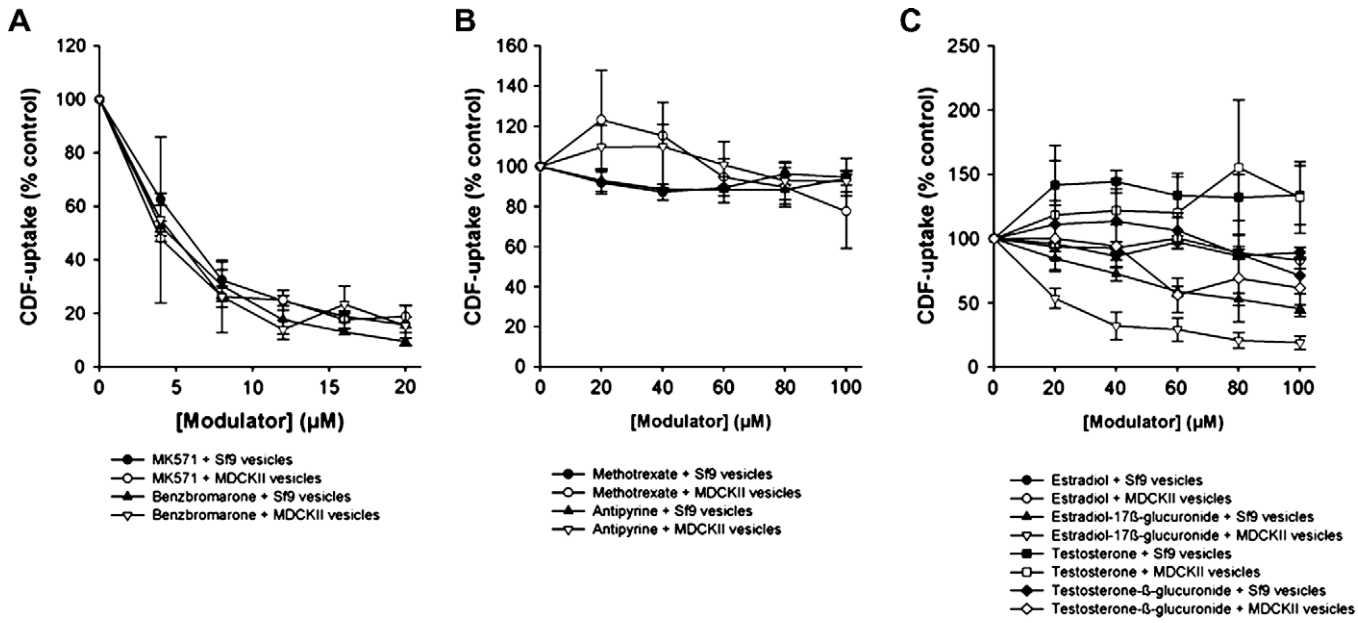

Fig. 7. Uptake of CDF into vesicles from MDCKII-MRPII cells and into vesicles from Sf9-MRP2-VT cells in the absence and in the presence of potential modulators of MRP2-mediated transport.

three compounds have only a moderate affinity to the export protein.

Ivermectin, a classical p-glycoprotein substrate [33,34] and methotrexate had only very little effect and antipyrine did not inhibit CDF uptake at all. In contrast to their parent compounds, estradiol-17β-glucuronide and testosterone-β-glucuronide exerted a quite strong effect, whereas coumarin and coumarin-glucuronide were both inactive.

In order to validate the assay performance all experiments were repeated with commercially available Sf9-MRP2-VT vesicles in the presence of ATP. The results obtained were very similar to those obtained with the MDCKII-MRP2-vesicles (Fig. 7, Table 3), indicating the validity of this assay.

As prediction of interactions with ABC-transport proteins is important in screening drug candidates with low bioavailability or low CNS activity, use of simple cell based assays may be helpful. Here, we showed the use of a CDF-based assay with MDCK-MRPII membrane vesicles. This assay can be used as an indirect assay measuring the inhibition of CDF transport by MRP2, but these vesicles may also be used for measuring the transport of unlabelled compounds with subsequent LC/MS analysis. Taken together, this test system proves to be a useful tool to study potential drug interactions with MRP2.

Table 3
Inhibition of CDF transport in MDCKII-MRP2 cells and Sf9-MRP2-VT vesicles.

Test compound	Vesicles	IC_{50} (μM)	Maximum inhibition (% of control)
MK571	MDCKII-MRP2	3.82 ± 0.18	82.22 ± 3.44
	Sf9-MRP2-VT	4.03 ± 0.10	84.19 ± 2.88
Benzbromarone	MDCKII-MRP2	1.97 ± 0.26	84.47 ± 7.66
	Sf9-MRP2-VT	3.87 ± 0.14	90.46 ± 1.34
Estradiol-17β-glucuronide	MDCKII-MRP2	19.89 ± 0.19	81.10 ± 5.24
	Sf9-MRP2-VT	40.01 ± 0.25	54.61 ± 3.10
Antipyrine	MDCKII-MRP2	–	7.33 ± 5.42
	Sf9-MRP2-VT	–	5.95 ± 3.33
Estradiol	MDCKII-MRP2	–	19.94 ± 5.24
	Sf9-MRP2-VT	–	10.99 ± 4.19

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References

[1] R.H. Ho, R.B. Kim, Transporters and drug therapy: implications for drug disposition and disease, Clin. Pharmacol. Ther. 78 (2005) 260–277.
[2] O. van Tellingen, The importance of drug-transporting p-glycoprotein in toxicology, Toxicol. Lett. 120 (2001) 31–41.
[3] T. Murakami, M. Takano, Intestinal efflux transporters and drug absorption, Expert Opin. Drug Metab. Toxicol. 4 (2008) 923–939.
[4] M. Huls, F.G. Russel, R. Masereeuw, The role of ATP binding cassette transporters in tissue defense and organ regeneration, J. Pharmacol. Exp. Ther. 328 (2009) 3–9.
[5] R.L. Oostendorp, J.H. Beijnen, J.H. Schellens, The biological and clinical role of drug transporters at the intestinal barrier, Cancer Treat. Rev. 35 (2009) 137–147.
[6] N.A. Colabufo, F. Berardi, M. Contino, M. Niso, R. Perrone, ABC pumps and their role in active drug transport, Curr. Top. Med. Chem. 9 (2009) 119–129.
[7] C. MacLean, U. Moenning, A. Reichel, G. Fricker, Closing the gaps: a full scan of the intestinal expression of p-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein 2 in male and female rats, Drug Metab. Dispos. 36 (2008) 1249–1254.
[8] C. Zimmermann, H. Gutmann, P. Hruz, J.P. Gutzwiller, C. Beglinger, J. Drewe, Mapping of multidrug resistance gene 1 and multidrug resistance-associated protein isoform 1 to 5 mRNA expression along the human intestinal tract, Drug Metab. Dispos. 33 (2005) 219–224.
[9] 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.
[10] G. You, Membrane transporters in drug disposition, Pharm. Res. 25 (2008) 441–443.
[11] R.A. van Aubel, P.H. Smeets, J.J. van den Heuvel, F.G. Russel, Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites, Am. J. Physiol. Renal Physiol. 288 (2005) F327–F333.
[12] C.A. Doige, F.J. Sharom, Strategies for the purification of p-glycoprotein from multidrug-resistant Chinese hamster ovary cells, Protein Expr. Purif. 2 (1991) 256–265.
[13] S. Chifflet, A. Torriglia, R. Chiesa, S. Tolosa, A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases, Anal. Biochem. 168 (1988) 1–4.
[14] M. Böhme, M. Müller, I. Leier, G. Jedlitschky, D. Keppler, Cholestasis caused by inhibition of the adenosine triphosphate-dependent bile salt transport in rat liver, Gastroenterology 107 (1994) 255–265.

[15] J. König, A.T. Nies, Y. Cui, I. Leier, D. Keppler, Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance, Biochim. Biophys. Acta 1461 (1999) 377–394.
[16] D. Keppler, J. König, Hepatic secretion of conjugated drugs and endogenous substances, Semin. Liver Dis. 20 (2000) 265–272.
[17] S.F. Zhou, L.L. Wang, Y.M. Di, C.C. Xue, W. Duan, C.G. Li, Y. Li, Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development, Curr. Med. Chem. 15 (2008) 1981–2039.
[18] D.W. Loe, K.C. Almquist, R.G. Deeley, S.P. Cole, Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport, J. Biol. Chem. 271 (1996) 9675–9682.
[19] J.C. Basham, A. Chabrerie, S.A. Kempson, Hypertonic activation of the renal betaine/GABA transporter is microtubule dependent, Kidney Int. 59 (2001) 2182–2191.
[20] R.A. van Aubel, M.A. van Kuijck, J.B. Koenderink, P.M. Deen, C.H. van Os, F.G. Russel, Adenosine triphosphate-dependent transport of anionic conjugates by the rabbit multidrug resistance-associated protein MRP2 expressed in insect cells, Mol. Pharmacol. 53 (1998) 1062–1067.
[21] 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) 6220–6228.
[22] Z.S. Chen, T. Kawabe, M. Ono, S. Aoki, T. Sumizawa, T. Furukawa, T. Uchiumi, M. Wada, M. Kuwano, S.I. Akiyama, Effect of multidrug resistance-reversing agents on transporting activity of human canalicular multispecific organic anion transporter, Mol. Pharmacol. 56 (1999) 1219–1228.
[23] A. Iliás, Z. Urbán, T.L. Seidl, O. Le Saux, E. Sinkó, C.D. Boyd, B. Sarkadi, A. Váradi, Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6), J. Biol. Chem. 277 (2002) 16860–16867.
[24] R. Evers R, M. Kool, L. van Deemter, H. Janssen, J. Calafat, L.C. Oomen, C.C. Paulusma, R.P. Oude Elferink, F. Baas, A.H. Schinkel, P. Borst, Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA, J. Clin. Invest. 101 (1998) 1310–1319.
[25] 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.
[26] H. Kouzuki, H. Suzuki, Y. Sugiyama, Pharmacokinetic study of the hepatobiliary transport of indometacin, Pharm. Res. 17 (2000) 432–438.
[27] R.A. van Aubel, P.H. Smeets, J.G. Peters, R.J. Bindels, F.G. Russel, The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP, J. Am. Soc. Nephrol. 13 (2002) 595–603.
[28] Y. Cui, J. König, 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. Pharmacol. 55 (1999) 929–937.
[29] I. Leier, J. Hummel-Eisenbeiss, Y. Cui, D. Keppler, ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2, Kidney Int. 57 (2000) 1636–1642.
[30] K. Ito, H. Suzuki, Y. Sugiyama, Charged amino acids in the transmembrane domains are involved in the determination of the substrate specificity of rat MRP2, Mol. Pharmacol. 59 (2001) 1077–1085.
[31] K. Heredi-Szabo, E. Kis, E. Molnar, A. Gyorfi, P. Krajcsi, Characterization of 5(6)-carboxy-2,′7′-dichlorofluorescein transport by MRP2 and utilization of this substrate as a fluorescent surrogate for LTC4, J. Biomol. Screen. 13 (2008) 295–301.
[32] F. Förster, A. Volz, G. Fricker, Compound profiling for ABCC2 (MRP2) using a fluorescent microplate assay system, Eur. J. Pharm. Biopharm. 69 (2008) 396–403.
[33] S. Nobmann, B. Bauer, G. Fricker, Ivermectin excretion by isolated functionally intact brain endothelial capillaries, Br. J. Pharmacol. 132 (2001) 722–728.
[34] G. Fricker, H. Gutmann, A. Droulle, J. Drewe, D.S. Miller, Epithelial transport of anthelmintic ivermectin in a novel model of isolated proximal kidney tubules, Pharm. Res. 16 (1999) 1570–1575.