European Journal of Pharmaceutics and Biopharmaceutics 75 (2010) 284–290

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

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

Christian Lechner [a], Valeska Reichel [a], Ursula Moenning [b], Andreas Reichel [b], Gert Fricker [a,*]

[a] Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, Heidelberg, Germany
[b] Bayer-Schering AG, Berlin, Germany

ARTICLE INFO

*Article history:*
Received 5 October 2009
Accepted in revised form 15 March 2010
Available online 20 March 2010

*Keywords:*
MRP2
ABCC2
Fluorescence assay
ABC protein
Carboxydichlorofluorescein (CDF)

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

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

0939-6411/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2010.03.008

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