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Research paper

A Caco-2 cell based screening method for compounds interacting with MRP2 efflux protein

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ABSTRACT

The aim of this work was to develop a screening method for MRP2 efflux substrates using the well-characterized, human-based intestinal Caco-2 cell model as a platform. MRP2 has a significant role in drug absorption and disposition and is known to co-operate with phase II metabolic enzymes. Caco-2 cells grown in a 96-well plate were loaded with non-fluorescent CDCFDA (diacetate ester of 5(6)-carboxy-2′,7′-dichlorofluorescein), which is hydrolyzed to fluorescent CDCF by intracellular esterases. De-esterification in Caco-2 was comparable to that in porcine liver esterases. CDCFDA enters the cells passively, while CDCF is effluxed out of the cells by the apically localized MRP2 and/or basolateral MRPs. The method was optimized with regard to several factors. In the concluding protocol, Caco-2 cells are grown on clear 96-well plates for 8 days. The loading conditions were optimized to 10 min incubation with 5 μM CDCFDA. The highest responses were obtained for samples taken at $t = 30$ min. The samples were analyzed in black 96-well plates with a fluorescence plate reader. The Caco-2 based method utilizing the probe pair CDCFDA/CDCF provides a fast screening tool for MRP2 substrates and/or inhibitors, along with compounds having metabolites formed in Caco-2 that interact with MRP2.

1. Introduction

The MRP2 (multidrug resistance protein 2) is a membrane efflux transporter and, although initially discovered in tumor tissues, it is also present in normal human intestinal epithelium, hepatic canalicular membranes, the blood–brain barrier, lung, kidney, etc. [1]. Its physiological function is to protect cells and tissues from endogenic compounds, toxins or xenobiotics such as bilirubin, arsenic or toxic metabolites of, e.g. acetaminophen by affecting their disposition [2,3]. At the same time, it can affect the bioavailability of several drugs (e.g. antibiotics, HIV protease inhibitors and anticancer agents) by the same mechanism, representing a potential risk for drug–drug interactions. Therefore, the possibility to predict whether a drug is a substrate or an inhibitor for MRP2 is pharmacologically and toxicologically important. MRP2 interacts with a wide structural and pharmacological range of compounds as well as phase II metabolites [2]. Thus, interplay between MRP2 and UGT (uridine diphosphate glucuronosyltransferase) isoenzymes has been observed [4].

The clinical importance of the above mentioned defence mechanisms has lead to the increased interest to develop *in vitro* methods that can be used during drug discovery and development in order to recognize, e.g. efflux protein substrates. The U.S. Food and Drug Administration (FDA) has published draft guidance for the industry on drug interaction studies, containing guidelines for the screening for P-glycoprotein (MDR1) substrates and inhibitors [5]. Methods such as the Calcein AM test and the ATPase assay have been developed for the rapid screening of MDR1 substrates, but there are concerns about the specificity of the methods, since other efflux proteins are involved in the kinetics of these probes as well [6,7].

Efflux protein substrates are often screened using vesicles or cells from cell lines transfected with a specific efflux protein (e.g. MDCKII-MRP2 of canine origin) [8,9]. However, if cells originating from other species are used, the possible interference of intrinsic efflux systems and their (dis)similarity to human cells/tissues have to be considered [10]. Furthermore, since many compounds do not interact directly with MRP2, but their intracellularly produced metabolites are substrates, the significance of the presence and functionality of the relevant metabolizing enzymes must be taken into account [11].

The Caco-2 is a widely used cell line derived from human colorectal carcinoma [12]. Permeability across the fully differentiated Caco-2 monolayers is considered to model intestinal absorption [13]. Caco-2 was chosen as a platform for this screening method, since it is relatively easy to culture and is well characterized, and the expression of MRP2 and UGT isoenzymes has been observed [14–16]. However, the Caco-2 batch used in the efflux studies

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should always be thoroughly characterized at the relevant passage and level of differentiation. In our cells, a higher mRNA level expression of MRP2 has been observed in the fully differentiated cells [17].

CDCF (5(6)-carboxy-2′,7′-dichlorofluorescein) has been used as a model substrate for MRP2- and MRP5-mediated efflux in hepatocytes and membrane vesicles of transfected HEK-cells [18,19]. Although it is also a substrate for MRP1 and MRP3, CDCF is specific to MRP2 considering the apical efflux transporters expressed in Caco-2 cells [20]. CDCF is actively transported into hepatocytes, but it can also be administered to the cells as a diacetate derivative, CDCFDA. The non-fluorescent diacetate form passively diffuses across the cell membrane and is thus susceptible to intracellular esterases capable of de-esterification of the CDCFDA into the fluorescent CDCF [21].

The objective of this work was to develop a rapid, higher-throughput, Caco-2 based early-stage screening method for the compounds that interact with MRP2 efflux protein, i.e. substrates and/or inhibitors. Protocol development was accomplished by culturing the Caco-2 cells on different 96-well plates for 7–15 days and then loading the cell monolayers with CDCFDA, varying the loading times and concentrations. The cells were then exposed to known MRP2 substrates, non-substrates and inhibitors. Efflux of the fluorescent CDCF was monitored either by measuring directly from the cell plate with a fluorescence plate reader at several time points or by taking samples from the cell plate and analyzing them on a separate plate. The observed fluorescence values were assumed to correlate with MRP2 efflux activity. Passive permeation properties of CDCF and de-esterification of CDCFDA in Caco-2 cell monolayers were also studied to validate the mechanistic assumptions.

2. Materials and methods

2.1. Reagents and materials

Cell culturing reagents were purchased from Euroclone (Pero, Italy) except for fetal bovine serum and HBSS 10× concentrate from Gibco Invitrogen Corporation (Carlsbad, CA, USA) and Hepes from Sigma (St. Louis, MO, USA). All the plasticware were obtained from Corning B.V. Life Sciences (Schiphol-Rijk, Netherlands). CDCF, CDCFDA, porcine liver esterase (PLE), acetaminophen, indomethacin and *p*-aminohippuric acid were purchased from Fluka (Buchs, Switzerland); probenecid, diclofenac and furosemide from Sigma–Aldrich (St. Louis, MO, USA); MK571 from Cayman Chemicals (Ann Arbor, MI, USA); and verapamil from MP Biomedicals (Aurora, OH, USA). All the other laboratory chemicals were of analytical grade.

2.2. Cell cultures

Caco-2 (wild type) cells obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained at +37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity. Cells were grown in a medium consisting of DMEM (Dulbecco's modified Eagle's medium, high glucose 4.5 g/l), 10% HIFBS (Heat Inactivated Fetal Bovine Serum, inactivation at +56 °C for 30 min), 1% NEAA (Non-Essential Amino Acids), 1% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The medium was changed three times a week. The cells were harvested weekly from plastic flasks (75 cm^2) with 0.25% trypsin. The cells were seeded at 6.8×10^4 cells/cm^2 in cell culture treated clear, black or clear-bottomed black 96-well plates for efflux experiments or onto polycarbonate filter membranes (pore size 0.4 μm, filter area 1.1 cm^2) in 12-well Transwell® insert plates for permeation studies. The cells were allowed to grow for 7–15 days for the efflux experiments and fully differentiate for 21–28 days for the permeation studies.

2.3. Permeation studies

Transport of the fluorescent efflux substrate CDCF was studied across Caco-2 monolayers both in apical to basolateral (A → B) and in basolateral to apical (B → A) direction at pH 7.4 and 6.5. The same pH was used in both compartments during each experiment. Cell monolayers were washed twice with HBSS–Hepes (10 mM Hepes, pH 7.4 or pH 6.5, respectively) and then equilibrated for 30 min in the washing buffer solution. The integrity of the monolayers was verified by measuring transepithelial electrical resistance (TEER) across each monolayer before and after the experiment using Millipore Millicell®-ERS (Bedford, MA, USA). TEER values above 250 Ω were considered acceptable.

In the experiments, 10 μM (pH 6.5) or 50 μM (pH 7.4) CDCF in HBSS–Hepes was placed in the apical or in the basolateral chamber. The initial concentration was verified by immediately taking a sample from the donor compartment, so that 500 μl (A) or 1.5 ml (B) of the solution was left at the beginning of the experiment. Samples were taken from the receiver compartment at 15 min intervals over 60 min and replaced immediately with buffer. Fluorescence of the samples was analyzed in 96-well plates using Wallac Victor® 1420 multilabel counter (Turku, Finland) at 485 nm excitation and 535 nm emission wavelengths. The analytical method was calibrated for each analysis over the concentration range 0.001–2 μM ($R^2 > 0.99$). Apparent permeability coefficients (P_{app}, cm/s) were calculated based on

$$P_{app} = \frac{(dQ/dt)}{(A * C_0 * 60)} \quad (1)$$

where dQ/dt is the cumulative transport rate (nmol/min), A is the surface area of the cell monolayer (1.1 cm^2) and C_0 is the initial concentration in donor compartment (nmol/ml).

2.4. De-esterification experiments

Caco-2 cells grown to confluency in a plastic flask (7–8 days, to mimic the cells grown in 96-well plates) or fully differentiated cell monolayers (at least 21 days) on polycarbonate filter membranes were washed four times with PBS solution, scraped off and disrupted in Milli-Q water (Millipore, Molsheim, France). The cells were exposed to four freeze-thawing cycles: rapidly frozen in liquid nitrogen and immediately thawed in 37 °C water bath. The disrupted cell suspension (100 μl/well, containing 10 μg of total protein) was pipetted into a black 96-well plate. One-hundred microliters of freshly prepared CDCFDA solution was added into each well and the fluorescence resulting from the de-esterification of CDCFDA (1 nM to 5 μM) into the fluorescent CDCF was monitored with the multilabel counter as described above. Spontaneous de-esterification in the study buffer was used as a negative control and de-esterification in the presence of porcine liver esterases (PLE) (0.25–250 μg/ml; 27 IU/mg) was used as a positive control.

2.5. Efflux experiments

Caco-2 cells grown in clear, black or clear-bottomed black 96-well plates for 7–15 days were used for these experiments. To ensure the viability of the cells over longer growth periods, Corning® CellBind® plates were used to improve the attachment of the cells during growth periods exceeding 9 days. The cells were washed twice with HBSS–Hepes (10 mM Hepes, pH 7.4) and then equilibrated for 15–20 min in the washing buffer solution. The washing solution was then removed, and the cells were loaded for 5–30 min

with 200 μl of 0.5–10 μM CDCFDA (all the solutions were prepared in HBSS–Hepes, pH 7.4). The loading solution was removed, and the cells were washed briefly with buffer. The solutions containing the studied compounds (probenecid, MK571, verapamil, acetaminophen, diclofenac, indomethacin, *p*-aminohippuric acid or furosemide) were pipetted into the wells. HBSS–Hepes, pH 7.4, was used as a control (100% efflux) in each experiment. All the incubations were conducted at +37 °C. In the experiments where the fluorescence was measured directly from the cell plate, the total volume was 200 μl in each well. Where a separate, fresh black plate was used for the analysis of samples, 200 μl of the total volume of 250 μl was collected from each well. In either approach, the fluorescence was determined as described above in the permeation studies.

The statistical quality of a screening method can be assessed with a value called *Z* factor (Eq. (2)). An ideal assay would have a *Z* factor of 1.0, which can never actually be achieved. Assays with *Z* factor values > 0.5 (or even 0.4 for cell-based assays) are considered excellent, while assays with *Z* factors < 0 are essentially useless [22].

$$Z\_\text{factor} = 1 - \frac{3 * (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (2)$$

μ is the mean, σ is the standard deviation, *p* are the positive controls, *n* are the negative controls.

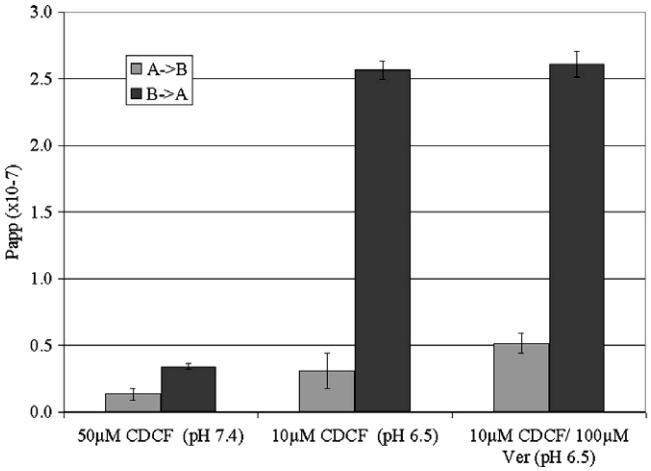

Fig. 1. The effects of pH and verapamil on the transport of CDCF (P_{app} ± SD, $n = 3$) across the fully differentiated Caco-2 monolayers.

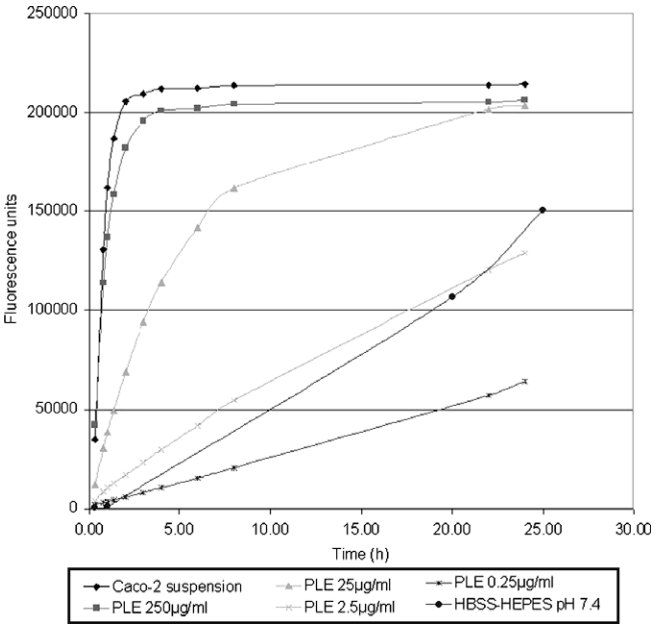

Fig. 2. Observed fluorescence of CDCF following the de-esterification of 1 μM CDCFDA in study buffer (HBSS–Hepes, pH 7.4), Caco-2 cell monolayer homogenate or PLE (porcine liver esterase) solutions.

3. Results

The permeation of CDCF across the Caco-2 monolayers was observed to be very low at pH 7.4, with $P_{app} < 10^{-7}$ cm/s both in apical to basolateral (A → B) and in basolateral to apical (B → A) direction (Fig. 1). Lowering the pH to 6.5 enhanced the permeation (more of the CDCF in unionized form), but it still remained quite low in both directions. Efflux activity was observed at pH 6.5, indicated by the B → A/A → B permeability ratio of 8.3; compared to the efflux ratio of 2.5 at pH 7.4. Verapamil did not affect the permeation or efflux ratio (Fig. 1).

De-esterification of CDCFDA in the Caco-2 cell suspensions resulted in a more than 100-fold CDCF formation compared to the study buffer without added cell suspension (Fig. 2). The intracellular enzymes present in the Caco-2 cells (monolayer homogenate) produced similar de-esterification kinetics to that observed in the reference esterase solution of the highest PLE concentration (250 μg/ml) with complete de-esterification of 1 μM CDCFDA within 2.5 h. Over the studied concentration range of CDCFDA (0.001–1.0 μM) and the different concentrations of PLE (0.25–250 μg/ml), de-esterification exhibited concentration dependent kinetics (Fig. 3). Similar concentration dependence was observed in Caco-2 cell homogenates (0.01–5.0 μM CDCFDA, $R^2 = 0.9993$). In conclusion, at the studied CDCFDA concentrations the de-esterification rates follow the linear part of the Michaelis–Menten curve, i.e. the intracellular esterases are not yet saturated.

During the method development, protocol variables (loading concentration of CDCFDA, sampling time, assay method, concentration of DMSO as a co-solvent) were optimized to improve the discriminating power of the method (Table 1). To that end, the lowest possible concentration of CDCFDA loading solution that produced clear responses was selected. Sampling time was balanced between the highest obtained responses, the smallest variation and the length of the experiment, since a screen should be as rapid as possible. The influence of low concentrations of DMSO as a co-solvent was tested due to the poor water solubility of many of the recent NCEs.

The final protocol (Fig. 4) is based on the initial set-up derived in Table 1 and the results of further refinement of variables are presented in Table 2. Preliminary tests were also performed, in part being the basis in the rationale of Table 1 (e.g. cell growth period, plate types for cell culturing and sample analysis, CDCFDA loading time, choice of study buffer; results not shown), and the conditions generating (yielding) the best reproducibility and the smallest variation were chosen for the protocol. The finalized protocol was evaluated for screening the compounds interacting with the MRP2 efflux protein (Fig. 5 and Table 2). In most cases, the method separated selectively the substrates or inhibitors from the non-substrates. The limitations, challenges and possible refinements of the method are further addressed in Section 4 (Table 3).

4. Discussion

The present results suggest that Caco-2 cells loaded with CDCFDA, followed by the efflux of fluorescent CDCF, provide a good

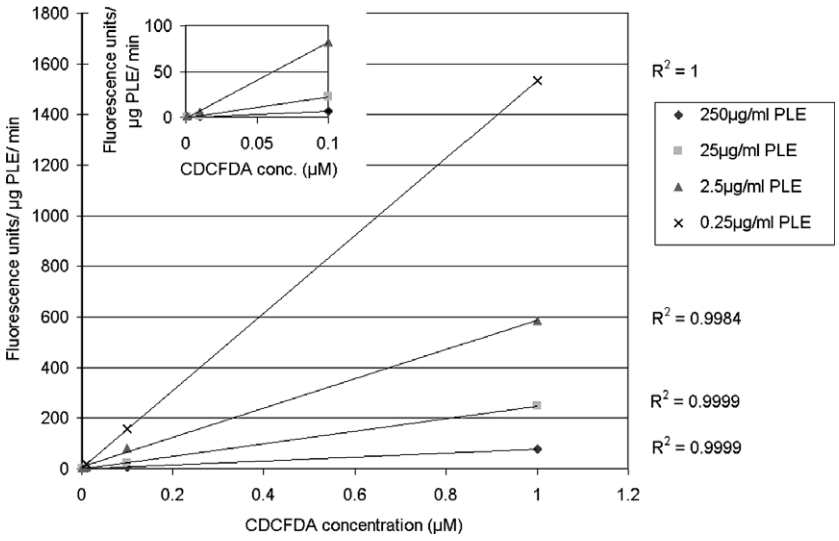

Fig. 3. Kinetics of de-esterification of 0.001–1 μM CDCFDA at different concentrations of PLE. The rates of de-esterification were calculated from five time points over the first 90 min of the incubation presented in Fig. 2. Fluorescence responses obtained for 0.001 μM CDCFDA with 0.25 μg/ml PLE were below (and with 2.5 μg/ml PLE close to) the limit of detection.

Table 1
The rationale for the selection of the initial experimental set-up of the MRP2 screening method

Variable	Considered options	Chosen option	Rationale
Cell line	• Caco-2 • MDCKII-MRP2	Caco-2	• Established expression of other transporters and phase II metabolizing enzymes possibly involved in MRP2 interactions
Culture plate	• 96-well cell culture plates: - Clear - CellBind® - Black - Black, clear bottom	Clear 96-well plates for cell culture. Black plates for fluorescence measurements of the samples	• Clear 96-well plates are the standard for HTS cell culturing: most affordable and allow for visual inspection of the cells • Black plates for fluorescence measurements: less background and cross-talk
Assay method	• Measurements directly from the cell plate • Sampling into a separate plate for analysis	Sampling into a separate plate followed by immediate analysis	• Greatly improved signal to noise ratio at measurements justifies an additional protocol step • Fluorescence reading immediately post-sampling to reduce the possible effect of spontaneous hydrolysis of residual CDCFDA
Cell growth period	• 8 days • 15 days	8 days	• Cells grown in 96-well plates (also CellBind®) for more than 8 days were more fragile during washing steps • Complete differentiation would require 21 days on a filter
CDCFDA loading time	• 10 min • 30 min	10 min	• Sufficiently long equilibration with CDCFDA solution to allow intracellular hydrolysis and accumulation of CDCF in the cells • Initially selected 30 min was later shortened to 10 min; reproducibility of the results was not affected

platform for rapid MPR2 interaction studies. The permeation of CDCF itself, studied across Caco-2 monolayers at pH 7.4, appeared to be quite low, giving little information on potential efflux activity. At 10 μM donor concentration in pH 7.4 buffer, the observed CDCF concentrations in the receiver compartment were below the limit of quantification, and a higher concentration (50 μM) was needed for the determination of P_{app} values. Being a dicarboxylic acid, CDCF is likely to be highly ionized at pH 7.4, and thus not

1. Seed wild type Caco-2 cells on regular, cell culture treated, clear 96-well plates.
2. Let the Caco-2 cells grow in the wells for 8 days. Change the medium every 2-3 days.
3. On the day of the experiment, remove the growth medium and wash the cells twice with 200µl/well of the experimental buffer (HBSS-HEPES pH 7.4). Incubate for 15-20 minutes at 37°C with the last washing solution.
4. Remove the washing solution and add 200µl of 5µM CDCFDA. Incubate for 10 minutes, remove the loading solution and wash briefly with buffer.
5. Pipette the experimental solutions (250µl/well) on the plate column by column at pre-set intervals (e.g. 10s) according to the plate map of your experiment.
6. At t = 30min, take 200µl samples from each column at pre-set intervals. Pipette the samples into a black 96-well plate and analyze with a fluorescence plate reader (λ_{ex} = 485nm and λ_{em} = 535nm).
7. Correct the fluorescence responses for the background fluorescence. Compare your results to known MRP2 substrates, non-substrates, inhibitors and compounds susceptible to glucuronidation.

Fig. 4. The final protocol for the MRP2 efflux screening method.

able to passively permeate cell membranes in efflux activity experiments. The pK_a values obtained for the carboxylic acid groups of CDCF by potentiometric titration (3.58 ± 0.04 and 4.86 ± 0.02) suggest that the distribution of CDCF into the Caco-2 cell membrane is indeed limited by the high extent of ionization at pH 7.4. Furthermore, lowering the pH to 6.5 enhanced somewhat the permeation, and efflux activity could be observed across the Caco-2 monolayers. CDCF has previously been used in hepatocytes, where the poor passive permeation properties of the highly ionized CDCF were circumvented by the active uptake of CDCF by organic anion-transporting polypeptides (Oatp) [18]. Oatp transporter activity was not evident in this study, although a few reports on the functionality/expression of Oatp transporters in Caco-2 cells have been published [30,31].

CDCF can, however, be administered into Caco-2 cells as the non-fluorescent diacetate derivative CDCFDA, which passively diffuses across the cell membrane [21]. According to our results, the hydrolysis of CDCFDA into CDCF by the intracellular esterases in Caco-2 cells was a rapid and complete process. Together with the poor passive permeability properties of the parent CDCF, this observation provides a solid mechanistic base for the utilization of the probe pair CDCFDA/CDCF as a screening tool for MRP2 efflux substrates in Caco-2 cells.

Several active transport proteins and metabolizing enzymes are expressed in Caco-2 cells. The specificity of any efflux activity screen has to be carefully considered, since a wide overlap is recognized in the specificity of substrates and inhibitors of, e.g. MDR1, BCRP and the MRPs [32]. In our studies, verapamil did not have any effect on the permeation of CDCF across the Caco-2 monolayers, which is consistent with the previous reports stating that verapamil is not an inhibitor of MRP2 at these concentrations [28]. However, verapamil is a known inhibitor of MDR1 and some of the other MRPs. Since MRP2 is the only MRP efflux protein presently known to be localized on the apical surface of the Caco-2 monolayers [20], we conclude that the described experimental setting is sufficiently specific for the screening of compounds interacting with MRP2. For the compounds that are transformed into MRP2 substrates after glucuronidation, the expression profile of the relevant UGT enzymes in Caco-2 cells should also be taken into account. Some UGTs are known to be expressed in Caco-2 cells, but the screening protocol would underestimate the MRP2 interaction, in case the UGT isoenzyme responsible for producing the glucuronide conjugate substrates was lacking [33].

During the development of the screening protocol, several factors needed to be considered and optimized. Most of these variables and their effects are described in Section 3 (Tables 1 and 2). The significance of some factors could only be determined by the visual observations of the cells. For example, several 96-well plate types were tested for varying growth periods and the fluorescence was measured either directly from the cell plate or from the fresh plates after withdrawing separate samples. While it was obvious that the background fluorescence caused by the black plate was the lowest, it was difficult to determine the viability of

Table 2
Inhibition of efflux of the fluorescent CDCF (%) compared to the fluorescence observed in the reference solution (HBSS–Hepes pH 7.4) following 10 min loading with CDCFDA

Protocol variable	Fluorescence response (% of reference)				Compound	
(A) Effects of the CDCFDA loading concentration (measurement/sampling at 30 min; Average ± SD, n = 6)						
CDCFDA concentration	0.5 µM	2 µM	5 µM			
Measurements directly from the black cell plate	124 ± 32	94 ± 15	45 ± 16		1 mM	Diclofenac
	96 ± 30	88 ± 19	71 ± 13		0.1 mM	
	85 ± 11	85 ± 19	87 ± 11		1 mM	Probenecid
Sampling into a separate plate for analysis	51 ± 3	54 ± 6	54 ± 8		1 mM	Diclofenac
	84 ± 20	89 ± 8	80 ± 16		0.1 mM	
	87 ± 32	85 ± 29	77 ± 19		1 mM	Probenecid
(B) Effects of the sampling time and use of DMSO as a co-solvent (loading with 5 µM CDCFDA, sampling into a separate plate for analysis)						
Sampling time	20 min	30 min	40 min	60 min		
(average ± SD, *n* = 6)	56 ± 9		47 ± 3	53 ± 17	1 mM	Indomethacin
	62 ± 3		62 ± 4	80 ± 11	0.1 mM	
	84 ± 10		87 ± 13	98 ± 12	0.01 mM	
(average ± SD, n = 48)		61 ± 13	70 ± 14		1 mM	Probenecid
DMSO concentration	0.5%	1%	2%			
(average ± SD, *n* = 6) (Sampling at 40 min, compared to 0% DMSO)	109 ± 19	103 ± 7	115 ± 4		0.5 mM	Indomethacin

DMSO used as a co-solvent at max 1% unless otherwise stated.

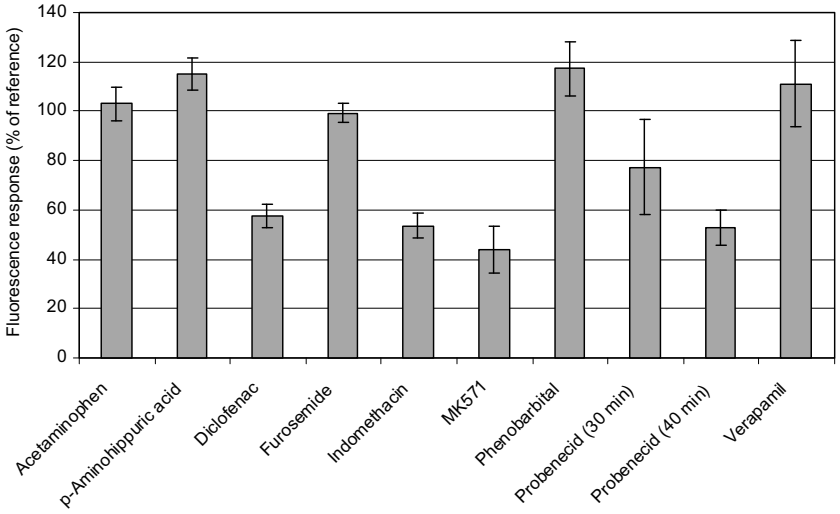

Fig. 5. Testing of the MRP2 screening method with known substrates, inhibitor and non-substrate: inhibition of efflux of the fluorescent CDCF compared to the fluorescence observed in the reference solution (HBSS–Hepes, pH 7.4; 100%) at the selected conditions: 10 min loading with 5 μM CDCFDA, 1 mM test compound concentration (except 100 μM MK571), sampling at 30 min (diclofenac, indomethacin, MK571, probenecid and verapamil) or at 40 min (all the other compounds), DMSO max 1%.

Table 3
Testing of the MRP2 screening method with known substrates, inhibitors and non-substrates

Compound	Substrate?	Results	Conclusion
Acetaminophen (Paracetamol)	Glucuronide conjugates are substrates of MRP2 and MRP3 [23]	Did not inhibit the efflux of CDCF. Log*P* = 0.34	Too hydrophilic to enter the cells or glucuronides not formed in Caco-2!
p-Aminohippuric acid	MRP2 substrate [24]	Did not inhibit the efflux of CDCF. Log*P* = −0.07	Too hydrophilic to enter the cells passively!
Diclofenac	Glucuronide conjugates are MRP2 substrates [25]	Inhibited the efflux of CDCF at 1 mM and 100μM (weaker response). Log*P* = 4.06	Substrate/inhibitor
Furosemide	Contradictory information on affinity to efflux proteins	Did not inhibit the efflux of CDCF. Log*P* = 3.00	Not a substrate/inhibitor
Indomethacin	Glucuronide conjugates are MRP2 substrates, but the parent compound is not [26]	Inhibited the efflux of CDCF at 1 mM and 100 μM (weaker response). Log*P* = 3.10	Substrate/inhibitor
MK571	Model inhibitor for MRPs	Inhibited the efflux at 100 μM. Log*P* = 5.93	Substrate/inhibitor
Phenobarbital	Glucuronide conjugates are MRP2 substrates [27]	Did not inhibit the efflux of CDCF. Log*P* = 1.67	Yet inconclusive
Probenecid	Inhibits several MRPs [28]	Inhibited the efflux of CDCF at 1 mM, 100 μM and 10 μM (weaker response). Log*P* = 3.30	Substrate/inhibitor
Verapamil	Inhibits MDR1, MRP1 and MRP3 but not MRP2 [29]	Did not inhibit the efflux of CDCF. Log*P* = 3.90	Not a substrate/inhibitor

Inhibition of efflux of the fluorescent CDCF compared to the fluorescence observed in the reference solution (HBSS–Hepes, pH 7.4; 100%) at the selected conditions: 10 min pre-loading with 5 μM CDCFDA, sampling at 30 min, DMSO max 1%.

the cells grown in black plates prior to and after each experiment as the only means for this was visual inspection (e.g. compared to measuring TEER values across cell monolayers on filters). Regular, clear 96-well plates allowing visual inspection of the cells were eventually chosen as the cell growth plates, whereas fluorescence measurements of separate samples should be performed on black plates. The cells should be used at the highest possible level of differentiation to ensure sufficient expression of the MRP2 efflux protein [17]. Still, the growth period of 8 days appeared to produce satisfactory efflux responses. Extension of the growth period to up to 15 days did not markedly improve the results, whereas the variation was increased due to the reduced viability of the cells (even in the CellBind® plates specially treated to improve attachment). A loading time of 30 min with CDCFDA solution was initially selected to allow for the accumulation of CDCF in the cells, following the intracellular hydrolysis of CDCFDA. At a later stage, the loading time was shortened to 10 min. However, a yet shorter loading time (e.g. 5 min) could be enough to produce reproducible results, but this would require further validation.

The developed protocol was evaluated with some substrates and inhibitors of MRP2 as well as with some non-substrates. While the protocol mostly succeeded in picking out the compounds known to interact with MRP2, the results also presented some challenges. A known issue is related to the use of cell-based screening methods for hydrophilic compounds: false negative responses due to the insufficient distribution into the cell membrane [8]. Another concern is related to the incomplete differentiation of Caco-2 cells cultured for screening purposes: in the absence of fully polarized monolayers, some uptake transporters may not be functional, limiting the entry of their substrates into the cells and thus the access to MRP2 binding site [34]. Nevertheless, most of the recent NCEs are quite lipophilic molecules, with more potential problems related to their solubility than permeability properties. The solubility issues can be addressed in the screening protocol by using co-solvents such as DMSO. Co-solvents can also be used to increase the concentrations of the screened substances, since quite high concentrations were needed for the statistically significant responses (*Z* factor).

The quality of the developed method as a screening assay was studied by determining the *Z* factor [22]. An assay with *Z* factor > 0.5 (or even 0.4 for cell-based assays) is considered excellent, while +/− type assays typically have *Z* factors ~ 0. When the response caused by 1 mM diclofenac was compared to the negative control (study buffer), a *Z* factor of 0.47 was obtained with the final protocol. However, +/− type responses sufficient for initial screening could also be obtained for other compounds studied, also at lower concentrations.

In order to determine the significance and application potential, the final protocol should be further tested and validated, preferably aided with an automated pipetting system. The possibility to dispense the study solutions and to take samples from all the 96 wells simultaneously would markedly reduce the systematic variation and sporadic errors caused by the delays due to manual pipetting. Furthermore, the spontaneous hydrolysis of CDCFDA into CDCF in the study buffer has to be taken into account when refining the method in terms of timing the pipetting sequences. The delay caused by manual pipetting was quite evident in some of our experiments; hence the use of, e.g. a 96-well pipettor would be well grounded.

In conclusion, the developed Caco-2 based method, utilizing the probe pair CDCFDA/CDCF, provides a promising screening tool for MRP2 substrates and inhibitors along with compounds, the glucuronides (and/or other phase II conjugates) of which are MRP2 substrates provided these are formed in the used Caco-2 cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.08.010.

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