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A 96-Well Efflux Assay To Identify ABCG2 Substrates Using a Stably Transfected MDCK II Cell Line

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Abstract: Human ABCG2 (breast cancer resistance protein, BCRP) is an important efflux transporter which exhibits broad substrate specificity and which is found in many tissues. The purpose of the present study was to develop a 96-Transwell assay using an MDCK II cell line stably transfected with ABCG2 (MDCK II/ABCG2) to identify ABCG2 substrates. In this assay, which also incorporates a high throughput mass spectrometry method for quantification, efflux activity of the MDCK II/ABCG2 cells was evaluated by monitoring the basolateral-to-apical/apical-to-basolateral (B to A/A to B) efflux ratio of several substrates. Mean MDCK II/ABGC2 efflux ratios for 2 μM prazosin, SN-38, and CI1033 were 2.8, 7.6, and 2.4, respectively, and the mean efflux ratio for 10 μM mitoxantrone was 5.0. Interday variability of the assay was low (CV = 10−29% for control compounds at 2 μM). Our data indicate that a compound tested at 2 μM can be considered a substrate of ABCG2 if its ratio of ratios (MDCK II/ABCG2 efflux ratio)/(MDCK II efflux ratio) is >1.2. This assay provides an efficient, high throughput means to identify ABCG2 substrates in drug discovery.

Keywords: ABCG2; BCRP; ATP-binding cassette, sub-family G, member 2; Transwell assay; efflux ratio

Introduction

The human ABCG2 protein is a recently discovered ABC half-transporter, which is a 72 kDa protein consisting of 655 amino acid residues that homodimerize to form an active transport complex.[1,2] ABCG2 mediates the extrusion of substrates toward the extracellular space through a process energized by ATP hydrolysis.[3] ABCG2 is expressed in a number of normal tissues, such as the canalicular membrane of hepatocytes, the apical membrane of intestinal epithelium, and the luminal surface of brain capillaries, as well as human placenta.[4−7] The ABCG2 transporter has a broad substrate specificity that overlaps with that of ABCB1 (MDR1) and ABCC2 (MRP2).[8]

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ABCG2 was originally isolated from a multidrug-resistant breast cancer cell line coselected with doxorubicin and verapamil (an ABCB1 inhibitor) in an effort to elucidate the non-ABCB1 mechanism of drug resistance.[2,9] ABCG2 is overexpressed in a variety of human multidrug resistant (MDR) cancer cell lines that exhibit either an ABCB1 or non-ABCB1 drug resistant phenotype. Overexpression of ABCG2 in these cell lines results in resistance to several anticancer drugs such as mitoxantrone, topotecan, daunorubicin, and bisantrene.[10]

Variants of ABCG2 cDNA have also been isolated from drug-selected cell lines displaying distinctly different MDR phenotypes. These variants exhibit different amino acids at position 482, which is presumed to be at the start of the third transmembrane helix. Wild-type ABCG2 contains an alkaline arginine at position 482 (R482) and is unable to efficiently transport the cationic dye rhodamine 123 and the anticancer drug doxorubicin,[1] whereas variant ABCG2 genes, with threonine (T482) or glycine (G482), are able to transport these compounds. These mutations confer different substrate specificities to anticancer drugs to these cell lines. Moreover, various human SNPs have been identified in the ABCG2-encoded gene.[10] These may affect drug absorption and distribution, with the potential of altering drug effectiveness in large populations;[11] in fact, inhibition of ABCG2-mediated transport has been shown to alter the pharmacokinetics of ABCG2 substrates, producing increased bioavailability in humans, and increased distribution across the placenta and decreased clearance in mice.[12,13]

In the present study, we describe the generation of a stable MDCK II/ABCG2 cell line. We have characterized the cell line by Western blot analysis and then evaluated the cell line by monitoring ABCG2-mediated efflux. Using the MDCK II/ABCG2 cell line, we developed a 96-Transwell high throughput assay which can be used in drug discovery to rapidly identify ABCG2 substrates.

Materials and Methods

Materials. Prazosin, triprolidine, daunorubicin, mitoxantrone, and topotecan were purchased from Sigma-Aldrich (St. Louis, MO). CI-1033, SN-38, and compound 1 were obtained from Pfizer Inc. (Groton, CT). High-performance liquid chromatography grade DMSO was obtained from Fisher Scientific Co. (Pittsburgh, PA) and used as the solvent for making stock solutions for all the compounds. MEM alpha medium, penicillin-streptomycin, FBS, nonessential amino acids, L-glutamine, trypsin-EDTA, phosphate-buffered saline (PBS), buffer B (Hanks balanced salt solution with added components, Invitrogen Cat. No. C17066), and Geneticin were purchased from Invitrogen (Carlsbad, CA). BODIPY-prazosin and Lysotracker Green were purchased from Molecular Probes Inc. (Eugene, OR). BD feeder trays and BD 96-well membrane inserts were purchased from Becton Dickinson BioScience (San Jose, CA), and 1.2 mL 96-well deep well plates were purchased from Marsh (Rochester, NY).

MDCK/ABCG2 cell line. The full-length cDNA clone of the human ABCG2 wild-type gene (R482) (acquired from Incyte Corp., Wilmington, DE) was subcloned into a pcDNA3.1/V5-His TOPO TA expression vector followed by RT-PCR. PCR reactions were carried out for 35 cycles as follows: denaturation for 2 min at 94 °C, annealing for 1 min at 57 °C, and extension for 7 min at 72 °C using the gene specific primers (forward, 5′ ctgctgctttaggaggtttgt; reverse, 5′ gaatacttcaatcaaagtgc).

The above ABCG2 plasmid DNA solution was diluted with Opti-MEM I reduced serum medium. The diluted DNA was combined with diluted Lipofectamine 2000 (ratio of DNA:lipofectamine = 1:3) and incubated for 20 min. The mixture was added to MDCK cells at 90−95% confluence, and cells were selected with 500 μg/mL Geneticin (G418) in complete MEM alpha media (500 mL of MEM alpha supplemented with 10% FBS, 1% nonessential amino acids, 1% penicillin-streptomycin, 1% L-glutamine) 2 days post-transfection. Cell colonies were expanded for further analysis 10 days post-transfection.

Selection of Single Cell Clone and Accumulation Analysis Using FACS (Fluorescence Activated Cell Sorting). The above expanded cell colonies were incubated with 5 μM of BODIPY-prazosin for 30 min. The reaction was stopped with ice-cold PBS, and cells were incubated with medium for 30 min. The cells were trypsinized in 0.25%

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trypsin-EDTA, then centrifuged for 10 min at 700 rpm and resuspended with PBS for flow cytometry analysis and sorting to select single cell clones. FACS was performed on a FACS VANTAGE SE (Becton Dickinson BioSciences, San Jose, CA). An excitation wavelength of 488 nm at 200 mW was used. Bodipy-prazosin fluorescence was detected through a 560 DMSP beam splitter and a 530/30 nm emission filter. A dual parameter dot-plot of forward light scatter and side light scatter were used to exclude debris and to identify and gate on viable cells. The per cell fluorescence distribution was displayed on a linear scale. Individual cells from the dimmest 1% of the bodipy-prazosin labeled population were sorted into each well of a 96-well flat bottom plate containing 200 μL of culture medium. The plates were then cultured in complete MEM alpha media for about 10 days to reach confluence. After expansion of the above selected cell clones, relative lower cellular accumulation of fluorescent compound in single cell clones (vs MDCK II cells) was determined and selected by analysis on a BD LSR 1 cytometer (BD BioScience, San Jose, CA). Excitation and emission wavelengths for BODIPY-prasozin were 480 and 530 nm, respectively. For efflux analysis of clones, viable cells were excluded from debris via application of a forward light scatter and side light scatter gate. Ten thousand cells were analyzed per sample. The fluorescence of accumulated substrate in the tested clones was quantified from histogram plots using the geometric mean of fluorescence.

Western Blotting Analysis. The single cell clones selected from FACS were expanded and then seeded on 6-well plates and cultured in complete MEM alpha medium with or without 2 mM sodium butyrate for 24 h. Sample buffer (500 μL) containing protease inhibitors (Roche, Indianapolis, IN) was used to lyse monolayer confluent cells. The cell lysate was placed on ice for 15 min and then aspirated and transferred to micro centrifuge tubes. One part of NuPage reducing agent and 9 parts of the lysate were mixed and then heated for 10 min at 70 °C. The sample was loaded at 20 μL/well to a 4−12% Bis-tris SDS PAGE gel in a Mini-protein II electrophoresis cell (Bio-rad, Hercules, CA). Standard Western blot procedure was followed. Briefly, proteins were electrotransferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) using MOPS buffer (Invitrogen, Carlsbad, CA). The membrane was blocked with TBST (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% dry milk for 1 h at room temperature. The membrane was incubated with monoclonal anti-ABCG2 antibody BXP-21 (CHEMICON International Inc.) at 1:500 dilution as primary antibody in TBST buffer for 1 h at room temperature. The blot was then incubated with secondary horseradish peroxidase-conjugated antibody (Bio-Rad, Herculers, CA) (1:2000 dilution, 1 h incubation at room temperature). The membrane was incubated with ECL plus detection buffer (Amersham Pharmacia Biotech, England, U.K.) for 5 min at room temperature. Visulization of ABCG2 expression was taken under a Lumi-Imager F1 (Roche, Mannheim, Germany).

MDCK II/ABCG2-Mediated Efflux Assay. FACS selected single cell clones were seeded on 96-well plates at a

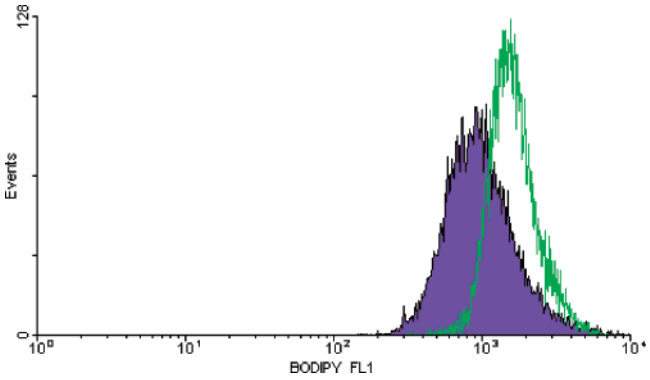
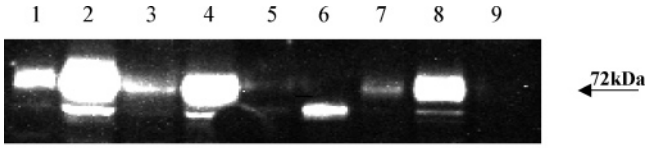


Figure 1. Selection of ABCG2-transfected MDCK II cells. Pooled (representing a mixture of multiple clones) cells were incubated with 5 μM BODIPY-prazosin for 30 min, the reaction was stopped with ice-cold PBS, and then cells were incubated with medium only for 30 min. The cells were then resuspended in PBS for flow cytometry analysis. The histogram shows the geometric mean of fluorescent intensity. Purple represents transfected cells, and green represents nontransfected MDCK II cells.



1 - clone 7 w/o sodium butyrate
2 - clone 7 treated with sodium butyrate
3 - clone 1 w/o sodium butyrate
4 - clone 1 treated with sodium butyrate
5 - Sf9/ABCG2 cell membrane protein (low concentration, 0.45 µg/mL)
6 - Sf9/ABCG2 cell membrane protein (high concentration, 4.5 µg/mL)
7 - ABCG2 pooled cells w/o sodium butyrate
8 - ABCG2 pooled cells treated with sodium butyrate
9 - MDCK II wild type cells

Figure 2. Western blot of ABCG2 protein in transfected MDCK II cells. Cells were treated with or without 2 mM sodium butyrate 24 h before whole cell lysis. Sf9/ABCG2 cell membrane protein was used as a positive control. ABCG2 expression in MDCK II cells was detected using ABCG2 monoclonal antibody BXP-21 (1:500 dilution). Protein was loaded at 25 μg/well. Positive control Sf9/ABCG2 membrane vesicles were provided by SOLVO Biotechnology (Budaörs, Hungary). Note: The lower molecular weight band represents nonglycosylation protein.[20]

cell density of 3×10^5 cell /mL on day 1. Different concentrations of Lysotracker Green DND-26 were added to cells on day 2. The cells were incubated with Lysotracker Green DND-26 for 30 min at 37 °C and the reaction was stopped with ice-cold PBS. Next, cells were washed twice with ice-cold PBS; then 100 μL of PBS was added per well. ABCG2-mediated efflux was measured with a Tecan Safire plate reader (Grödig/Salzburg, Austria). Excitation and emission wavelengths for Lysotracker Green were set at 485 and 535 nm, respectively.

96-Transwell Assay. On day 1, ABCG2 transfected cells were seeded at a cell density of 2.5×10^5 cells/mL in complete MEM alpha media on Falcon/BD 96-well mem-

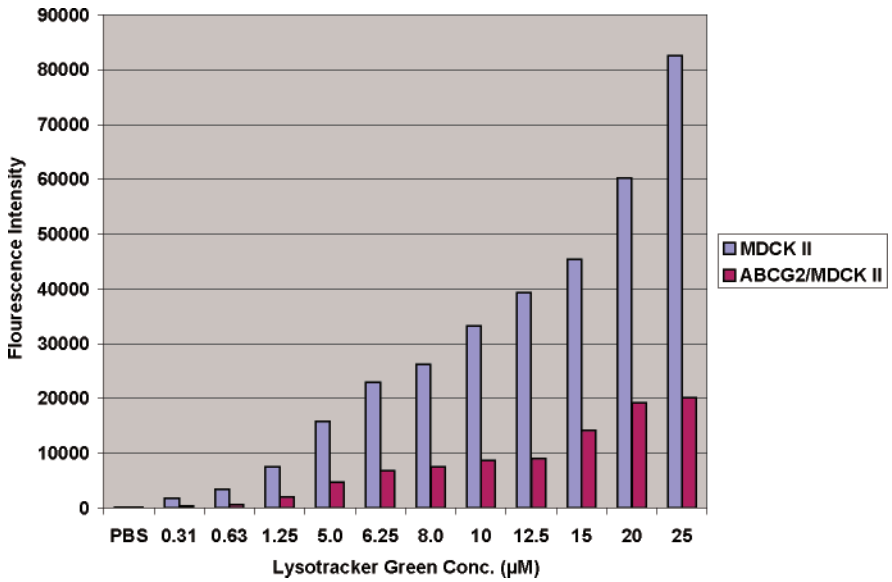


Figure 3. Lysotracker Green efflux mediated by MDCK II/ABCG2 cells. MDCK II/ABCG2 cells [clone #B7, cell passage number 3; 3×10^5 cells/well] were cultured in a medium containing 2 mM sodium butyrate for 48 h. The cells were incubated with Lysotracker Green for 30 min at 37 °C. The reaction was stopped with ice-cold PBS. Then cells were washed with ice-cold PBS twice, 100 μL of PBS per well was added, and cells were read with a plate reader (excitation at 485 nm and emission at 535 nm). Data came from a single experiment.

brane inserts, placed into BD feeder trays containing 37 mL of complete MEM alpha growth media, and incubated (37 °C, 95% humidity, 5% CO_2) for 5 days. Transwell studies were conducted on day 5.

Apical to Basolateral Studies. On day 5, medium was removed from the insert, 75 μL of fresh buffer B containing test substrates was added using a 96-well pipettor (Apricot Designs, CA), and the entire insert was placed into a 96-well collection plate containing 250 μL of buffer B per well, covered, and then incubated at 37 °C, 95% humidity, and 5% CO_2. After 5 h, 150 μL was removed from each well of the collection plate and transferred to a fresh 1.2 mL 96-well deep well plate. The remaining 100 μL of buffer B in the collection plate was removed and replaced with 100 μL of acetonitrile containing an internal standard. After mixing, 60 μL of acetonitrile was removed and added to the 150 μL buffer B sample previously removed. All samples were analyzed by MS/MS detection.

Basolateral to Apical Studies. Medium from the 96-well insert was removed and replaced with 75 μL of fresh buffer B. The insert was then placed into a fresh 96-well collection plate containing 250 μL of test compound in buffer B per well and incubated for 5 h. Afterward, 50 μL of sample from each well of the insert was removed and placed into a 1.2 mL deep well block containing 100 μL of fresh buffer B and 60 μL of acetonitrile containing an internal standard. All samples were analyzed by MS/MS detection.

Liquid Chromatography. A Gilson 215 multiprobe chromatography system (Gilson Instruments, Middleton, WS) was used during this study. The system was equipped with a custom-made autosampler deck that was configured to inject samples from 96-well plates. Sample injection volume was 25 μL. Chromatography was performed using Opti-Lynx C_{18}, 1 × 15 mm columns that contained particles with a pore size of 20 μm (Optimize Technologies, Oregon City, OR). The columns were set up in a dual column/column-switching format as described previously.[14] Specifically, the columns were configured to allow rapid on-line desalting of sample followed by elution of analyte to the mass spectrometer for detection. Column switching timing was set up such that total liquid chromatography cycle time was 20 s per sample. Two binary mobile phase systems were used during this study. Each system contained an aqueous mobile phase for sample loading and an organic mobile phase for compound elution. The first mobile phase system (pH = 6.4) consisted of an aqueous solvent system A, 5:5:90 (v/v/v %) methanol:acetonitrile:2 mM ammonium acetate, and organic solvent system B, 45:45:10 (v/v/v %) methanol:acetonitrile:2 mM ammonium acetate. The second mobile phase system (pH = 3.5) consisted of an aqueous solvent system A, 5:5:90:0.05 (v/v/v/v %) methanol:acetonitrile:5 mM ammonium formate: formic acid, and organic solvent system B, 45:45:10:0.05 (v/v/v/v %) methanol:acetonitrile:5 mM ammonium formate: formic acid. The mobile phase used during sample analysis was chosen on the basis of the system that gave optimal chromatographic parameters for the analyte(s). Two HPLC pumps (model PU-1580, Jasco Inc., Easton, MA) delivered mobile phase at a flow rate of 2 mL/min.

Electrospray Ionization—Tandem Mass Spectrometry. An API-3000 triple quadrupole mass spectrometer equipped

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Table 1. Summary of P_{app} Values ($\times 10^{-6}$ cm/s) and Efflux Ratios among Control Compounds[a]

prazosin (μM)		ABCG2 B to A P_{app}	ABCG2 A to B P_{app}	ABCG2 efflux ratio	MDCK II B to A P_{app}	MDCK II A to B P_{app}	MDCK II efflux ratio	ABCG2 ratio/ MDCK II ratio	95% CI for ABCG2 ratio/MDCK II ratio
0.5	mean	21	8.2	2.7	12	15	0.8	3.2	3.0−3.5
	% CV	13	16	20	18	19	23	12	
2	mean	23	8.4	2.8	13	14	1.0	2.9	2.8−3.1
	% CV	7.4	21	19	15	23	23	7.5	
10	mean	17	8.8	2.1	10	10	1.0	2.0	1.8−2.2
	% CV	16	22	21	12	17	21	15	
50	mean	9.6	8.2	1.4	7.5	13	0.7	2.1	1.5−2.8
	% CV	14	34	34	17	39	39	45	

compound 1 (μM)		ABCG2 B to A P_{app}	ABCG2 A to B P_{app}	ABCG2 efflux ratio	MDCK II B to A P_{app}	MDCK II A to B P_{app}	MDCK II efflux ratio	ABCG2 ratio/ MDCK II ratio	95% CI for ABCG2 ratio/MDCK II ratio
0.5	mean	19	6.4	3.2	8.0	14	0.6	5.6	4.7−6.5
	% CV	17	30	28	20	29	23	26	
2	mean	18	5.7	3.4	8.1	13	0.7	5.3	4.5−6.1
	% CV	12	26	29	12	29	25	24	
10	mean	15	7.3	2.3	8.3	14	0.6	3.6	2.8−4.4
	% CV	15	30	37	15	22	26	37	
50	mean	13	12	1.1	9.6	16	0.6	1.7	1.5−2.0
	% CV	19	27	30	18	17	25	22	

triprolidine (μM)		ABCG2 B to A P_{app}	ABCG2 A to B P_{app}	ABCG2 efflux ratio	MDCK II B to A P_{app}	MDCK II A to B P_{app}	MDCK II efflux ratio	ABCG2 ratio/ MDCK II ratio	95% CI for ABCG2 ratio/MDCK II ratio
0.5	mean	15	22	0.7	14	25	0.6	1.2	1.1−1.3
	% CV	11	14	14	13	16	12	11	
2	mean	16	24	0.7	15	25	0.6	1.1	1.0−1.2
	% CV	7.4	10	12	9.2	9.5	10	10	
10	mean	19	28	0.7	19	30	0.6	1.1	1.0−1.1
	% CV	28	23	13	28	22	14	7.8	
50	mean	22	31	0.7	22	32	0.7	1.1	1.0−1.1
	% CV	43	41	16	47	40	16	8.9	

[a] Data are from 9−11 individual studies. Cell passage numbers between 6 and 27 were used since no significant effect of cell passage number was seen for these three compounds.

with a turbo ion-spray electrospray ionization (ESI) interface (PE Sciex, Ontario, Canada) was used during this study. Mobile phase flow was split approximately 1:4 to reduce flow rate to approximately 400 μL/min prior to entering the ESI source. Positive ion electrospray was achieved using a spray voltage of 5000 V, and nebulization/solvent evaporation was aided using a nebulizer gas flow of 12, curtain gas flow of 12, and probe temperature of 400 °C. Negative ion electrospray was achieved used a spray voltage of −4500 V (all other ESI parameters the same). Ring voltage was 22 V. All other mass spectrometer parameters were optimized for each analyte used in this study. Selected reaction monitoring (SRM) scanning was used throughout this study to simultaneously monitor for analyte and internal standard in each sample. Optimal precursor ($[MH]^+$, $[M - H]^-$) to product ion ($[MH - x]^+$, $[M - H - x]^-$) transitions for each analyte were determined using Q3-product ion scanning. Q2 collision induced dissociation (CID) parameters (e.g., offset voltage and gas pressure) were optimized for each analyte.

Data Processing. The following equations were used to determine apparent permeability and efflux ratios:

$$P_{app}(\text{cm/s}) = ((((\text{receiver units} - \text{blank})\cdot\text{receiver cm}^3\cdot\text{dilution factor})\cdot\text{donor cm}^3)/\text{s})/((\text{membrane surface area (cm}^2)\cdot(T = 0 \text{ donor units} - \text{blank})\cdot\text{dilution factor}\cdot\text{donor cm}^3)) \quad (1)$$

$$\text{efflux ratio} = \text{“basolateral to apical” } P_{app}/\text{“apical to basolateral” } P_{app} \quad (2)$$

$$\text{ratio of ratios (R/R)} = (\text{MDCK II/ABCG2 efflux ratio})/(\text{MDCK II efflux ratio}) \quad (3)$$

Results

Selection and Characterization of a Single Cell MDCK II/ABCG2 Clone. The wild-type ABCG2 gene was trans-

fected into MDCK II cells to generate a stable cell line, and BODIPY-prazosin, a fluorescent ABCG2 substrate, was used to select cell clones expressing ABCG2. The transfected cell clones that showed lower fluorescence intensity were selected (Figure 1). The ABCG2/MDCK II cell clones selected from FACS were then expanded, and whole cell lysates prepared from these clones were subjected to immunoblotting for analysis of ABCG2 expression. As seen in Figure 2, transfected MDCK II cell clones exhibited ABCG2 expression, whereas nontransfected MDCK II wild-type cells showed no ABCG2 expression. ABCG2 expression was enhanced in transfected cells by treatment with 2 μM sodium butyrate, after which a single ABCG2/MDCK II cell clone was isolated and characterized for efflux activity using the ABCG2 substrate Lysotracker Green. The single cell clone efficiently effluxed this substrate out of the cells. A 4−5-fold difference was obtained between the ABCG2 cell clone and MDCK II cells (Figure 3).

96-Transwell High Throughput Assay. A high-throughput 96-Transwell assay was developed to identify ABCG2 substrates using the MDCK II/ABCG2 stable cell line without sodium butyrate pretreatment. Initially, prazosin and Pfizer compound 1 were used as positive controls, and triprolidine was used as a negative control. (Compound 1 is a proprietary Pfizer compound that was identified as an ABCG2 substrate by comparison to the efflux ratios obtained with ABCB1-transfected MDCK II cells and ABCC2-transfected MDCK II cells (see Figure 6).) As shown in Table 1, basolateral to apical directed transport (B to A P_{app} values) for 0.5 − 10 μM prazosin or compound 1 with MDCK/ABCG2 cells was higher than apical to basolateral directed transport (A to B P_{app} values), resulting in efflux ratios ranging from 2.1 to 2.8 for prazosin, and 2.3 to 3.4 for compound 1. Corresponding efflux ratios in MDCK II cells ranged from 0.8 to 1.0 and 0.6 to 0.7, respectively. The ABCG2/MDCK II efflux ratio/MDCK II efflux ratio (ratio of ratios, R/R) for all concentrations of prazosin ranged from 2.1 to 3.2, whereas the corresponding ratios for compound 1 showed a range of 1.7−5.6. These results are consistent with these two compounds being substrates for ABCG2. The R/R for the negative control triprolidine ranged from 1.1 to 1.2 at all concentrations tested. With the exception of some parameters for the highest tested concentration of prazosin and triprolidine, the reproducibility of the assay was good as evidenced by the low range of % CV values (7.4−37%) at 0.5, 2, and 10 μM substrate concentrations.

A series of dose response studies were also conducted with the control compounds. For prazosin, the difference between B to A P_{app} values and A to B P_{app} values in ABCG2-transfected cells was larger than that observed in MDCK II cells (Figure 4). ABCG2 B to A P_{app} values were greater than A to B P_{app} values at all concentrations tested. A to B P_{app} values were relatively constant over the concentration range tested, but B to A P_{app} values decreased at concentrations above 5 μM. Efflux ratios of prazosin ranged from ∼2−4 and declined at concentrations above 2 μM (Figure 4). The data suggest that saturation of efflux for prazosin occurs at concentrations greater than 25 μM.

For Pfizer compound 1, ABCG2 B to A P_{app} values were greater than A to B P_{app} values at all concentrations tested (Figure 5). B to A P_{app} values decreased at concentrations greater than 2 μM. ABCG2 A to B P_{app} was relatively constant between 0.1 and 10 μM, but increased at higher concentrations. The ABCG2 efflux ratio ranged from 1.5 to 4.9 over the concentration ranged tested. ABCG2 efflux ratios were high, yet variable, between 0.1 and 2 μM and declined at concentrations above 2 μM, whereas the efflux ratio was relatively constant for MDCK II cells. Despite the variability in ABCG2 efflux data, when each study was considered separately the same relationship between MDCK efflux and ABCG2 efflux was seen (data not shown). The data suggest that saturation of efflux occurs at a concentration between 25 and 50 μM.

The additional ABCG2 substrates mitoxantrone, daunorubicin, topotecan, CI1033, and SN-38 were also tested in the high throughput assay. Most substrates showed good efflux activity in ABCG2 cells, as shown in Table 2. (Doxorubicin was also tested in the assay. However it did not show efflux activity in ABCG2/MDCK II cells (data not shown). This is consistent with reports that doxorubicin is not an efficient substrate of wild-type (R482) ABCG2.[16,17]) Peak ABCG2-mediated efflux appeared at 2 and 10 μM with daunorubicin. For mitoxantrone, significant efflux activity was seen at 10 and 50 μM. CI1033 showed efflux ratios that decreased with dose. CI1033 has been reported to be an ABCG2 inhibitor as well, with a low IC_{50} (3.7 μM).[15] This might explain why efflux activity decreased at high concentrations. SN-38 showed very high efflux ratios with ABCG2 cells at all concentrations. Efflux ratios of daunorubicin in MDCK II cells were relatively high (2.2−5.9), a finding consistent with this compound being an ABCB1 substrate and the fact that MDCK II cells contain endogenous canine Mdr1.

The effect of cell passage on the vectorial transport of these substrates was also evaluated (Table 2). No effect of cell passage number on mitoxantrone or CI1033 transport was observed. There were some effects of cell passage on topotecan and SN-38, with A to B P_{app} values being lower in high passage cells compared to lower passage cells. However, regardless of the passage number, the efflux activity

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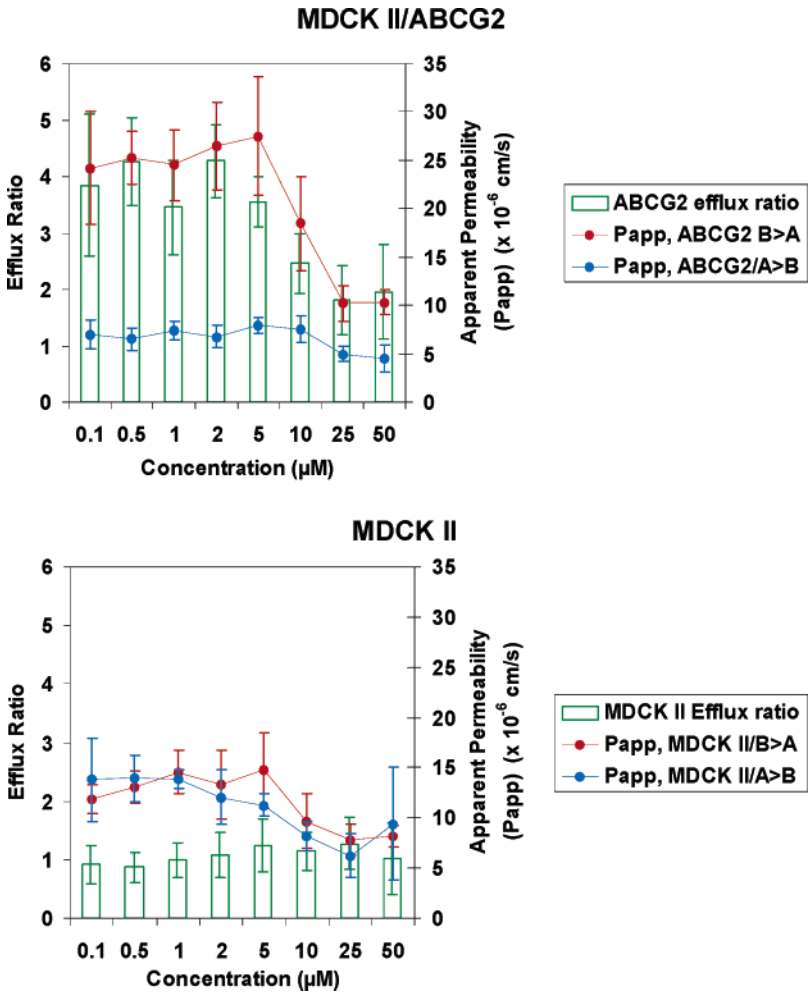

Figure 4. Apparent permeability (P_{app}) and efflux ratios (B to A/A to B) of prazosin from ABCG2 and MDCK II cells. Compounds were tested at concentrations of 0.1, 0.5, 1, 2, 5, 10, 25, and 50 μM. Results are the means; the error bars indicate the standard deviations from at least four experiments.

was consistent with these compounds being substrates for ABCG2. (For example, for topotecan the ABCG2/MDCK II efflux ratio and the R/R value of 4.3 and 5.4, respectively, are similar to those observed by Pavek et al. (7.4 and 3.1, respectively).[18]) Daunorubicin efflux was most affected by cell passage number. A higher cell passage number was associated with a smaller A to B P_{app} value such that high efflux ratios and high R/R values were only associated with high passage cells.

Identification of ABCG2 Substrates Using the 96-Transwell Assay. As an initial assessment to assess the utility of using the ABCG2 HTS to identify substrates, ~150 Pfizer discovery compounds, including some known ABCG2 substrates such as prazosin and topotecan,[8] were tested in the assay and compared to results obtained in analogous 96-Transwell assays for ABCB1 and ABCC2. As shown in Figure 6, several compounds exhibited overlapping substrate specificity with ABCG2, ABCB1, and ABCC2. These results are consistent with previous results that this transporter exhibits substrate overlap with ABCB1 and ABCC2.[8] In addition, generally there was a positive relationship between ABCG2 efflux and ABCB1 efflux for many of the compounds. However, ABCG2 appeared to be the major transporter for three compounds, as indicated in Figure 6. One of these three compounds is Pfizer compound 1, which has an ABCG2 efflux ratio of >10 compared to ABCB1 or ABCC2 ratios of <2.

Discussion

ABCG2 is an important transporter, which can impact the disposition of drugs in humans. This transporter also is expressed in various cancer cells and is a contributor to cancer cell resistance to drug therapy. In order to easily test for ABCG2 substrates, polarized MDCK II cells expressing wild-type ABCG2 were generated and used to develop a novel 96-Transwell HTS assay capable of screening hundreds of compounds each week. Although the generation of MDCK II/ABCG2 cells has recently been reported,[18] this is the first report showing the development a high throughput format and its subsequent utility in identifying ABCG2 substrates in a drug discovery setting. On the basis of results with the negative control compound triprolidine, our data suggest that a compound tested at 2 μM cannot be considered an ABCG2 substrate if the MDCK II/ABCG2 efflux ratio is <0.9. (This

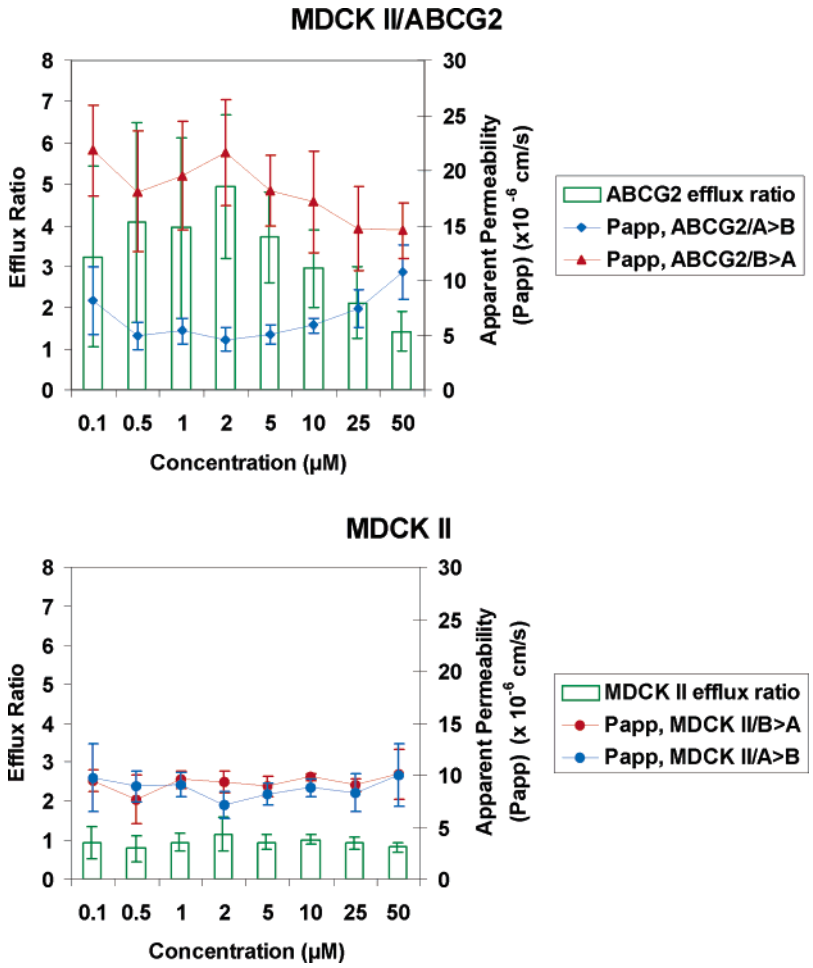

Figure 5. Apparent permeability (P_{app}) and efflux ratios (B to A/A to B) of compound 1 from ABCG2 and MDCK II cells. The concentrations tested were 0.1, 0.5, 1, 2, 5, 10, 25, and 50 μM. Results are the means; the error bars indicate the standard deviations from at least four experiments.

criterion is more strictly defined as being 2σ (mean $\pm$ 2 SD) higher than the mean efflux ratio of triprolidine (using data from Table 1).) Moreover, on the basis of the 95% confidence interval of the R/R values for triprolidine, a compound can be categorized as an ABCG2 substrate if it has an R/R value >1.2 at a test concentration of 2 μM.

MDCK II/ABCG2 cell passage number did not affect the vectorial transport of the control compounds prazosin, compound 1, and triprolidine in 9−11 separate studies (data not shown). However, we did observe that for some substrates cell passage did affect transport, yet the reason for this is unclear. For the substrates daunorubicin, topotecan, and SN-38, higher cell passage was always associated with a larger ABCG2 efflux ratio and a larger R/R value. In all instances, this higher efflux ratio was due to a lower MDCK II/ABCG2 A to B P_{app} value, while the B to A ratio was unaffected. This would indicate that ABCG2 expression is not affected by cell passage, although we have not yet measured ABCG2 protein levels. (In contrast, the expression of ABCG2 in Caco-2 cells has been shown to be dependent on cell passage number.[19]) The decrease of A to B P_{app} in our system suggests that an uptake transporter for the affected compounds may be downregulated with increasing cell passage. If this were true, one would expect to see some attenuation of A to B P_{app} (due to saturation) at high substrate concentrations. However, such attenuation is not seen (Table 2). Further studies are necessary to understand the effect of cell passage on A to B P_{app} for these substrates; however, in order to best identify ABCG2 substrates we recommend conducting this assay with high passage cells.

Although our assay is very useful in identifying substrates of ABCG2, caution is needed when testing compounds that

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Table 2. Summary of Mean P_{app} Values ($\times 10^{-6}$ cm/s) and Efflux Ratios for Various Substrates Tested in the MDCK II/ABCG2 96-Transwell Assay[a]

Daunorubicin ($N = 4$)[b]

concn (μM)	mean MDCK P_{app} A>B	B>A	mean MDCK ratio	mean ABCG2 P_{app} low A>B	low B>A	high A>B	high B>A	mean ABCG2 ratio low	high	mean ABCG2 ratio/MDCK ratio low	high
0.5	1.1	5.9	5.4	1.4	7.6	0.8	8.4	5.4	11	1.0	2.0
2	1.2	7.1	5.9	1.3	9.3	0.6	9.9	7.2	17	1.2	2.9
10	1.5	7.2	4.8	1.7	10.4	0.7	11	6.1	16	1.3	3.3
50	1.9	4.1	2.2	1.9	6.9	0.9	9.2	3.6	10	1.6	4.5

Mitoxantrone ($N = 3$)[c]

concn (μM)	mean MDCK P_{app} A>B	B>A	mean MDCK ratio	mean ABCG2 P_{app} low A>B	low B>A	high A>B	high B>A	mean ABCG2 ratio low	high	mean ABCG2 ratio/MDCK ratio low	high
0.5	nd[d]		nd	nd		nd		nd	nd	nd	nd
2	nd		nd	nd		nd		nd	nd	nd	nd
10	1.1	1.2	1.1	0.3	2.0	0.4	2.0	6.7	5.0	6.1	4.5
50	1.0	2.3	2.3	0.8	3.0	0.6	3.4	3.8	5.7	2.0	2.5

CI-1033 ($N = 3$)[e]

concn (μM)	mean MDCK P_{app} A>B	B>A	mean MDCK ratio	mean ABCG2 P_{app} low A>B	low B>A	high A>B	high B>A	mean ABCG2 ratio low	high	mean ABCG2 ratio/MDCK ratio low	high
0.5	0.8	1.1	1.1	0.6	2.2	0.7	2.2	3.7	3.1	2.6	2.2
2	1.2	1.3	1.1	0.7	2.2	1.0	2.4	3.1	2.4	2.8	2.2
10	2.0	1.5	0.80	1.0	1.6	1.8	1.8	1.6	1.0	2.0	1.3
50	2.7	1.3	0.50	2.7	1.3	2.4	1.4	0.50	0.60	1.0	1.2

Topotecan ($N = 2$)[f]

concn (μM)	mean MDCK P_{app} A>B	B>A	mean MDCK ratio	mean ABCG2 P_{app} low A>B	low B>A	high A>B	high B>A	mean ABCG2 ratio low	high	mean ABCG2 ratio/MDCK ratio low	high
0.5	5.7	3.9	0.70	2.6	5.2	1.4	5.2	2.0	3.7	2.9	5.3
2	4.7	3.7	0.80	2.5	5.2	1.3	5.5	2.1	4.3	2.6	5.4
10	4.8	3.6	0.80	2.2	5.1	1.3	5.3	2.3	4.1	2.9	5.1
50	5.3	3.5	0.70	2.4	4.7	1.2	5.0	2.0	4.2	2.9	6.0

SN-38 ($N = 2$)[g]

concn (μM)	mean MDCK P_{app} A>B	B>A	mean MDCK ratio	mean ABCG2 P_{app} low A>B	low B>A	high A>B	high B>A	mean ABCG2 ratio low	high	mean ABCG2 ratio/MDCK ratio low	high
0.5	6.6	5.3	0.80	2.2	9.3	1.1	8.8	4.3	8.3	5.4	10
2	6.2	5.7	0.90	1.4	8.6	1.3	9.5	6.8	7.6	7.6	8.4
10	5.0	3.7	0.70	2.1	5.8	1.2	6.1	2.7	5.1	3.9	7.3
50	1.1	1.1	1.1	0.4	2.1	0.3	2.1	5.1	6.8	4.6	6.2

[a] The number of studies is indicated next to each substrate name. Data is shown for both low and high passage cells. [b] Low = 6, 8, 10, and 11. High = 21, 22, 25, and 27. [c] Low = 6, 8, and 11. High = 22, 25, and 27. [d] Not determined due to poor detection sensitivity. [e] Low = 9, 10, and 11. High = 19, 21, and 22. [f] Low = 10 and 11. High = 21 and 22. [g] Low = 10 and 11. High = 21 and 22.

may have low permeability. As with all cell-based Transwell assays (such as those using MDCK II, Caco-2 or LLC-PK1), the compound being tested must be able to readily enter the cell in order to generate meaningful data to assess vectorial transport. Therefore it is very important to always run the nontransfected MDCK II cells along with the transfected-MDCK II cells in a Transwell assay. A very low A to B P_{app} value in MDCK II cells may indicate low permeability; consequently a very high B to A/A to B ratio in transfected cells should be interpreted with caution since this could merely indicate a very low A to B P_{app} value due to poor permeability. This same caution needs to be applied when testing potential inhibitors of ABCG2 in our model as well, since poor permeability of a potential inhibitor could lead to a false negative effect. Supplementing ABCG2 Transwell data with chemical/physical properties and perme-

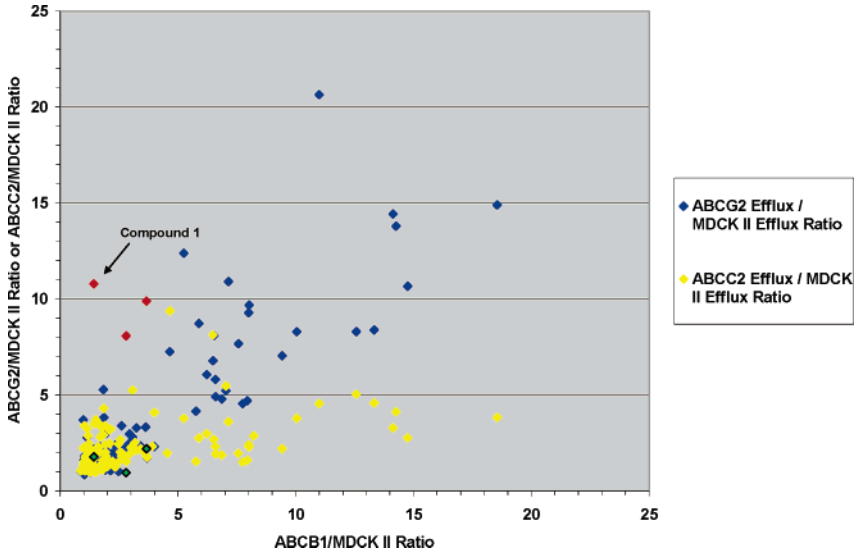

Figure 6. Comparison of ABCG2 efflux ratios to ABCB1 and ABCC2 efflux ratios for 151 compounds. A large set of proprietary discovery compounds from various therapeutic areas was tested in Transwell assays containing either MDCK II/ABCG2, MDCK II/ABCB1 or MDCK II/ABCC2 cells. Assay conditions for each cell line were as described for MDCK II/ABCG2 in Materials and Methods, with the exception that the mobile phase system consisted of an aqueous solvent system A, 98% (v/v) 2 mM ammonium acetate/2% (v/v) methanol, and an organic solvent system B, 10% (v/v) 2 mM ammonium acetate/90% (v/v) 50/50 (acetonitrile/methanol). All compounds were tested at a concentration of 2 μM. Three putative ABCG2 specific substrates are indicated with red symbols, and the corresponding ABCC2 efflux ratio values for these three compounds are indicated as green symbols. The putative ABCG2 specific substrate Pfizer compound 1 is indicated with an arrow.

ability data (from PAMPA studies) for the test compounds of interest will also help identify poorly permeable compounds.

In summary, we have successfully transfected the human breast cancer resistant protein ABCG2 into MDCK II cells and used this stable cell line to develop a novel 96-Transwell HTS assay format for easily identifying substrates of this important human transporter. The assay has the capacity to test hundreds of compounds per week due to the incorporation of a high throughput method for quantification by mass spectrometry. Importantly, in addition to testing for ABCG2 substrates, this assay can be used in concert with other MDCK II transfected cell lines, such as MDCK II/ABCB1 and MDCK II/ABCC2 to allow one to identify putative selective substrates (or inhibitors) for ABCG2. Due to increased evidence supporting the important role of ABCG2 in drug disposition in humans, the availability of a high throughput assay to identify ABCG2 substrates could have a significant impact on drug discovery in the pharmaceutical industry.

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