**High-Activity P-Glycoprotein, Multidrug Resistance Protein 2, and Breast Cancer Resistance Protein Membrane Vesicles Prepared from Transiently Transfected Human Embryonic Kidney 293-Epstein-Barr Virus Nuclear Antigen Cells**

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**ABSTRACT:** Membrane-bound transporter proteins play an important role in the efflux of drugs from cells and can significantly influence the pharmacokinetics of drug molecules. This study describes the production of large amounts of high-activity transporter membrane vesicles from human embryonic kidney 293-Epstein-Barr virus antigen cells transiently transfected using a Gateway-adapted pCEP4 plasmid. Transfections were scaled up to 10-liter cell cultures, and vesicle preparations were optimized using ultracentrifugation with a sucrose cushion, which enabled us to produce hundreds of milligrams of membrane vesicles expressing human efflux transporter proteins P-glycoprotein (P-gp)/multidrug resistance 1 (ABCB1), multidrug resistance protein 2 (MRP2) (ABCC2), and breast cancer resistance protein (BCRP) (ABCG2). Assays were developed and optimized for analyzing the ATP-dependent functionality of the transporters using probe substrates and specific inhibitors. Excellent signal/noise ratios of ATP-stimulated uptake for P-gp, MRP2, and BCRP vesicles were obtained, indicating high expression of functioning transporters. The uptake kinetics of the transporters was determined by determining $K_m$ and $V_{max}$ using the model substrates N-methylquinidine (P-gp), estradiol-17β-glucuronide (MRP2), and estrone-3-sulfate (BCRP). The ATP-dependent transport was inhibited by the model inhibitors verapamil (P-gp), benzbromarone (MRP2), and sulfasalazine (BCRP). The vesicles are thus well suited to screen for possible substrates and inhibitors in high throughput systems or are used for detailed mechanistic investigations of transporter kinetics of specific substances.

**Abbreviations:** ABC, ATP-binding cassette; MDR1, multidrug resistance 1; P-gp (ABCB1, MDR1), permeability glycoprotein; MRP2 (ABCC2), multidrug resistance protein 2; BCRP (ABCG2), breast cancer resistance protein; S9, Spodoptera frugiperda ovarian; HEK, human embryonic kidney; EBNA, Epstein Barr virus nuclear antigen-1; PEI, polyethylenimine; AMP-PNP, adenosine 5'-[(β, γ-imido)triphosphate; $E_17\beta$G, estradiol-17β-glucuronide; $E_2S$, estrone-3-sulfate; $[^3H]E_17\beta$G, [14,15,19,20-3H]estradiol-17βG; $[^3H]E_2S$, [6,7-3H(N)] estrone sulfate ammonium salt; NMQ, N-methylquinidine; DHI, Iscove’s modified Dulbecco’s medium; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction.
proteins. The most commonly used cell line for expressing transporter protein and preparing vesicles is insect Sf9 cells (Glivanas et al., 2008). However, the membrane composition in the insect Sf9 cell plasma membrane is different from that for mammalian plasma membranes and has been shown to significantly affect transporter functionality (Pil et al., 2007). In addition, Sf9 insect cell membranes express endogenous efflux transporters, which could confound the results.

The purpose of the present study was to develop a system to produce large quantities of high-activity efflux transporters in mammalian membrane vesicles. Human embryonic kidney 293 (HEK293) cells constitutively expressing the Epstein-Barr virus nuclear antigen (HEK293-EBNA) were used to overexpress the human efflux transporters P-gp, MRP2, and BCRP. The HEK293-EBNA cell line was chosen because it exhibits a low background of both uptake and efflux transporters (Ahlin et al., 2008, 2009) and has been used widely for expression of various recombinant proteins (Wurm, 2004). Moreover, this cell line is amenable to large-scale polyethyleneimine (PEI)-mediated transient transfection for large-scale expression of recombinant protein (Tuveson et al., 2008). By using this method, high-activity membrane vesicles containing P-gp, MRP2, or BCRP were produced in large quantities from transiently transfected HEK293-EBNA cells.

Materials and Methods

Materials. ATP disodium salt, adenosine 5'-triphosphate tetra- lithium salt hydrate (AMP-PNP), benzamorone, estradiol-17β-glucuronide (E217G), estrone-3-sulfate (E3S), dimethyl sulfoxide, glutathione, potassium chloride, magnesium chloride, MOPS, sucrose, sulfosalazine, Tris base, and verapamil HCl were purchased from Sigma-Aldrich (Stockholm, Sweden); sodium chloride was from Merck Chemicals Ltd. (Beeston, UK); and [14,15,19,20,21H]E217G (1H[E217G]), [6,7-3H(H)]estrone sulfate ammonium salt (1H[ES]), and Optiphase HiSafe 2 were from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]-methylquinidine (1H[MQ]) was obtained from RC Tritec Ltd. (Teufen, Switzerland), and unlabeled N-methylquinidine was prepared by the Department of Medicinal Chemistry, AstraZeneca R&D Mölndal (Mölnadal, Sweden).

Expression of BCRP and MRP2 from Adherent Stable Cell Lines. The pT-REx-BCRP vector for cell transfection was generated by recombination of the entry vector from in-house collection and the pT-REx-DEST30 Gateway vector (Invitrogen, Stockholm, Sweden). HEK293 cells (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were grown in DHI medium (custom version made by SAFCE Biosciences, Andover, UK) supplemented with 2 mM glutamine, 10% fetal bovine serum, 500 μg/ml G418 (all from Invitrogen), and 1.8 mM CaCl2. The Madin-Darby canine kidney II (MDCKII) cell line (Beeston, UK) and has been shown to significantly affect transporter functionality (Pa´l et al., 2008). By using this method, high-activity membrane vesicles containing P-gp, MRP2, or BCRP were produced in large quantities from transiently transfected HEK293-EBNA cells.

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**Expression of MRP2, P-gp, and BCRP from Transient Suspension Cell Cultures.** Plasmid construction and preparation. cDNA for MRP2 was obtained from OriGene Technologies (Rockville, MD). The BCRP Entry vector, P-gp cDNA, and the pCEP4GW-EGFP plasmid, used in control transfections, were obtained from in-house collection of vector constructs. Gateway-adapted pCEP4 (pCEP4GW) and pEAK10 (pEAK10GW) destination vectors were obtained from an in-house vector collection (Davies et al., 2005). MRP2 and P-gp were amplified, and att sites were added by PCR (Table 1). att-adapted

**TABLE 1**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers and PCR conditions for amplification and adaption of MRP2 and P-gp</th>
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<tr>
<td>MRP2</td>
<td><strong>Forward:</strong> 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCCACCATG-3’ <strong>Reverse:</strong> 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTGGCGCTTTGTTCCAGCCTGGAC-3’</td>
<td>95°C for 2 min (94°C for 30 s, 55°C for 30 s, 68°C for 5 min)</td>
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<tr>
<td>P-gp</td>
<td><strong>Forward:</strong> 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCCACCATG-3’ <strong>Reverse:</strong> 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTGGCGCTTTGTTCCAGCCTGGAC-3’</td>
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PCR products were purified by electrophoresis on a 1% agarose gel (Bio-Rad Laboratories, Sundbyberg, Sweden), and products of the correct size were excised and extracted using a StrataPrep gel extraction kit (Stratagene, La Jolla, CA). *att*-adapted MR2, BCRP, and P-gp PCR products were subcloned into the pCEP4GW and pEAK10GW destination vectors using Gateway technology (Invitrogen) (Fig. 1). For construct verification Entry vectors containing the *att*-adapted MR2 and P-gp genes were sequenced using gene-specific primers and the CEQ DTCS Quick Start Kit (Beckman Coulter, Bromma, Sweden). Sequencing was performed using a CEQ8000 Genetic Analysis System (Beckman Coulter). DNA sequence analysis and alignments were performed using Lasergene SeqMan II software (version 5.07; DNASTAR, Madison, WI). In silico Gateway cloning and construction of restriction maps were performed using Vector NTI Advance 9 (Invitrogen).

Chemically competent *Escherichia coli* XL1 blue strain organisms (recA1 endA1 gyrA96 thi-1 hsdRI7 supE44 relA1 lacI [F’proAB lacZAM15 Tn10 (TetR)’]; Stratagene) were transformed with plasmids (Novagen’s TB009 user protocol; http://www.emdbiosciences.com). Pure cultures were generated, and glycerol stocks were maintained at −80°C. Production scale cultures were performed in batch mode in BIOSTAT C vessels (5-liter working volume) (Sartorius BBI, Melsungen, Germany). Fermenter cultures were run at 37°C with dissolved oxygen concentrations no less than 30%. Culture medium was TB (24 g/l Trypton, 12 g/l yeast extract, 17 mM KH$_2$PO$_4$, and 72 mM K$_2$HPO$_4$), and pH was monitored but not controlled. Cultures were harvested in stationary phase, determined by constant OD$_{600}$ or declining respiration rate. Plasmid purification was performed using the NucleoBond AX 10000 kit (Macherey-Nagel, Düren, Germany). The manufacturer’s instructions were modified as follows to accommodate the low plasmid content in the cell pellet: a larger amount of cell pellet (30 g) was used for plasmid preparation with the total volume of lysate being 1 liter. Centrifugation (8000g for 10 min) was used before filtration for separation of flocs, and subsequent steps were carried out as described in the manual (Macherey-Nagel).

**Suspension growth adaptation and cell growth.** The adherent cell line HEK293-EBNA (Invitrogen) stably expressing the Epstein-Barr virus nuclear antigen-1 gene was adapted to suspension growth (Davies et al., 2005) in DHI medium supplemented with 4 mM glutamine, 2% (v/v) ultra-low IgG fetal bovine serum, 250 μg/ml G418 and 0.1% (w/v) Pluronic F68 (Sigma-Aldrich) for a maximum of 20 passages. For expansion of the seeding culture the cells were grown in plastic shake flasks at 37°C in 5% CO$_2$ atmosphere at 115 rpm in an orbital shaking incubator (Infors AG, Bottmingen, Switzerland). The cells were routinely passaged when they reached a density of 2 × 10$^6$ cells/ml. Cell density and viability were measured using a Cedex automatic cell counter (Innovatis AG, Bielefeld, Germany).

**Screening of expression conditions.** Screening for optimal expression conditions was carried out using 125-ml shake flasks (Corning B.V. Life Sciences) with 40 ml of medium as described previously (Davies et al., 2005). Conditions investigated were vector type (pCEP4GW or pEAK10GW), concentration of DNA-PEI complex (Table 2), and time of harvest (2, 3, or 4 days after transfection).

Expression was evaluated by Western blotting of the whole-cell lysates. Cell pellets from 2 ml of culture broth was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA) with 5 μl of sample per well and run at 150 V for 80 min. Western transfer was performed with a NC membrane kit and iBlot equipment (both from Invitrogen). Blocking was performed by incubation for 1 to 2 h with 5% (w/v) nonfat milk. Primary antibodies, mouse anti-MRP2 (Sigma-Aldrich), mouse anti-P-gp (Sigma-Aldrich), and mouse anti-BCRP (Sigma-Aldrich) were diluted 1:1000 in 5% (w/v) nonfat milk, and blots were incubated for at least 2 h. Secondary antibody anti-mouse horseradish peroxidase conjugate (Cayman Chemical Company, Ann Arbor, MI) was diluted 1:4000 in 5% (w/v) nonfat milk, and blots were incubated for at least 2 h. Chemiluminescence was induced by the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Chemical, Rockford, IL) and detected using VersaDoc (Bio-Rad Laboratories).

**Transfection procedure and production in Wave bioreactors.** Large-scale cultures were carried out in 20L Wave bioreactors (Wave Biotech AG, Tagelswangen, Switzerland) with 10 liters of culture volume. Cells were split to a density of 1 × 10$^6$ cells/ml 1 day before transfection.

Cell culture for the production of MR2 and BCRP was performed as follows. Cells for inoculum (4.5 × 10$^7$ cells) were centrifuged (300g, 15 min), resuspended in 0.5 liter of fresh DHI medium and transferred to the Wave bioreactor with 4 liters of DHI medium, resulting in a cell density of 1 × 10$^6$ cells/ml. Cells were allowed to adapt for 2 h before transfection. Transfection mixture (0.5 liter total) was prepared with 2 mg of plasmid DNA and 5 mg of PEI (Tuvesson et al., 2008) and transferred to the culture. Four hours after transfection 5 liters of supplemented DHI medium was added to the culture. Cells were harvested by centrifugation (1500g for 15 min) after 96 h (MRP2) or 72 h (BCRP).

Cell culture for production of P-gp was performed using a simplified protocol. Inoculum was prepared as described above, but the initial volume in the Wave bioreactor was 9 liters with an initial cell density of 0.5 × 10$^6$ cells/ml. After incubation for 2 h, the culture was transsected by addition of 1 liter of transfection cocktail containing 8 mg of DNA and 20 mg of PEI. Cell pellets were harvested by centrifugation after 72 h and were stored at −80°C until used in the vesicle preparation.

**Immunofluorescence microscopy.** For immunostaining of HEK293-EBNA cells transiently transfected with transporter and mock HEK293-EBNA cells transfected with vector alone, cells were attached to Adcell 12-well slides (Thermo Fisher Scientific, Histolab, Sweden). Cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature, washed twice with ice-cold PBS, permeabilized with 0.5% saponin in PBS, washed with PBS (three times) for 5 min, and then blocked (30 min at room temperature) with PBS containing 5% fetal calf serum. Cells were incubated with primary

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| **Concentrations of DNA and PEI for small-scale expression studies**
| (40-ml cultures) |
| Concentration of DNA-PEI Complex | pCEP4GW | pEAK10GW |
| DNA | PEI  | µg/ml |
| **“Low”** | 0.4 | 0.5 | 1 |
| **“High”** | 0.8 | 1.0 | 2 |
antibody for P-gp (mouse anti-human IgG1, clone F4; Sigma-Aldrich), MRP2 (mouse anti-human IgG2a, M2 III-6; Abcam Inc., Cambridge, MA), or BCRP (mouse anti-human IgG2a, BXP-21; Abcam Inc.) for 60 min at room temperature (all three antibodies were diluted 1:20 with PBS). Cells were washed (three times) for 5 min at room temperature and then blocked (30 min at room temperature) with PBS containing 10% goat serum. Secondary antibodies (diluted 1:1000 in PBS containing 1% goat serum) were added to the cells and incubated for 60 min at room temperature [for P-gp, goat anti-mouse IgG2a (γ2a), Alexa 488-conjugated (Invitrogen); for MRP2 and BCRP, goat anti-mouse IgG1 (γ1), Alexa 568-conjugated (Invitrogen)]. After incubation, cells were washed three times with PBS for 5 min at room temperature and then once with distilled water. ProLong Gold antifade reagent with 4.6-diamidino-2-phenylindole was added for nuclei staining (Invitrogen). As negative controls for the specific reactivity of the antibodies slides were prepared with secondary antibody added in the absence of primary antibody. Cells were imaged using an Olympus AX70 fluorescence microscope with a 40× or 63× water objective lens. Photographs were taken using a Sony 3CCD video camera.

Membrane Vesicle Preparation. Vesicle preparation was performed based on the method described by Keppler et al. (1998), modified for larger-scale production as described below. Harvested cells were resuspended in lysis buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.0, and Complete Protease Inhibitor). Lysis was performed using an Ultra-Turrax T25 homogenizer (IKA Works, Wilmington, NC). The resulting lysate was centrifuged for 10 min at 12,000g. The pellet was resuspended in lysis buffer and homogenized again. Postnuclear supernatant was pooled and centrifuged at 83,000g for 90 min or for 38,000g for 3 h (depending on volume). Pellets were resuspended in incubation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and EDTA-free Complete Protease Inhibitor), resulting in a crude membrane preparation. This solution was layered on top of a sucrose cushion in a 1:1.8 ratio [38% (w/v) sucrose, and 5 mM HEPES-KOH] and centrifuged for 17 h at 28,000 g. The pellet was resuspended in incubation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and EDTA-free Complete Protease Inhibitor), resulting in a crude membrane preparation. This solution was layered on top of a sucrose cushion in a 1:1.8 ratio [38% (w/v) sucrose, and 5 mM HEPES-KOH] and centrifuged for 17 h at 28,000 rpm (SW28 rotor, Optima L-100K centrifuge; Beckman Coulter). The turbid interface layer was collected, diluted to 180 ml, and centrifuged for 2.5 h at 82,000g. Pellets were resuspended in incubation buffer and passed through a 27-gauge needle 10 times. The protein concentration was determined using the BCA Protein Assay Kit (Pierce Chemical). All work was performed on ice or at 4°C whenever possible. Cell quantities and solution volumes are given in Table 3. During the course of preparation of this article the large-scale production method for the vesicles has been repeated with good reproducibility.

Transport Studies with Membrane Vesicles. The vesicular transport activity was measured using a rapid filtration technique with a 12-vial system (Millipore rapid filtration apparatus and 1225 sampling manifold) or on 96-well plates (MultiScreen HTS FB plate; Millipore Corporation, Billerica, MA). After a preincubation period of 5 to 10 min, membrane vesicles (50 μg of protein/75-μl final reaction volume) were incubated at 37°C (for MRP2 and P-gp) or 32°C (for BCRP) in the presence or absence of 4 mM ATP in assay buffer containing radiolabeled probe substrate (3 μCi/ml) for the indicated times. The uptake reaction was stopped by adding 1 ml of ice-cold washing buffer to the membrane suspension and was then rapidly filtered through a GF/F glass fiber filter (diameter 25 mm, pore size 0.7 μm; Whatman, Clifton, NJ) or filter plate. Filters were washed with 2 × 5 ml of ice-cold washing buffer. Filters were transferred to large glass scintillation vials (20 ml) and left to dry at room temperature for at least 2 h before scintillation liquid (10 ml) was added, and the radioactivity retained on the filter was measured using a WinSpectral 1414 liquid scintillation counter (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland). For experiments using a 96-well system, the uptake reaction was stopped by adding cold washing buffer, immediately transferring the vesicles to the filter plate, and washing the filter with washing buffer. The filter plate was dried, and radioactivity was measured with a scintillation counter (TopCount NXT; PerkinElmer Life and Analytical Sciences). ATP-dependent transport (picomoles of minute per milligram of protein) was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP. Assays were performed, at minimum, in triplicate. In control experiments, ATP was replaced by an equal concentration of the nonhydrolyzable ATP analog AMP-PNP.

For vesicular transport experiments with MRP2-expressing vesicles, the assays were performed in assay buffer consisting of 47 mM MOPS-Tris, 65 mM KCl, and 7.0 mM MgCl2 (pH 7.0), which was supplemented with 2 mM glutathione. The washing buffer consisted of 40 mM MOPS-Tris and 70 mM KCl (pH 7.0). Uptake in MRP2 vesicles of [3H]E217β (50 μM) was measured at 37°C for 8 min. Benzylxanthone was used as the positive control inhibitor of MRP2-mediated transport in the membrane vesicles.

For vesicular transport experiments with BCRP- and P-gp-expressing vesicles, the assays were performed in assay buffer consisting of 10 mM Tris-HCl, 250 mM sucrose, and 10 mM MgCl2 (pH 7.0), respectively. The washing buffer consisted of 10 mM Tris-HCl, 250 mM sucrose, and 100 mM NaCl (pH 7.0). Uptake in P-gp vesicles of [3H]NMQ (1 μM) was measured at 37°C for 2 min. Uptake in BCRP vesicles of [3H]E3S (1 μM) was measured at 32°C for 1 min. Verapamil and sulfasalazine were used as positive control inhibitors of the transport in the P-gp and BCRP membrane vesicles, respectively. The effect of medium osmolality was investigated by measuring the ATP-dependent uptake in the presence of increasing sucrose concentrations (increasing medium osmolality).

Data Analysis. Curve-fitting was performed using XLfit 4.2.2 software. $K_{m}$ and $V_{max}$ values from vesicle transport experiment were calculated using the Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot S}{K_m + S}$$

where $V$ is the uptake velocity (picomoles of substrate per minute per milligram of protein), $V_{max}$ is the maximum uptake velocity, $S$ is the substrate concentration (micromolar), and $K_m$ is the Michaelis-Menten constant. The transport of estradiol-17β-glucuronide in MRP2 vesicles did not follow simple Michaelis-Menten kinetics and in this case the Hill equation for sigmoidal transport kinetics was used:

$$V = \frac{V_{max} \cdot S^n}{K_{m,s} + S^n}$$

where $K_{m,s}$ is the half-maximal rate and $n$ is the Hill slope factor indicating the degree of cooperativity and suggesting the presence of more than one binding site.

$IC_{50}$ values for different transporter control inhibitors were calculated using the following equation:

$$V = \frac{100}{1 + \left(\frac{x}{IC_{50}}\right)^n}$$

This equation fits inhibition data to a two-parameter equation, where the lower data limit is 0 and the upper limit is 100. $IC_{50}$ is the concentration of the inhibitor leading to half-maximal inhibition, $x$ is the inhibitor concentration, and $n$ is the slope factor.

Results

**att** Adaption of Transporters and Entry Vector Sequence Analysis. An adapted two-step PCR process (Kagawa et al., 2004) was required for the att adaption of the MRP2 and P-gp. The att-adapted constructs were sequenced and found to be in agreement with published amino acid sequences (NM_004827, NM_000927, and...
NM_000392 for BCRP, P-gp, and MRP2, respectively) with the exception of MRP2 for which the amino acid change V417I was observed. This is a conservative amino acid change and is not expected to have any effect on the transporter activity.

Expression Tests in Small-Scale Suspension Cultures. The two expression vectors (pCEP4GW and pEAK10GW), the DNA/PEI concentration, and the optimal time of harvest were initially tested in small-scale suspension cultures. Evaluation by Western blot showed that for all transporters pCEP4GW-based constructs gave higher expression levels compared with pEAK10GW-based constructs. Expression in pCEP4GW cultures resulted in a high level of expression after 48 h and remained constant until 96 h after transfection, thus providing a wide window for harvest. A representative result for BCRP expression is shown in Fig. 2.

Cell growth and viability were negatively affected by transfection in all cultures. Whereas untransfected controls exhibited 99% viability, the cells in transfected cultures declined to approximately 50% viability in the worst case (Fig. 3). To discriminate between the effect of recombinant protein expression and PEI alone we included a control with PEI only (2 μg/ml). The result shows that toxicity of PEI was a major reason for slower cell growth and decline in viability (Fig. 3). Recombinant protein expression accounted for a smaller additional effect. Western blots revealed that higher DNA/PEI levels improved P-gp expression, but led to decreased levels of MRP2 and BCRP expression.

The conditions selected for large-scale cultures were pCEP4GW-based constructs: lower concentrations of DNA (0.4 μg/ml) and PEI (1 μg/ml) for BCRP and MRP2, but higher concentrations of DNA (0.8 μg/ml) and PEI (2 μg/ml) for P-gp. Because the expression levels did not significantly change between 48 and 96 h, the harvest could be performed between these times.

Expression of P-gp, MRP2, and BCRP in transiently transfected HEK293-EBNA cells was further analyzed by immunofluorescence microscopy. Transfected HEK293-EBNA cells immunostained specifically for P-gp (clone F4), MRP2 (M2 III-6), or BCRP (BXP-21) showed intense fluorescence signal, which appeared to be predominantly localized to the plasma membrane (Fig. 4). This observation was further supported using confocal laser scanning microscopy (data not shown). Under the same conditions, the endogenous expression of P-gp, MRP2, or BCRP were not detectable in the mock HEK293-EBNA cells transfected with the vector alone (Fig. 4).

BCRP and MRP2 were expressed in adherent stable cell lines using 10-layer cell stacks. The expression levels in these cells were compared with the best conditions for transiently transfected cells. Western blotting revealed a higher expression of both MRP2 and BCRP in transiently transfected cells compared with stable transfections (data not shown). The higher expression levels in transiently transfected cells and the potential for scale-up of suspension cell cultures encouraged us to continue with the transient transfection system.

Large-Scale Transient Transfection Suspension Cultures. The cultures with MRP2 and BCRP were performed according to the large-scale protocol published earlier (Tuveson et al., 2008). The transfection was carried out at a cell density of 1.0 × 10^6 cells/ml in half of the final volume for several hours, after which the remaining medium was added. The rationale behind this protocol is that transfection at a higher cell density is more efficient and at the same time reduces the required plasmid quantity by half, which is important for large-scale cultures. Although this method has achieved satisfactory results in the past we discovered that expression levels of MRP2 and BCRP in large-scale cultures were lower compared with small-scale expression tests (Fig. 5). This result showed that cell density at transfection is a very important parameter, and the subsequent cultures with P-gp were scaled-up, strictly following the best conditions achieved in a small scale, i.e., at a cell density of 0.5 × 10^6 cells/ml. As a result, the expression level of P-gp in the large scale exceeded that of small-scale cultures (Fig. 5).

Vesicle Preparation. Expressed transporters localized in the plasma membrane were recovered using established cell fractionation techniques (Huber et al., 2003). The postnuclear supernatant, which was obtained by homogenization and short centrifugation to remove intact cells and nuclei, contained membranes and other organelles. Membrane-bound transporter proteins were recovered by high-speed centrifugation as a crude preparation. Ultracentrifugation on a sucrose cushion was used to fractionate preparations to enrich lower-density lipid-rich fractions, including endoplasmic reticulum membranes. The interface fraction on top of the 38% (w/v) sucrose cushion was added to the final buffer. The recovery of transporter protein in the preparation method was evaluated by Western blotting of the fractions and showed essentially the same result for all three transporters. Densitometric analysis of Western blots showed that the recovery of the transporter proteins in the final product ranged from 48 to 70% of the amount detected in the whole-cell lysate (data not shown).

Functional Transporter Activity. The vesicles prepared from HEK293-EBNA cells transiently transfected with P-gp, MRP2, or BCRP showed a prominent ATP-dependent substrate uptake, indicating high functional transporter activity (Fig. 6). In contrast, no significant ATP-dependent uptake of the probe substrates was observed in vesicles from mock-transfected cells (Fig. 6). In a series of control experiments, uptake in the transporter vesicles and mock vesicles was measured when ATP was replaced by an equal concentration of ATP.
AMP-PNP, a nonhydrolyzable analog of ATP (Fig. 7). For both transporter vesicles and mock vesicles, AMP-PNP did not stimulate uptake of the probe substrates, and there was no difference in uptake rate values in the presence of AMP-PNP compared with uptake rates obtained in the absence of ATP (Fig. 7). Thus, rates of ATP-dependent uptake of the probe substrates in the P-gp, MRP2, and BCRP vesicles, calculated by subtracting values in the absence of ATP or presence of AMP-PNP as control from those in the presence of ATP, were equal. These results indicated that the measured ATP-dependent transport in the vesicles was an active transporter-mediated process requiring hydrolysis of ATP and rule out binding of substrates to membrane and transporters themselves in the presence of ATP. To confirm that the observed ATP-dependent uptake of probe substrates reflects transport into a vesicular space, the effect of extravesicular medium osmolarity on the uptake was investigated (Fig. 8). The ATP-dependent transport of probe substrates in P-gp, MRP2, or BCRP vesicles prepared from stably transfected MDCKII and HEK293 cells was found to be 50- and 6-fold lower, respectively, than that obtained in the transiently transfected HEK293-EBNA cells. Control mock-transfected cells (right panels) were not significantly stained under the same conditions. The magnification is the same for all panels.

All activity measurements were optimized to obtain linear conditions for time and amount of vesicles in the incubations. With use of the optimized experimental conditions, excellent signal/background ratios (ratio of transport + ATP/−ATP) and level of ATP-dependent uptake were measured for the probe substrates (Table 4). The ATP-dependent uptake of probe substrates in MRP2- and BCRP-expressing membrane vesicles prepared from stably transfected MDCKII and HEK293 cells was found to be 50- and 6-fold lower, respectively, than that observed in the transiently transfected HEK293-EBNA cells (Fig. 9).

The concentration dependence of the ATP-dependent transport of model substrates in the P-gp, MRP2, and BCRP transporter vesicles...
was characterized (Fig. 10). NMQ was transported in the P-gp vesicles with a $K_m$ value of 2.9 $\mu$M and a $V_{max}$ value of 1449 pmol/mg/min. The transport kinetics for E3S in the BCRP vesicles showed a $K_m$ value of 11 $\mu$M and a $V_{max}$ value of 1643 pmol/mg/min. E217G showed sigmoidal concentration-dependent uptake kinetics with a Hill slope factor of 2.1, which suggests the involvement of more than one binding site on the transporter protein. E217G was transported in the

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**Fig. 6.** Uptake of substrates into transporter membrane vesicles from HEK293-EBNA cells transiently transfected with P-gp (A), MRP2 (B), and BCRP (C) in the absence or presence of ATP compared with uptake in mock vesicles. P-gp vesicles were incubated for 2 min with 1 $\mu$M [3H]NMQ, MRP2 vesicles were incubated for 8 min with 50 $\mu$M [3H]E217G, and BCRP vesicles were incubated for 1 min with 1 $\mu$M [3H]E3S. Values are means ± S.D. ($n = 4$–8).

**Fig. 7.** Effect of AMP-PNP on the uptake of probe substrates into membrane vesicles from HEK293-EBNA cells transfected with P-gp (A), MRP2 (B), and BCRP (C) compared with uptake into vesicles from mock-transfected HEK293-EBNA cells. Membrane vesicles were incubated with the probe substrates in the presence of ATP (4 mM), in the absence of ATP, or in the presence of the nonhydrolyzable ATP-analog AMP-PNP (4 mM). P-gp vesicles were incubated for 2 min with 1 $\mu$M [3H]NMQ, MRP2 vesicles for 8 min with 50 $\mu$M [3H]E217G, and BCRP vesicles were incubated for 2 min with 1 $\mu$M [3H]E3S. Values are means ± S.D. ($n = 4$).
MRP2 vesicles with a $K_{0.5}$ value of 146 nM and a $V_{\text{max}}$ value of 8266 pmol/mg/min.

Well characterized transporter inhibitors abolished the ATP-dependent uptake of the probe substrates. Transport of NMQ (1 μM) was inhibited by the P-gp inhibitor verapamil with an IC$_{50}$ value of 2.7 μM, the MRP2 inhibitor benzbromarone inhibited E$_{217}$G (50 μM) transport with an IC$_{50}$ value of 106 μM, and the BCRP inhibitor sulfasalazine inhibited E$_3$S (1 μM) with an IC$_{50}$ value of 0.6 μM (Fig. 11).

**Discussion**

The main objective of this study was to develop a system to produce large quantities of high-activity efflux transporters in mammalian membrane vesicles. The results from the present study show that the HEK293-EBNA cell line is suitable for the overexpression by transient transfection of and for the production of large quantities of human P-gp, MRP2, and BCRP membrane vesicles. The transporter vesicles showed high activity toward model substrates and exhibited saturable uptake kinetics, and uptake was sensitive to known inhibitors of each respective transporter. The transport of substrates in the vesicles was shown to be an active transporter-mediated process requiring hydrolysis of ATP. Any binding of substrates to membrane and transporters themselves in the presence of ATP was ruled out. The drug transporters were also found to be expressed at the cell surface as shown by immunofluorescence microscopy, indicating correct expression and position of the transporters.

Two widely used methods for production of recombinant proteins in mammalian cells are the modified baculovirus (BacMam virus) process and transient transfection using nonviral plasmid vectors (Wurm and Bernard, 1999). Both BacMam and plasmid-based transient transfection share the advantage that a variety of proteins can be expressed using different stocks of virus or plasmids, whereas only a single mammalian cell line has to be maintained. Plasmid DNA can be bulk-produced and stored long-term (several years) ready for transfection, whereas storage of baculovirus is limited to a few months at 4°C. Transient transfection mediated by FuGENE6 reagent has been successfully used for small-scale BCRP production (Mohrmann et al., 2005). Recent advances in large-scale PEI-mediated transient transfection (Tuvesson et al., 2008) enabled us to apply this technique for the large-scale expression of human ABC transporters.

The HEK293 cells express low endogenous levels of transporters, which make them suitable for overexpression of target transporters (Ahlin et al., 2008, 2009). These cells could therefore be used as a common cell system for the overexpression of not only uptake transporters but also efflux transporters. Membrane proteins expressed in Sf9 cells are underglycosylated, and it has been suggested that the

![Fig. 8. Osmolarity dependence of ATP-dependent uptake of [3H]NMQ in P-gp vesicles (A), [3H]E$_{17}$G uptake in MRP2 vesicles (B), and [3H]E$_3$S uptake in BCRP vesicles (C). ATP-dependent uptake of [3H]NMQ (1 μM, 2 min), [3H]E$_{17}$G (50 μM, 8 min), and [3H]E$_3$S (1 μM, 2 min) in transporter-containing membrane vesicles (■) and mock vesicles (□) was measured in the presence of sucrose concentrations ranging from 250 mM (isotonic condition) to 1000 mM. ATP-dependent transport was plotted against the reciprocal sucrose concentration. Values are means ± S.D. (n = 4).

![Fig. 9. ATP-dependent uptake of [3H]E$_{17}$G (50 μM) in MRP2 vesicles (A) and [3H]E$_3$S (1 μM) in BCRP vesicles (B) prepared from stably or transiently transfected cells. MRP2 vesicles were prepared from stably transfected MDCKII cells or transiently transfected HEK293-EBNA cells. BCRP vesicles were prepared from stably transfected HEK293 cells or transiently transfected HEK293-EBNA cells.

**TABLE 4**

**Summary of transport properties of P-gp-, MRP2-, and BCRP-expressing membrane vesicles prepared from transiently transfected HEK293-EBNA cells**

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Incubation (min)</th>
<th>Substrate</th>
<th>Mean Response (+ATP/ATP) (pmol/mg/min)</th>
<th>ATP-Dependent Transport*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>2</td>
<td>[3H]NMQ (1 μM)</td>
<td>18.5</td>
<td>300 ± 10</td>
</tr>
<tr>
<td>MRP2</td>
<td>8</td>
<td>[3H]E$_{17}$G (50 μM)</td>
<td>36.8</td>
<td>1761 ± 187</td>
</tr>
<tr>
<td>BCRP</td>
<td>1</td>
<td>[3H]E$_3$S (1 μM)</td>
<td>5.4</td>
<td>283 ± 72</td>
</tr>
</tbody>
</table>

*Values are means ± S.D.; n = 8.

MRP2 vesicles with a $K_{0.5}$ value of 146 μM and a $V_{\text{max}}$ value of 8266 pmol/mg/min.

Well characterized transporter inhibitors abolished the ATP-dependent uptake of the probe substrates. Transport of NMQ (1 μM) was inhibited by the P-gp inhibitor verapamil with an IC$_{50}$ value of 2.7 μM, the MRP2 inhibitor benzbromarone inhibited E$_{217}$G (50 μM) transport with an IC$_{50}$ value of 106 μM, and the BCRP inhibitor sulfasalazine inhibited E$_3$S (1 μM) with an IC$_{50}$ value of 0.6 μM (Fig. 11).
membrane composition influences the transporter function (Telbisz et al., 2007). Plasma membranes of insect cells such as Sf9 contain low levels of endogenous cholesterol in comparison with mammalian cells. Cholesterol is suggested to be an important component in the plasma membrane for transporter function. Telbisz et al. (2007) found that cholesterol loading of Sf9 cells enhanced the BCRP transporter activity, whereas cholesterol depletion of intact mammalian cells significantly reduced BCRP-dependent transport activity. In our study, the functional activity of the transporter vesicles produced from transiently transfected HEK293-EBNA cells was excellent as shown by a signal/background ratio (ratio of transport in the absence and presence of ATP) in the range of 5 to 40 and a high level of ATP-dependent transport. The functional activity was found to correlate well with the expression level detected by Western blotting, indicating that it can be used as a quality control measure during vesicle production. Samples of vesicles were removed throughout the preparation process and the uptake activity in the vesicle fractions was analyzed. The results of this analysis demonstrated that the sucrose gradient centrifugation step was critical to obtain enrichment of vesicles with high uptake activity (data not shown). The efflux transporter

![Fig. 10. Kinetics of ATP-dependent transport of probe substrates in transporter membrane vesicles. Concentration dependence of transport of NMQ in P-gp vesicles (A), E17βG in MRP2 vesicles (B), and E3S in BCRP vesicles (C). Each point represents the mean ± S.D. (n = 3). K_m and V_max values presented in figures represent fitted parameter estimate ± S.E.](image1)

![Fig. 11. Inhibition of ATP-dependent transport of [3H]NMQ (1 μM) by verapamil in P-gp vesicles (A), [3H]E17βG (50 μM) by benzbromarone in MRP2 vesicles (B), and [3H]E3S (1 μM) by sulfasalazine in BCRP vesicles (C). Each point represents the mean ± S.D. (n = 3). IC_{50} values presented in figures represent fitted parameter estimates.](image2)
assays presented in this study are all based on transporter transfections with a verified amino acid sequence that corresponds to the wild-type sequence. The functional uptake activity in vesicles prepared from the transiently transfected cells was higher than that of vesicles prepared from stably transfected cells. In our hands, the level of activity in these vesicles as indicated by $V_{max}$ values (picomoles per minute per milligram of protein) for probe substrates used in this study is even higher than that reported for MRP2 (Zelcer et al., 2003; Telbisz et al., 2007; Pedersen et al., 2008; Herédí-Szabó et al., 2009), BCRP (Pál et al., 2007, Xia et al., 2007), and P-gp (Hooveld et al., 2002) vesicles prepared from Sf9 cells. The transient transfection approach and large-scale production technologies described in this study should be generally applicable for other ABC efflux transporters of interest, e.g., MRP3, MRP4, and bile salt export pump.

In this study, the transport kinetics for $E_{17}BG$ uptake by MRP2 was sigmoidal with an estimated half-maximal transport rate ($K_{0.5}$) of 146 µM and a Hill number of 2.1. These results are in agreement with previous reports proposing that MRP2 contains two interacting binding sites (Bodo et al., 2003; Zelcer et al., 2003). Results similar to our data were reported by Zelcer et al. (2003) and Herédí-Szabó et al. (2009), who had $K_{0.5}$ values of 120 and 150 µM, respectively, for $E_{17}BG$ transport in MRP2 membrane vesicles prepared from baculovirus-infected Sf9 cells. The $K_m$ value for transport of NMQ in our P-gp vesicles was 2.9 µM, which is lower than the $K_m$ value of 14.7 µM reported for the transport of this substrate into membrane vesicles prepared from P-gp-overexpressing SF21 insect cells (Hooveld et al., 2002). The observed differences in affinity might be related to the effect of membrane cholesterol on the P-gp transporter because the Sf9 insect cell membranes contain less cholesterol than the mammalian HEK293 cell membranes (Eckford and Sharon, 2008).

The membrane vesicles can be used in vesicle transport assays to assess whether the drug is a substrate and/or inhibitor of the transporter. High specific transporter activity of the vesicles is important to be able to identify transporter substrates. P-gp substrates are often lipophilic and highly membrane-permeable, resulting in high passive uptake into the vesicles, masking the contribution of active P-gp transport. The results of this study support previously reported data indicating that NMQ, a low permeability cationic compound, is a suitable P-gp substrate to use in a vesicle-based P-gp inhibition assay (Hooveld et al., 2002).

In addition to investigating whether compounds may be substrates, the membrane vesicles are also suitable for use in inhibition assays. The radiolabeled probe substrates used in our characterization studies showed excellent sensitivity and known efflux inhibitors inhibited the uptake. Investigations of possible inhibition of specific transporters by drug candidates could predict drug transporter interactions. Transporter drug interactions are a relatively new area of investigation and for the efflux transporters attention has been focused mainly on P-gp (Zhang et al., 2008). The results from the present study indicate that the high-activity human P-gp membrane vesicles could potentially be used as an alternative to the commonly used P-gp inhibition assays in Caco-2 or MDCK-MDR1 cell monolayers with digoxin as the probe substrate.

The vesicle system can be easily applied in a high-throughput format, screening for both substrate and inhibitors of the individual transporter. By using vesicles the investigated drug is in direct contact with the efflux transporter, which implies that the determination of kinetic parameters, $K_m$ values for substrates, and $K_{IC_{50}}$ values for inhibitors are more accurate (closer to true values) than the corresponding values determined in the cell monolayer systems, in which there is a basolateral membrane to traverse to reach the transporter protein of interest.

In conclusion, this study shows that high-activity membrane vesicles containing P-gp, MRP2, or BCRP can be produced in large quantities from transiently transfected HEK293-EBNA cells. This approach for the preparation of membrane vesicles should be generally applicable to the production of vesicles of other human ABC efflux transporters that are expressed in a human cell membrane.

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References

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