Pharmaceutical Research, Vol. 24, No. 7, July 2007 (© 2007)
DOI: 10.1007/s11095-007-9244-1

Research Paper

Expression of the ATP-binding Cassette Membrane Transporter, ABCG2, in Human and Rodent Brain Microvessel Endothelial and Glial Cell Culture Systems

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Received October 15, 2006; accepted January 11, 2007; published online March 23, 2007

Purpose. The function of ABCG2 (BCRP), a member of the ATP-binding cassette (ABC) superfamily of membrane-associated drug transporters, at the blood-brain barrier remains highly controversial. This project investigates the functional expression of endogenous ABCG2 in cultures of human and rodent brain cellular compartments.
Materials and Methods. RT-PCR, western blot and fluorescent immunocytochemical analyses were performed on ABCG2-overexpressing human breast cancer (MCF-MX100) cells, human and rat brain microvessel endothelial (HBEC and RBE4, respectively), and rat glial cells.
Results. RT-PCR analysis detected ABCG2 mRNA in all the cell culture systems. Western blot analysis with anti-ABCG2 monoclonal BXP-21 antibody detected a robust band at ~72 kDa in the ABCG2-overexpressing MCF-MX100 cell line, whereas low expression was found in human and rat brain cell systems. Immunofluorescence microscopy detected predominant plasma membrane localization of ABCG2 in MCF-MX100 cells but weak signal in all brain cellular compartments. In the presence of ABCG2 inhibitors, the accumulation of ^{3}H-mitoxantrone and pheophorbide A, two established ABCG2 substrates, was significantly increased in MCF-MX100 cells but not in the human and rodent brain cell culture systems.
Conclusions. Our data show low endogenous ABCG2 protein expression, localization and activity in cultures of human and rat brain microvessel endothelial and glial cells.

KEY WORDS: ABCG2 (BCRP); brain; glial cells; microvessel endothelial cells; mitoxantrone; transport.

INTRODUCTION

The ABCG2 protein, initially named the breast cancer resistance protein (BCRP), was first cloned in 1998 from a highly doxorubicin-resistant human breast cancer cell line (MCF-7/AdrVp) (1). Orthologs of human ABCG2 were then cloned from mouse fibroblasts (Bcrp1) (2) as well as from porcine brain capillary endothelial cells (brain multidrug resistance protein, BMDP) (3). Recently, rat ABCG2 cDNA was isolated from rat brain capillary fraction and was found to have amino acid sequence of 81, 93 and 81% identity with human, mouse and porcine, respectively (4).

ABCG2 belongs to the ATP-binding cassette (ABC) superfamily of transporters that includes the drug efflux transporters, P-gp and the Multidrug Resistance-Associated Proteins (MRPs). Structural and sequence analysis have shown this protein to be a member of the *ABCG* gene subfamily, containing the Drosophilia *white*, *brown*, and *scarlet* genes and the human *white* homologue, ABCG1 (5). Unlike P-gp and MRP1, which are full transporters with 12 transmembrane spanning domains and two ATP-binding domains, ABCG2 is a half transporter comprised of only six putative transmembrane spanning domains and a single ATP-binding region (1). ABCG2 is believed to function as a dimer similar to other half-transporters such as ABCG5 and ABCG8 that dimerize to form functional homo- or heterodimers (6). ABCG2 has broad substrate specificity and recognizes a wide selection of sulfoconjugated organic anions, hydrophobic and amphiphilic compounds, as well as physiologic substrates such as steroid hormones and folic acid (7).

Similar to P-gp and MRP1, ABCG2 is not only expressed in tumor cell lines but also in a variety of normal human tissues such as placental syncytiotrophoblasts, liver canaliculi, colon, thyroid, heart, small intestine, pancreas, a specific population of hematopoietic stem cells, as well as the brain (8). Recent studies have provided evidence for ABCG2

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; HBEC, human brain endothelial cells; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein.

expression in the central nervous system, particularly along the luminal side of the blood-brain barrier (9). Zhang *et al.* (10) reported the gene and protein expression of ABCG2 in primary cultures of human brain microvessel endothelial cells and human fetal astrocytes. Over-expression of human ABCG2 in an immortalized rat brain endothelial cell line resulted in enhanced vectorial transport of ABCG2 substrates, mitoxantrone and rhodamine-123, in the abluminal-to-luminal direction (10). In the porcine brain, ABCG2 appears to be predominantly expressed in brain microvessel endothelial cells and to a lesser extent in choroid plexus epithelial cells and pericytes (3). Immunohistochemical analysis in rat brain tissue showed that ABCG2 is localized along the luminal membrane of rat brain capillaries (4). A recent study by Lee *et al.* (11) also reported mouse *ABCG2* gene and protein expression along the luminal side of the mouse brain capillaries.

The emerging evidence for the expression of ABCG2 at the blood-brain barrier suggests a possible role for this efflux transporter in restricting the accumulation of potentially harmful xenobiotics in the central nervous system. However, data on the functional activity of ABCG2 at this site remain inconclusive. Although *in vitro* systems of human and rat brain microvessel endothelial cells have been used to characterize the functional activity of ABCG2 at the blood-brain barrier, it is important to note that these cell systems were transfected to over-express the exogenous protein of interest (4,10). Data from recent *in vivo* studies in ABCG2 knockout mice are controversial. Some studies suggest that ABCG2 lacks functional activity at the blood-brain barrier (11,12) while others suggest a functional ABCG2 protein (13,14).

Evidence for a possible link between multidrug resistance in various neurological disorders (brain neoplasias and epilepsy) and the functional expression of P-gp in brain compartments has been proposed (15,16). This however, remains to be further investigated for ABCG2. The purpose of this project is to investigate the functional expression of ABCG2 in human and rat brain cell culture systems that have not been selected in drug or transfected to over-express ABCG2.

MATERIALS AND METHODS

Materials

(^{3}H) Mitoxantrone (3 Ci/mmol) and (^{14}C) D-mannitol (55 mCi/mmol) were purchased from Moravek Biochemicals Inc (Brea, California, USA). Pheophorbide A was purchased from Frontier Scientific (Logan, UT, USA). PSC 833 was a generous gift from Novartis Pharma (Basel, Switzerland). Fumitremorgin C (FTC) was a kind gift from the laboratory of Dr. S. Bates (Bethesda, Maryland, USA) and the tetracyclic analog of FTC, Ko143, was a generous gift from the laboratory of Dr. A. Schinkel (Amsterdam, The Netherlands). The anti-BCRP monoclonal antibodies, BXP-21 and BXP-53 were purchased from Kamiya Biomedical Company (Seattle, WA, USA) and Alexis Biochemicals (Lausen, Switzerland), respectively. MK571 was purchased from Biomol Research Labs Inc (Plymouth Meeting, PA, USA).

Cell Culture Systems

Primary Cultures of Human Brain Microvessel Endothelial Cells (HBEC)

Primary cultures of human brain microvessel endothelial cells (HBEC) were kindly provided by the laboratories of Dr. Jack Antel (Montreal Neuroimmunological Institute, McGill University, Montreal, Quebec) and Dr. Alexander Prat (CHUM research center, Notre-Dame Hospital, Montreal, Quebec). Currently, all brain tissue is obtained from the Neurosurgery Unit at the Notre-Dame Hospital (CHUM research center, Montreal, Quebec) from consented patients. Adult brain tissue was primarily retrieved from temporal lobe resections for intractable epilepsy ($n = 14$) and some trauma cases ($n = 3$). The tissue we obtained consisted of healthy tissue (isolated from the 'passage' made in order to resect the diseased parts). The isolation and initial culture of HBEC were performed and maintained in the laboratory of Dr. A. Prat as described in Prat *et al* (17). Briefly, tissue was minced and homogenized in phosphate buffer saline (PBS) and filtered once through a Nitex mesh (350 μm) and then twice through a 112 μm mesh. The filtrate was collected, centrifuged and digested for 10 min with collagenase type IV (Sigma, Mississauga, ON, Canada) at 37°C. Suspension was centrifuged and seeded on 0.5% gelatin coated flasks in M199 media (GIBCO-BRL, Burlington, ON, Canada) supplemented with 10% human normal serum, insulin-transferrin-selenium, endothelial growth supplement, and 10% fetal calf serum (all from Sigma). Visible colonies were collected at day 10 and expanded in fresh media. These cells have shown antigens specific to brain endothelial cells (Factor VIII related antigen, UAE-1 binding sites, HT-7 antigen, occluding, ZO-1 and-2, junctional adhesion molecule-1) for up to eight passages. Contamination by astrocytes and smooth muscle cells was estimated to be less than 1% since immunoreactivity for α-myosin and glial fibrillary astrocytic factor could not be detected in the culture after three passages. Subsequently, T-75-cm^2 flasks (Sarstedt) containing primary cultures of HBEC were grown in our laboratory and plated on to 0.5% gelatin-coated (Sigma) 48-well plates (Becton-Dickinson) for functional studies (passages 3–5). HBEC cultures are grown in M199 media (Invitrogen Canada Inc), 5% human serum type AB (Sigma), 10% fetal bovine serum, 20% mouse melanoma conditioned media (American Type Culture Collection, Manassas, VA, USA), 0.1% Endothelial cell growth supplement (VWR International), and 0.2% insulin-transferrin-selenium premix (Sigma) at 37°C, 5% CO_2 and 95% humidified air.

Immortalized Rat Brain Microvessel Endothelial Cell Line (RBE4)

The immortalized rat brain endothelial cell line (RBE4) was kindly provided by the laboratory of Dr. Francoise Roux (Paris, France). This cell line was developed from primary cultures of rat brain microvessel endothelial cells transfected with an E1A adenovirus gene that confers immortalization without oncogenic transformation (18). The RBE4 cell line is a well-characterized system that displays some morphological and biochemical characteristics of brain microvessel endothelial

cells *in vivo* (i.e., RBE4 cells grow into a confluent monolayer, presence of tight junctions, express Factor VIII-related antigen and express specific brain microvessel enzymes such as alkaline phosphatase) (18,19). This cell line was grown as monolayers on T-75 cm^2 polystyrene tissue culture grade flasks (Sarstedt, St. Leonard, PQ, Canada) and 48-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) pre-coated with Type 1 rat-tail collagen (Sigma-Aldrich, Oakville, Ontario, Canada) at 37°C, 5% CO_2, and 95% humidified air. RBE4 culture medium consists of α-minimal essential medium/Ham's F10 (1:1), supplemented with 2 mM glutamine, 10% fetal calf serum, 1 ng/ml basic fibroblast growth factor, 0.3 mg/ml geneticin and 0.05 mg/ml gentamicin (all ingredients from Invitrogen Canada Inc, Burlington, ON, Canada). Culture medium was changed every 2 days and confluent monolayers are subcultured with 0.25% trypsin-EDTA (Invitrogen Canada Inc).

Primary Cultures of Rat Astrocytes

Primary cultures of rat astrocytes were isolated and cultured as previously described (20). All procedures were performed in accordance with the University of Toronto Animal Care Committee and the Province of Ontario Animals for Research Act. Briefly, neonatal Wistar rats (2–3 day old, Charles River Laboratory, St Constant, PQ, Canada) were sacrificed by cervical dislocation. Whole brains were removed and cerebral cortices were dissected and digested for 30 min in minimum essential medium with serum containing 2 mg/ml trypsin and 0.005% DNase 1. The tissue was mechanically disrupted using a standard cell dissociation kit (Sigma-Aldrich) and the cell suspension was centrifuged for 10 min at 1000 g and resuspended in fresh culture media (minimum essential medium supplemented with 5% fetal bovine serum, 5% horse serum and 50 μg/ml gentamicin). The cells were plated onto T-75 cm^2 tissue culture flasks (Sarstedt) and incubated in medium at 37°C, 5% CO_2 and 95% humidified air for 7–10 days until confluent. The culture was placed on an orbital shaker at 100 rev/min overnight to remove any contaminating microglia cells. The microglia were poured off into another T-75 cm^2 flask and grown in fresh culture medium. These cells were then used to isolate RNA for RT-PCR or used for crude membrane preparations for western blot analysis. The remaining astrocytes were harvested with 0.1% trypsin/EDTA in Hank's Balanced Salt Solution and plated onto 48-well plates (Becton-Dickinson).

Continuous Rat Brain Microglia Cell Line (MLS-9)

The continuous rat brain microglial cell line (MLS-9) was isolated and cultured from the neopallia of 2 or 3-day old Wistar rats and provided by Dr. Lyanne Schlichter (University Health Network, Toronto Western Hospital, Toronto, Ontario). As previously described by Lee *et al.* (21), microglial cultures that were >98% pure (positive labeling with isolectin B4, Sigma) were induced to proliferate by adding colony-stimulating factor-1. After a few weeks some colonies were harvested and grown in the absence of growth factor and from one of these colonies the MLS-9 cell line was established. MLS-9 cells were grown as a monolayer on T-75 cm^2 tissue culture flasks or 48-well plates (Sarstedt) at 37°C, 5% CO_2 and 95% humidity. Media were changed every 2 days with minimum essential medium, pH 7.2, supplemented with L-glutamine, D-glucose, 5% fetal bovine serum, 0.5% penicillin/streptomycin and 5% horse serum (all obtained from Invitrogen Canada Inc). Confluent cultures were subcultured with sodium citrate containing 130 mM NaCl, 15 mM sodium citrate, 10 mM glucose and 10 mM HEPES, pH 7.4.

Human Breast Cancer Cell Lines, MCF-WT and MCF-MX100

MCF-7 cell lines were a generous gift from Dr. Susan Bates (Bethesda, Maryland, USA). The MCF-MX100 cell line is a human breast cancer cell line which over express wild-type (R482) ABCG2 when cells were selected in 100 ng/ml mitoxantrone (22). The cells were grown as a confluent monolayer on T-75 cm^2 polystyrene tissue culture grade flasks (Sarstedt) and 48-well plates (Becton-Dickinson) at 37°C, 5% CO_2, and 95% humidified air. MCF-7 wild type (MCF-WT) cultures were maintained in medium containing RPMI 1640 media (Medicorp Inc, Montreal, Quebec), 10% fetal bovine serum, 1% L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin G. MCF-MX100 cells were grown in the same medium supplemented with 100 nM mitoxantrone. Two weeks prior to the undertaking of functional assays, drug treatment with 100 nM mitoxantrone was discontinued in the MCF-MX100 cultures. Control western blot studies undertaken in our laboratory showed that ABCG2 protein expression levels did not change in MX100 cultures once the drug treatment was discontinued for up to 3 weeks (*data not shown*). In addition, these cells do not express P-gp or MRP1 protein (23).

RT-PCR Analysis

All cell culture systems used in this study were lysed using TRIZOL reagent (Invitrogen Canada Inc). The cell lysates were incubated at room temperature for 3 min in chloroform/isoamyl alcohol (49:1) to remove any genomic contamination. The lysates were centrifuged at 11,500×*g* (4°C, 15 min) for separation of the organic and aqueous phases. The aqueous phase was incubated in isopropyl alcohol and centrifuged at 11,500×*g* (4°C, 10 min) to isolate cellular RNA. RT was performed by preparing a cDNA reaction mixture of 0.5 μg oligo(dt) (12–18) primer and 2 μg RNA in a 20 μl reaction mixture containing 20 mM Tris-HCl, ph 8.4, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM dNTP, 2 U DNaseI and 200 U of SuperScript II reverse transcriptase (Invitrogen Canada Inc). PCR reaction was performed with 1 mM $MgCl_2$, 0.5 mM dNTP, 0.5 μM forward and reverse primers, 2.5 U platinum Taq DNA polymerase (Invitrogen Canada Inc) and 10% of the RT product using a GeneAmp 2400 Thermocycler (Perkin Elmer, Mississauga, ON, Canada).

The sequences of human *ABCG2* gene (hABCG2, ~172 bp) were: forward primer 5′- TGC-CCA-GGA-CTC-AAT-GCA-ACA-G-3′ and reverse primer 5′- ACA-ATT-TCA-GGT-AGG-CAA-TTG-TG-3′. The *hABCG2* gene was co-amplified with the housekeeping gene human β2-microglobulin. The human β2-microglobulin (~120 bp) sequences were: forward primer 5′- ACC-CCC-ACT-GAA-AAG-

ATG-A-3′ and reverse primer 5′- ATC-TTC-AAA-CCT-CCA-TGA-TG-3′. The primer sequences for the rat *ABCG2* gene (rABCG2) were the same as those used for the hABCG2 described above. The *rABCG2* gene was co amplified with rat β-actin. Rat β-actin (~349 bp) sequences were: forward primer 5′- TGG-AAT-CCT-GTG-GCA-TCC-ATG-AAA-C-3′ and reverse primer 5′- TAA-AAC-GCA-GCT-CAG-TAA-CAG-TCC-G-3′. Amplified DNA products were resolved on 1.7% agarose gels, stained with 1 mg/ml ethidium bromide and visualized by UV transilluminescence.

For cDNA sequencing, primers flanking the amino acid 482 region of interest were created for human (forward primer: 5′- GGG-TTC-TCT-TCT-TCC-TGA-3′ and reverse primer: 5′- CAC-TCT-GAC-CTG-CTG-CTA-3′; GenBank accession no. *NM_004827*) and rat (forward primer: 5′- CAG-TGT0GTC-AGC-TGT-GGA-3′ and reverse primer: 5′-GCT-TTG-GCC0TGC-GGC-TA-3′; GenBank accession no. *AB105817*). ABCG2 cDNA sequencing was performed by The Centre for Applied Genomics (Toronto Medical Discovery Tower, Medical and Research Science Centre, Toronto, Canada)

Western Blotting

Crude membranes from all cell culture systems used in this study were prepared by centrifuging the cell suspension at 400 g (4°C, 10 min). The resulting supernatant was discarded and the pellet lysed for 30 min at 4°C in 250 mM sucrose buffer containing 1.0 mM EDTA and 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). The cell suspension was homogenized with a Dounce homogenizer at 10,000 rev/min for three cycles of 10 s each. Homogenates were centrifuged at 3,000 g for 10 min to eliminate cellular debris. The supernatant was collected and centrifuged at 100, 000 g for 1 h at 4°C. The pellet was resuspended in 10 mM Tris buffer, pH 8.8, aliquoted and kept frozen at −20°C until further use. Protein concentrations of the crude membrane preparations were determined with the Bradford protein assay.

For immunoblotting studies, 35–50 μg of crude membrane proteins were mixed with Laemmeli buffer and resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel. The gel was then electro-transferred onto a polyvinylidene difluoride (PVDF) membrane and protein transfer was verified by Ponceau S staining. The membranes were blocked overnight at 4°C in Tris-buffered saline (15 mM Tris-HCL, 150 mM NaCl, pH 7.6) containing 0.05% (v/v) Tween-20 and 5% (m/v) dry skim milk powder. The membranes were incubated with the mouse anti-BCRP monoclonal antibody BXP-21 (1:100) or rat BXP-53 (1:100) in 2.5% blocking solution for 2 hr at room temperature. BXP-21 reacts with an internal epitope of BCRP (amino acids 271–396; GenBank accession no. *AF098951*) and has shown specificity for human and porcine species (Kamiya Biomedical Company, Seattle, WA, USA). BXP-53 monoclonal antibody reacts with an internal epitope of BCRP corresponding to amino acids 221–394 and is specific for human and mouse (GenBank accession no. *AF098951*). The membranes were incubated with anti-mouse and anti-rat (Serotec Inc, France) horseradish peroxidase-conjugated secondary antibody (1:1000) in 2.5% milk blocking buffer for 1 h at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for approximately 3–5 min. The ABCG2 over expressing drug resistant cell line, MCF-MX100, was used as a positive control. In each blot, β-actin was detected to control for loading using anti-actin monoclonal antibody, AC-40 (Sigma-Aldrich, 1:500) and anti-mouse secondary antibody (1:2,500).

The relative protein and mRNA expression of ABCG2 in the various brain compartments is provided in units based on semi-quantitative densitometric measurements of the 10% sodium dodecyl sulfate-polyacrylamide immunoblot and ethidium-stained PCR gels, respectively. Units were first normalized with their respective house-keeping genes and then normalized to the expression level in the RBE4 cells, which is arbitrarily set to 1 U.

Immunofluorescence Microscopy

All brain cell culture systems were plated onto sterile glass cover slips (22 × 22, Fisher Scientific Int). Coverslips were coated with 0.5% gelatin for HBEC and with Type 1 rat-tail collagen for RBE4. Immunofluorescence was performed on 7 day old cultures (culture age at which functional studies are performed). Cells were fixed in 4% paraformaldehyde for 12 min at room temperature and washed three times with PBS. Cover slips were then incubated for 30 min at room temperature in PBS supplemented with 0.5% (v/v) normal goat serum and 0.2% (v/v) TritonX-100. Subsequently, cover slips were incubated for 60 min at room temperature with primary anti-BCRP monoclonal antibody (BXP-21, 1:30 and 1:50, Kamiya Biochemical Company) in 0.5% (v/v) goat serum and 0.2% (v/v) TritonX-100 in PBS. They were then washed 5x with PBS and incubated with secondary Alexa Flour 594 conjugated goat anti-mouse secondary antibody (1:1000, Molecular Probes, Eugene OR, USA) in 0.5% (v/v) goat serum and 0.2% (v/v) TritonX-100 in PBS for 45 min. Cover slips were washed 5x with PBS and mounted onto a glass slide using Prolong Gold Antifade Reagent (Molecular Probes) and allowed to cure for 2 hr. All coverslip washes were done at 5 min intervals. Cells labeled with anti-ABCG2 BXP-53 monoclonal antibody (1:30) were fixed in acetone at −20 °C for 10 min and blocked in PBS and 0.5% goat serum (no Triton-X was added). Cells were then incubated with Alexa Flour 594 conjugated goat anti-rat secondary antibody (1:1000, Molecular Probes, Eugene OR, USA) in PBS and 0.5% goat serum. For negative controls, samples were incubated only with the secondary antibody. Samples were examined at ×10 or ×40 magnification with a Nikon Eclipse E1000R fluorescent microscope (Nikon, Tokyo, Japan) equipped with a TRITC filter tube (excitation filter 528–553 nm; dichromatic mirror 565 nm; barrier filter 600–660 nm), CCD camera (Hamamatsu) and Simple PCI imaging software.

Functional Studies

Radiolabeled transport assays were performed on 7 day old confluent monolayers of primary cultures of HBEC (passages 3–5), RBE4 cells (passages 36–45), MLS-9 (passages 13–25), primary cultures of rat astrocytes (passage 1), MCF-MX100 ($p+1$ to $p+7$) and MCF-WT cells ($p+1$ to $p+7$) grown on 48-well plates (Becton-Dickinson). The cellular accumulation of ^{3}H-mitoxantrone, an established ABCG2

substrate (24) was determined by a modified radiolabeled transport assay protocol previously described in our laboratory (21). In brief, the cells were washed and equilibrated for 30 min at 37°C in Earles Balanced Saline Solution, pH 7.4, (20 mM Hepes, 5.5 mM D-glucose, 1.0 mM Na_2HPO_4, 138 mM NaCl, 0.8 mM $MgSO_4$ anhydrous, 5.4 mM KCl, and 1.8 mM $CaCl_2$). Cell cultures were then incubated for the desired time with 20 nM ^{3}H-mitoxantrone, a concentration used previously to detect for ABCG2 activity in radiolabeled functional assays (25,26), in the presence or absence of various standard inhibitors for ABCG2 (i.e., 1–5 μM Ko143, 5–10 μM FTC), P-gp (1 μM PSC833) and MRP (5 μM MK571). PSC833 is a non-immunosuppressive cyclosporin A analogue and MK571 is a leukotriene D4 receptor antagonist. At the end of the desired time point, the incubation medium was aspirated and uptake reaction was terminated with the addition of ice-cold PBS. The cells were washed once with ice-cold PBS, solubilized with 2.5 ml 1.0 M NaOH for 30 min and transferred to 7.0 ml scintillation vials (1.25 ml 2.0 M HCl added to neutralize NaOH). ^{3}H-mitoxantrone cellular accumulation was measured by a Beckman liquid scintillation counter with automated quench correction. Samples were corrected for 'zero-time' and background radioactivity. The accumulation of ^{3}H-mitoxantrone was standardized to the protein concentration (mg/ml) as determined by the Bradford colorimetric assay using bovine serum albumin (Sigma-Aldrich) as the standard. Cellular ^{3}H-mitoxantrone concentrations were expressed as picomole per milligram of protein (pmol/mg protein).

Accumulation of pheophorbide A (10 μM), an established substrate for ABCG2 but not for P-gp or MRP1 (23), was determined in confluent monolayer cells by a fluorescent transport assay. Briefly, the cells were washed and incubated at 37°C for 30 min in Earle's Balance Saline Solution (same composition as that used in the radiolabled transport assay). The cells were incubated with 10 μM pheophorbide A for the desired time points in the presence or absence of ABCG2 inhibitors (Ko143 1 μM, FTC 10 μM). At the end of each time point, the incubation medium was aspirated and the reaction was terminated with 500 ml of ice-cold PBS. The cells were then solubilized with 100 ul of 1% Triton-X-100 solution for 15 min. The cellular accumulation of pheophorbide A was determined quantitatively by fluorescence spectrophotometry. Aliquots (50 ml) of solubilized cell solutions were collected in a 96-well fluorescence plate and measured using a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) microplate spectrofluorometer (excitation wavelength 420 nm; emission wavelength 670 nm). The concentration of pheophorbide A in each sample was determined by the fluorescence measurements obtained from a standard curve. Accumulation of pheophorbide A by the cell monolayers was standardized to the amount of total cellular protein (mg/ml) as determined by the Bradford colorimetric method using bovine serum albumin as the standard and expressed as nanomoles per milligram protein (nmol/mg protein).

Data Analysis

Each set of experiments was repeated at least three times in the immortalized and continuous cell lines pertaining to different passages. In primary cultures, experiments were performed in at least three independent sets of isolated cultures. In each individual experiment, each data point represents quadruplicate trials. Results are reported as a mean ± SD from a minimum of three independent experiments. For unpaired experimental data, the Student's *t*-test was used to determine statistical significance. For multiple comparison, the test of repeated measures ANOVA and/or the post hoc multiple comparison Bonferroni *t*-test was employed. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

ABCG2 mRNA Expression

RT-PCR analysis was used to detect ABCG2 mRNA expression in human and rat brain microvessel endothelial and rodent glial cells. Using specific primers for the *ABCG2* gene (as described in "MATERIALS AND METHODS"), a band for ABCG2 (~ 172 bp) was detected in HBEC (Fig. 1, lane 1), in RBE4 cells (Fig. 2, lane 6) as well as rat glial compartments (Fig. 2) i.e., primary cultures of rat microglia (lane 3), MLS-9 (lane 4), primary cultures of rat astrocytes (lane 5). The ABCG2 over-expressing cell line, MCF-MX100, was used as a positive control (Fig. 2, lane 2). As expected, no band was detected in our negative controls (Fig. 1, lane 2; Fig. 2, lane 1), which contained RT product prepared in the absence of mRNA. The housekeeping genes β2-microglobulin (~120 bp) (Fig. 1) and β-actin (~349 bp) (Fig. 2) were co-amplified with human and rat samples, respectively. Semi-quantitative densitometric analysis in rat brain cellular compartments shows that mRNA levels are highest in RBE4 cells followed by primary cultures of rat astrocytes and microglia. Compared to the RBE4 cell line, mRNA levels were significantly lower in the MLS-9 cell line (Fig. 2).

Since it has been proposed that amino acid substitutions at position 482 (with arginine, glycine or threonine in humans and arginine, methionine and serine in mice) could be a

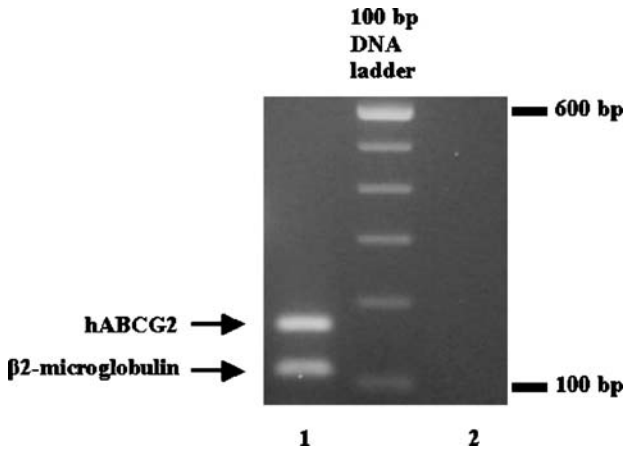

Fig. 1. RT-PCR analysis of ABCG2 mRNA in primary cultures of human brain microvessel endothelial cells (HBECs). An ethidium bromide stained gel shows amplification of a specific band for human ABCG2 (~172 bp) in HBEC (*lane 1*). The housekeeping gene β2-microglobulin (~120 bp) was co-amplified with the *ABCG2* gene. *Lane 2* represents the negative control where water was added to the PCR reaction in place of cDNA.

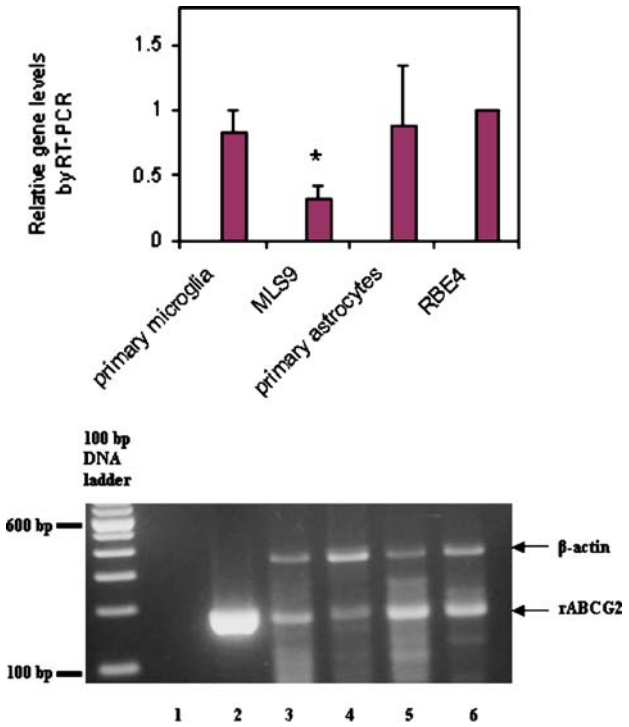

Fig. 2. RT-PCR analysis of ABCG2 mRNA in rat brain glial and microvessel endothelial cell culture systems. An ethidium bromide stained gel shows mRNA expression of rABCG2 (~172 bp) in primary cultures of rat brain microglia (*lane 3*), a continuous rat brain microglia cell line, MLS-9 (*lane 4*), primary cultures of rat astrocytes (*lane 5*) and the RBE4 cell line (*lane 6*). MCF-MX100 cells were used as a positive control (*lane 2*). The housekeeping gene β-actin (~349 bp) was co-amplified with the *ABCG2* gene. *Lane 1* represents the negative control, where water was added to the PCR reaction in place of cDNA. Semi-quantitative densitometric analysis from three separate gels is shown with the gel. *Asterisks* represent data points that are significantly different from RBE4. $*p < 0.05$.

determinant of ABCG2 substrate specificity (27), we sequenced the cDNAs of our brain cell culture systems and found that the wild-type form of ABCG2 is present in all our brain cultures (*data not shown*). In addition, we confirmed the expression of wild-type ABCG2 in the MCF-MX100 cell line as previously reported by Honjo *et al.* (27).

ABCG2 Protein Expression

Western blot analysis was used to detect ABCG2 protein in human and rat brain compartments using the anti-ABCG2 monoclonal antibody, BXP-21. This antibody interacts with an internal epitope of ABCG2 and does not cross-react with P-gp or MRP1 (23). Figure 3 shows a robust band at approximately 72 kDa for the ABCG2 over-expressing MCF-MX100 cells (lane 1), a molecular weight previously reported for ABCG2 (8). Bands were also detected in HBEC (lane 2) and RBE4 (lane 3) at the same molecular weight with much lower intensity bands observed in primary cultures of rat astrocytes (lane 4), MLS-9 (lane 5) and primary cultures of rat microglia (lane 6). Semi-quantitative densitometric analysis shows that ABCG2 protein levels are significantly greater in HBEC compared to rat brain cellular systems (Fig. 3). Within the rat brain compartments, RBE4 cells express significantly higher levels of ABCG2 protein compared to MLS-9 and primary cultures of rat astrocytes. Western blot analysis also shows that the anti-ABCG2 BXP-53 monoclonal antibody recognizes the 72 kDa band for ABCG2 in human cells (MCF-MX100 and HBEC) but not in the RBE4 cell line (*data not shown*).

ABCG2 Detection by Immunofluorescence Microscopy

The cellular and subcellular localization of ABCG2 in our human and rat brain cell culture systems were studied by immunofluorescence analysis applying fluorescence microscopy on cells 7 days in culture (culture age at which functional studies were performed) using BXP-21 or BXP-53 antibodies (Fig. 4).

MCF-MX100 cells revealed strong plasma membrane ABCG2 localization with the use of both BXP-21 (Fig. 4a) and BXP-53 antibodies (Fig. 4b). In addition, some cytoplasmic staining was observed with BXP-21 antibody. MCF-WT parent cell line showed extremely weak labeling with both antibodies (Fig. 4c and d). Primary cultures of HBEC showed overall weak labeling with the BXP-21 (Fig. 4e) and BXP-53 antibodies (Fig. 4f). Similar to the MCF-MX100 cells, some cytoplasmic staining was observed with both antibodies.

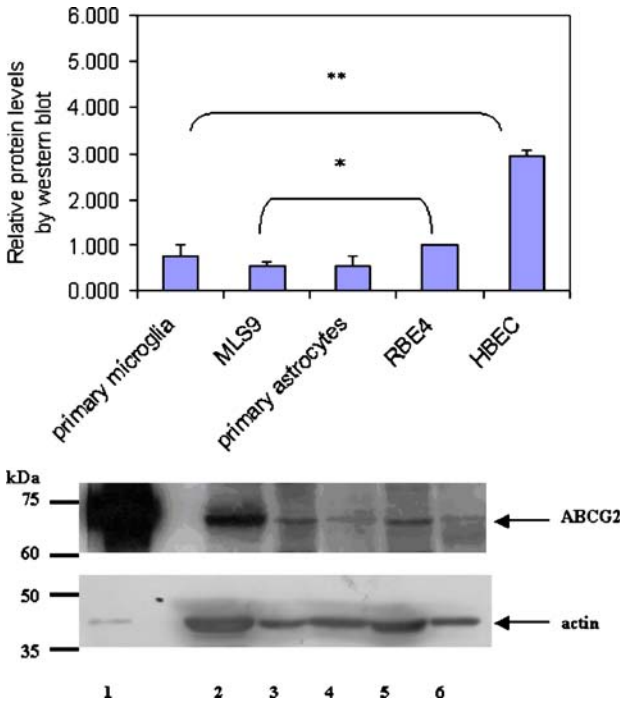

Fig. 3. Western blot analysis of ABCG2 protein in primary cultures of human brain microvessel endothelial cells and in rat brain glial and microvessel endothelial cell culture systems. Crude membrane proteins were resolved on a 10% SDS polyacrylamide gel. *Lane 2* (primary cultures of human brain endothelial cells, HBEC 35 μg), *lane 3* (RBE4, 35 μg), *lane 4* (primary cultures of rat astrocyte, 35 μg), *lane 5* (MLS-9, 35 μg) and *lane 6* (primary cultures of rat microglia, 35 μg). MCF-MX100 cell line (*Lane 1*, 2 μg) was used as a positive control. The blot was incubated with anti-ABCG2 BXP-21 primary antibody (1:100 dilution) and secondary anti-mouse antibody (1:1,000 dilution). Semi-quantitative densitometric analysis of three separate blots is shown with the western blot. *Asterisks* represent data points that are significantly different from HBEC and RBE4. $*p < 0.05$, $**p < 0.01$.

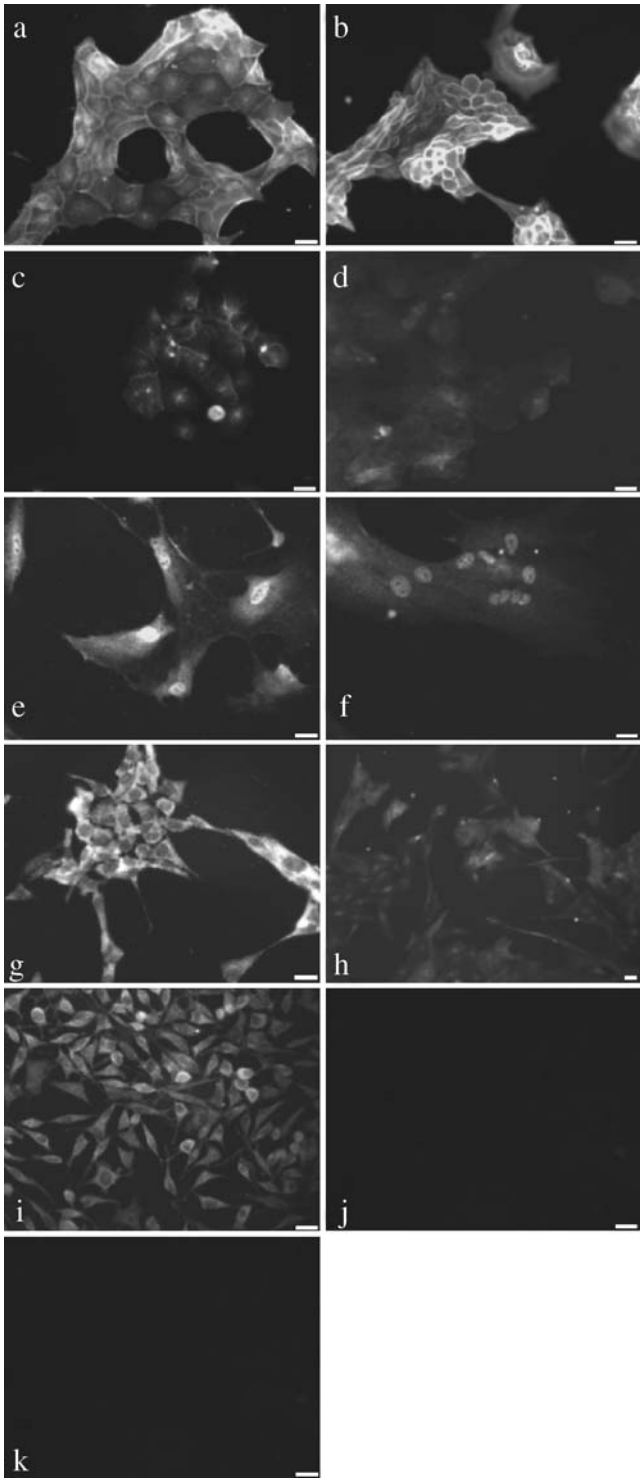

Fig. 4. Immunocytochemical localization of ABCG2 in brain cell systems at 7 days in culture. Cells cultured on glass coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.2% TritonX-100 (for BXP-21) or fixed with acetone at −20°C (for BXP-53). ABCG2 protein was localized with anti-ABCG2 BXP-21 monoclonal antibody (1:30 or 1:50) and TRITC conjugated secondary anti-mouse antibody (1:1,000) and/or anti-ABCG2 BXP-53 monoclonal antibody (1:30) and TRITC conjugated secondary anti-rat antibody (1:1,000). MCF-MX100 cells show strong plasma membrane and minor nuclear labeling with **a**, BXP-21 and **b**, BXP-53. *MCF-WT cells* show weak overall labeling with **c**, BXP-21 and **d**, BXP-53. HBEC show overall weak labeling and some nuclear staining with **e**, BXP-21 and **f**, BXP-53. Weak labeling is observed in RBE4 **g**, primary cultures of rat astrocytes (×10 magnification) (**h**) and MLS-9 (**i**). For the negative controls, only the TRITC conjugated secondary antibodies (1:1,000) were added. No labeling is observed with anti-mouse (**j**) and anti-rat (**k**) secondary antibodies. ×40 magnification unless stated otherwise.

RBE4 cells also showed overall weak labeling and minor intracellular labeling with the BXP-21 antibody (Fig. 4g). Immunocytochemical studies in RBE4 cells using the BXP-53 antibody showed lack of specific labeling (as was observed in our western blot) compared with the BXP-21 antibody (*data not shown*). Due to this lack of specificity for rat ABCG2, further immunocytochemical studies in rat cell cultures were only performed with the BXP-21 antibody. Primary cultures of rat astrocytes (Fig. 4h) and MLS-9 cells (Fig. 4i) showed a similar pattern of weak labeling with the BXP-21 antibody. Cells incubated with only Alex Flour 594-conjugated goat anti-mouse or anti-rat secondary antibody (1:1,000) served as negative controls. Representative images for all negative controls using anti-mouse and anti-rat antibodies are shown in Fig. 4j and k, respectively.

Functional Studies

To investigate the functional activity of endogenous ABCG2 the accumulation of ^{3}H-mitoxantrone (20 nM), an established ABCG2 substrate, was measured at 37°C in confluent monolayers of MCF-WT, MCF-MX100, primary cultures of HBEC, RBE4, MLS-9, and primary cultures of rat astrocytes. Since all our cell culture systems are known to express both P-gp and MRP1 (10,20,21,28,29) and some reports have identified mitoxantrone to be a substrate for P-gp and MRP1 (24) ABCG2 specific inhibitors FTC and Ko143 were used to distinguish ABCG2-mediated transport activity. FTC is a chemosensitizing agent effective at reversing mitoxantrone resistance ($K_i \sim 1$ μM) in ABCG2 over-expressing cell lines and does not interact with P-gp or MRP1 (22). Ko143 is a synthetic tetracyclic analog of FTC and potently inhibits ABCG2 mediated mitoxantrone transport ($IC_{50} \sim 0.1$ μM) but does not seem to alter P-gp or MRP1 mediated efflux (30).

Time profiles of ^{3}H-mitoxantrone accumulation by parent MCF-WT and MCF-MX100 cells show an increase in drug uptake over 30 min with a steady-state reached by 2 h (Fig. 5a and b). As expected, the overall cellular accumulation of ^{3}H-mitoxantrone by the MCF-MX100 cells, an ABCG2 over-expressing drug resistant cell line that does not express P-gp or MRP1 (23), was lower than in the wild-type. Specificity studies at 2 h in MCF-MX100 cells confirmed that FTC (2.2-fold increase) and Ko143 (1.7-fold increase) are specific against ABCG2 transport activity whereas PSC 833 (a known P-gp inhibitor) and MK571 (a known MRP inhibitor) did not have any effect on mitoxantrone accumulation (Fig. 6b). The inhibitors did not significantly alter mitoxantrone accumulation in wild-type cells under similar conditions (Fig. 6a). Mitoxantrone cellular accumulation by RBE4 (Figs. 7 and 8) and HBEC cells (Figs. 9 and 10) was similar to MCF-WT cells and transport was not significantly altered in the presence of Ko143 or FTC. In addition,

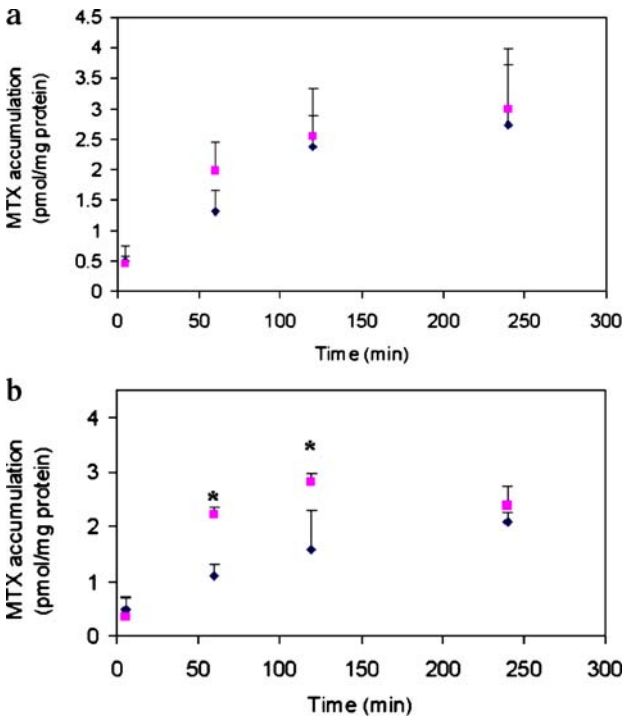

Fig. 5. Time profile of ^{3}H-mitoxantrone accumulation by **a** MCF-WT monolayer cells and **b** MCF-MX100 monolayer cells. Accumulation of 20 nM ^{3}H-mitoxantrone was measured at 37°C in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 1 μM (*squares*). Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements. *Asterisks* represent data points that are significantly different from control. *$p < 0.05$.

mitoxantrone accumulation by HBEC and RBE4 cells was not significantly increased in the presence of varying concentrations (5, 10 and 25 μM) of FTC and Ko143 (*data not shown*). Similarly, a lack of ABCG2-mediated transport of mitoxantrone in the presence of ABCG2 specific inhibitor Ko143 was observed in both MLS-9 cell line, as well as primary cultures of rat astrocytes (Figs. 11 and 12).

In order to confirm the observation on the lack of detectable ABCG2-mediated transport of radiolabeled mitoxantrone in our brain culture systems, we measured the accumulation of pheophorbide A, a specific substrate for ABCG2 but not for P-gp or MRP1 (31), in RBE4 and HBEC cell cultures. Using a fluorescent transport assay, we observed a significant increase in the accumulation of pheophorbide A in the presence of Ko143 and FTC in MCF-MX100 cells (Figs. 13 and 14) but not in the RBE4 (Figs. 15 and 16) or HBEC cells (Figs. 17 and 18).

DISCUSSION

ABCG2 possesses broad substrate specificity for compounds that include physiological substrates (i.e., sulfated estrogens, conjugates of folic acid, chlorophyll-derived phototoxins) and a diverse range of inhibitors (i.e., flavonoids, antiretrovirals, chemotherapeutics) (7). Studies in ABCG2 knockout mice suggest a possible physiological role in preventing the transport of toxic compounds across the maternal-fetal barrier as well as protection from diet-induced skin phototoxicity (32). Although it has been suggested that ABCG2 may play a role in restricting the permeation of xenobiotics across the blood-brain barrier, conflicting results from *in vitro* brain microvessel systems and some recent in vivo studies exist. The objective of this project was to investigate the functional expression of endogenous ABCG2 in human and rat brain cell culture systems.

In our studies, RT-PCR analysis demonstrates that ABCG2 mRNA is present in human and rat brain microvessel endothelial cells as well as in rat glial compartments (i.e., astrocytes and microglia) with highest expression levels in the RBE4 cells and lowest in MLS-9 cells. Western blot analysis using the anti-ABCG2 BXP-21 monoclonal antibody detected a band at the expected molecular weight range of 70–72 kDa (25) in our human and rat brain cell culture systems. As expected, the level of protein was much lower in the HBEC and RBE4 cells when compared to the ABCG2 over-expressing MCF-MX100 cells. Semi-quantitative densitometric analysis showed that protein levels were significantly higher in HBEC compared to rat brain cellular compartments. Among the rat brain compartments, protein levels are highest in RBE4 cells compared to glial cells. Recently, Aronica *et al* . (33) reported low ABCG2 protein expression in isolates from the cortex and white matter of normal human brain tissues. Interestingly, this protein expression was significantly increased in primary glial tumors from patients with refractory epilepsy compared to controls (33). Zhang *et al.* (10) also reported an increase in ABCG2 mRNA expression in primary brain tumors compared to normal

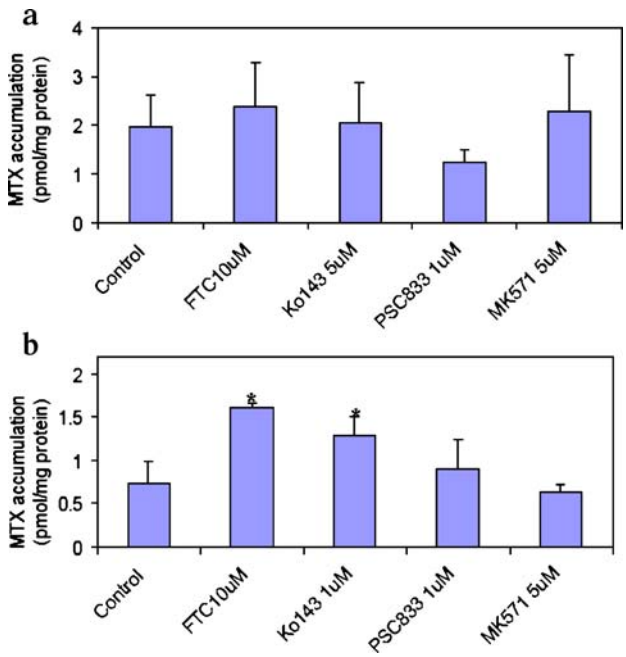

Fig. 6. Specificity of ABC transport inhibitors in **a** MCF-WT monolayer cells and **b** MCF-MX100 monolayer cells. Accumulation of ^{3}H-mitoxantrone (20 nM) at 2 h was measured in the absence (control) or presence of ABCG2 inhibitors (Ko143 1 and 5 μM or FTC 10 μM), or P-gp inhibitor (PSC 833 1 μM) or MRP inhibitor (MK571 5 μM) at 37°C. Results are expressed as mean ± SD of four separate experiments with each data point in an individual experiment representing quadruplicate measurements. *Asterisks* represent data points that are significantly different from control. *$p < 0.05$.

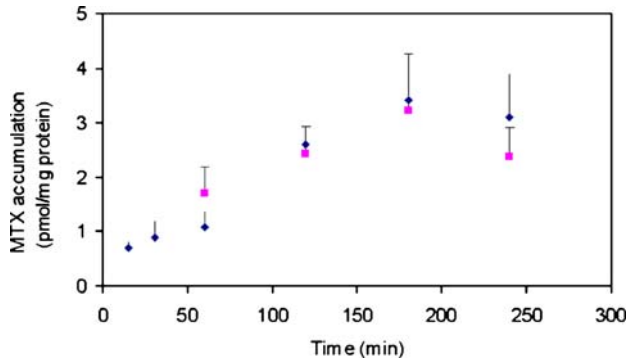

Fig. 7. Time profile of ^{3}H-mitoxantrone accumulation by RBE4 monolayer cells. The accumulation of 20 nM ^{3}H-mitoxantrone over time by RBE4 cells was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 5 μM (*squares*) at 37°C. Results are expressed as mean ± SD of four separate experiments with each data point in an individual experiment representing quadruplicate measurements.

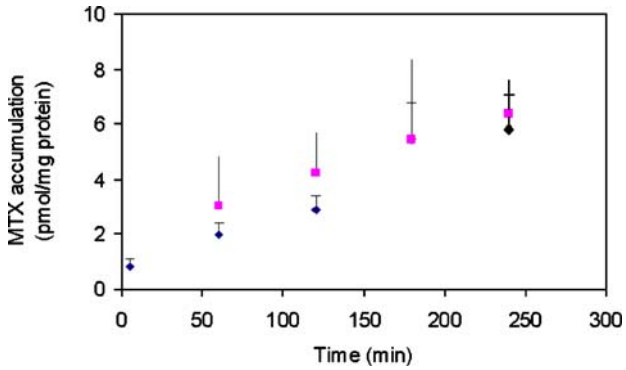

Fig. 9. Time profile of ^{3}H-mitoxantrone accumulation by HBEC monolayer cells. The accumulation of 20 nM ^{3}H-mitoxantrone over time by primary cultures of HBEC was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 1 μM (*squares*) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements.

brain tissues, although P-gp expression was unaltered. This low level of protein expression in normal brain tissue parallels our observations and suggests that ABCG2 expression may be increased in neuropathological conditions.

Immunofluorescence microscopy studies reveal predominant ABCG2 plasma membrane localization in MCF-MX100 cells. This is in agreement with results observed in various drug-resistant ABCG2 over-expressing cells (8,34). In contrast to the MCF-MX100 cells, the brain cell culture systems showed weak labeling and lack of plasma membrane staining. We also observed some intracellular labeling at the nucleus and cytosol of MCF-MX100 and HBEC confirming previous reports in MCF-7 cells as well as in rat cerebral cortex (4,34). The overall low level of ABCG2 protein localization parallels the low protein expression observed in these brain cellular systems.

Results from functional studies in the MCF-MX100 cells demonstrate FTC and Ko143 sensitive ^{3}H-mitoxantrone (2.2- and 1.7-fold difference, respectively) accumulation by the monolayer cells. Similarly, we observed FTC and Ko143 sensitive accumulation of pheophorbide A, a specific ABCG2 fluorescent substrate, by MCF-MX100 cells. Since MCF-MX100 cell line does not express detectable levels of P-gp or MRP1 (23), mitoxantrone and pheophorbide A transport in these cells is most likely ABCG2-mediated. In contrast, FTC and Ko143 did not have an effect on mitoxantrone or pheophorbide A transport in the MCF-WT parent cell line or the human and rat brain cell culture systems. This lack of activity compared to the MCF-MX100 cells appears to correlate with the low protein expression and lack of plasma membrane localization observed in the brain cell culture systems. Although we do not detect ABCG2 function in the brain cell culture systems, we would like to emphasize that previous studies including those from our laboratory have demonstrated activity of other ABC efflux drug transporters such as P-gp and MRP1, 4 and 5 in these cell culture systems (20,21,28,29,35,36).

It is believed that changes in substrate specificity for ABCG2 may be a result of single amino acid substitutions at position 482, a position thought to be involved in substrate binding. Although all three variants (R482, R482T, R482G) are able to transport mitoxantrone, wild-type ABCG2 is somewhat less effective in mitoxantrone extrusion than the other variants (37). cDNA sequencing revealed that all our brain cell culture systems expressed the wild-type form of

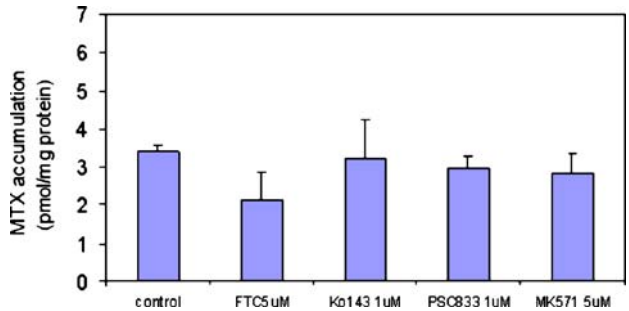

Fig. 8. Specificity of inhibitors in RBE4 monolayer cells. Accumulation of ^{3}H-mitoxantrone (20 nM) by RBE4 cells at 3 h was measured in the absence (control) or presence of inhibitors for ABCG2 (1 μM Ko143 or 5 μM FTC), P-gp (1 μM PSC 833) and MRP (5 μM MK571) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements.

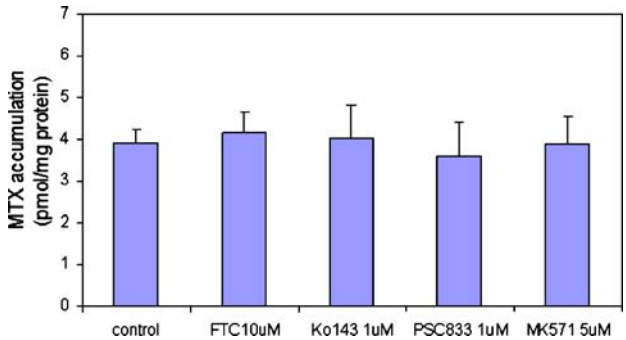

Fig. 10. Specificity of inhibitors in primary cultures of HBEC cells. Accumulation of ^{3}H-mitoxantrone (20 nM) at 3 h by primary cultures of HBEC was measured in the absence (control) or presence of inhibitors for ABCG2 (1 μM Ko143 or 10 μM FTC), P-gp (1 μM PSC 833) and MRP (5 μM MK571) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements.

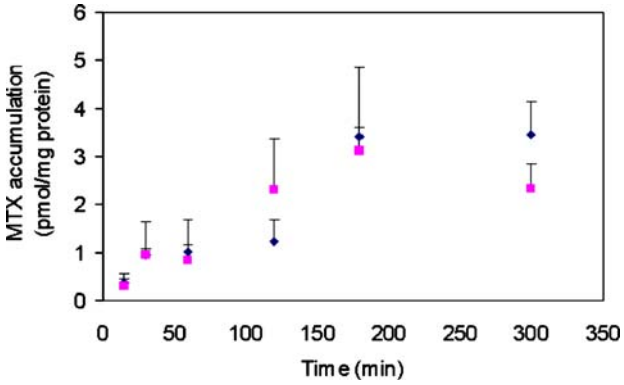

Fig. 11. Time profile of ^{3}H-mitoxantrone accumulation by a continuous rat brain microglia cell line, MLS-9. The accumulation of 20 nM ^{3}H-mitoxantrone by MLS-9 cells was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 1 μM (*squares*) at 37°C. Results are expressed as mean ± SD of 3 separate experiments with each data point in an individual experiment representing quadruplicate measurements.

ABCG2 (R482). In addition, MCF-MX100 cells cultured in our laboratory also express wild-type ABCG2, confirming previous results of Honjo *et al.* (27). In light of the observations made by Ozvegy *et al.* (37), the expression of wild-type ABCG2, along with the low protein expression and lack of plasma membrane localization, may be some important factors contributing to the lack of detectable mitoxantrone transport activity in our brain cell culture systems.

To date, results from *in vitro* transport assays have suggested a functionally active protein in ABCG2 over-expressing brain microvessel endothelial cell culture systems. Immortalized rat brain endothelial cells transfected with wild-type human ABCG2 protein showed reduced mitoxantrone accumulation compared with empty-vector controls (10). Hori *et al.* (4) demonstrated ABCG2-mediated transport of mitoxantrone in human HEK293 cells transfected with rat ABCG2. It is important to note that these cell systems were transfected to over-express ABCG2 protein and do not provide information on the functional activity of endogenously expressed ABCG2 in its native cellular environment. In primary cultures of porcine brain microvessel

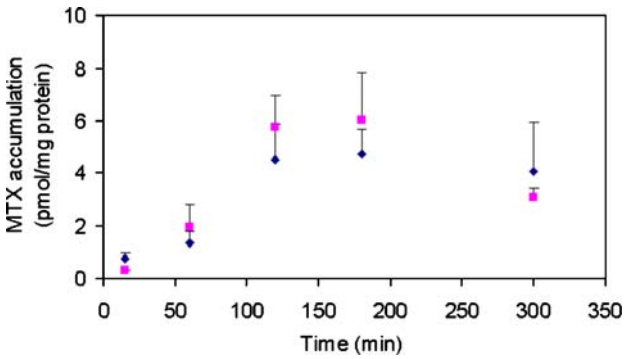

Fig. 12. Time profile of ^{3}H-mitoxantrone accumulation by primary cultures of rat astrocytes. The accumulation of 20 nM ^{3}H-mitoxantrone by primary cultures of rat astrocytes was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 1 μM (*squares*) at 37°C. Results are expressed as mean ± SD of 3 separate experiments with each data point in an individual experiment representing quadruplicate measurements.

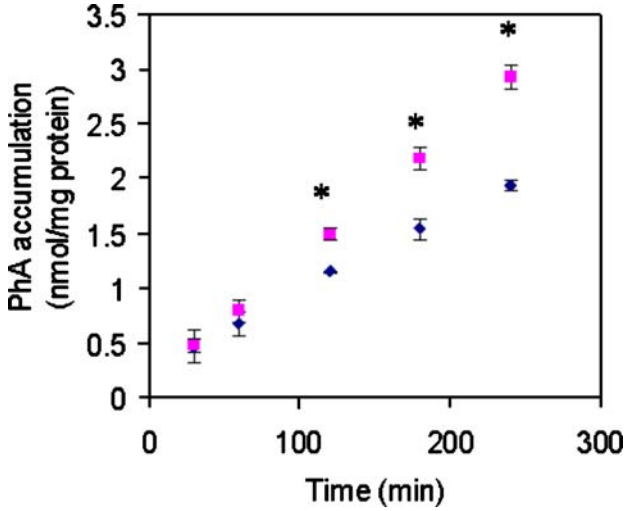

Fig. 13. Time profile of pheophorbide A accumulation by MCF-MX100 monolayer cells. The accumulation of 10 μM pheophorbide A (PhA) by MCF-MX100 cells was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor Ko143 1 μM (*squares*) at 37°C. Results are expressed as mean ± SD of 3 separate experiments with each data point in an individual experiment representing quadruplicate measurements. *Asterisks* represent data points that are significantly different from control. *$p < 0.05$.

endothelial cells, treatment with GF120918, a P-gp and ABCG2 inhibitor, reduced vectorial transport of daunorubicin compared to non-treated cells (3). These porcine brain endothelial cultures were grown in the presence of hydrocortisone and reported 30-fold greater ABCG2 mRNA expression compared to P-gp. Bauer *et al.* (38) recently reported that glucocorticoids such as dexamethasone are able to up-regulate the functional expression of P-gp at the blood-brain barrier. It is possible that the greater ABCG2 mRNA expression and detectable activity in these primary cultures

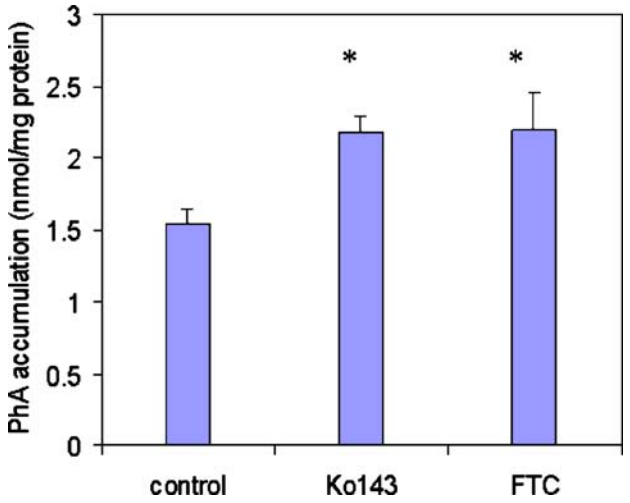

Fig. 14. Specificity of ABCG2 inhibitors in MCF-MX100 monolayer cells. The accumulation of 10 uM pheophorbide A at 3 h by MCF-MX100 cells was measured in the absence (control) or presence of inhibitors for ABCG2 (1 μM Ko143 or 10 μM FTC) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements. *Asterisks* represent data points that are significantly different from control. *$p < 0.05$.

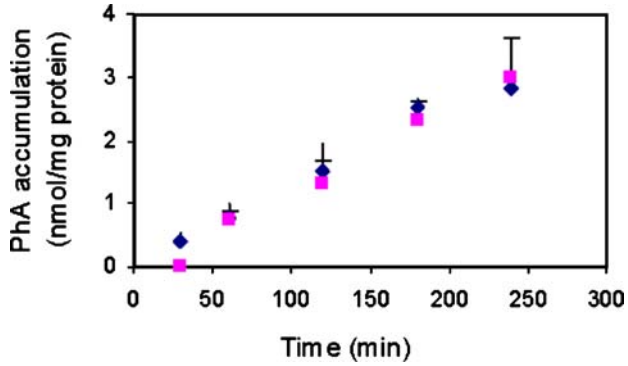

Fig. 15. Time profile of pheophorbide A accumulation by RBE4 monolayer cells. The accumulation of 10 μM pheophorbide A over time by RBE4 cells was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor 1 μM Ko143 (*squares*) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements.

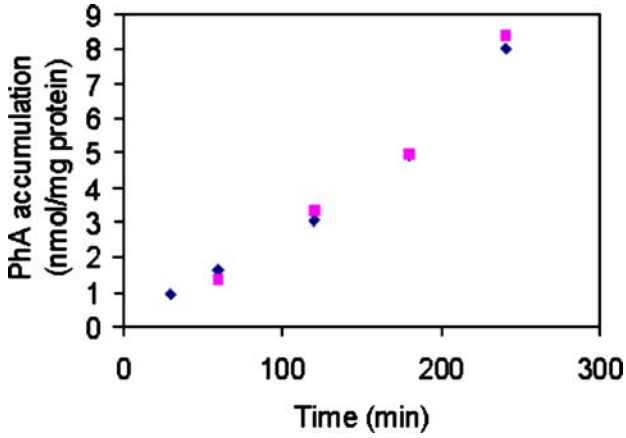

Fig. 17. Time profile of pheophorbide A accumulation by primary cultures of HBEC. The accumulation of 10 μM pheophorbide A over time by HBEC was measured in the absence (control, *diamonds*) or presence of ABCG2 inhibitor 1 μM Ko143 (*squares*) at 37°C. Results are expressed as a combination of two separate experiments with each data point in an individual experiment representing quadruplicate measurements.

may be due to ABCG2 up-regulation in the presence of hydrocortisone and may not reflect the normal physiological state of these cells.

In vivo data from ABCG2 and P-gp knockout mice models suggest that ABCG2 activity is minimal at the mouse blood-brain barrier. Cisternino *et al.* (13) reported that brain microvessels from mdr1a deficient mice expressed 3 times more ABCG2 mRNA than in the microvessels of P-gp wild-type mice. In addition, the accumulation of ABCG2 substrates, mitoxantrone and prazosin, was significantly increased in the presence of GF120918 in both wild-type and P-gp deficient mice but not in the presence of PSC 833. Whether or not this increased expression and activity of ABCG2 is a compensatory mechanism for the lack of mdr1a at the mouse blood-brain barrier remains to be elucidated. Recently, Breedveld *et al.* (14) reported a 2.5-fold increase in the brain penetration of imatinib (an antitumor agent) in Bcrp1 knockout mice and a 3.6-fold increase in P-gp knockout mice compared to controls. This suggests that mouse Bcrp1 limits the brain penetration of imatinib at the blood-brain barrier, but to a lower extent than P-gp. A study by Lee *et al.* (11) demonstrated increased brain accumulation of two ABCG2 substrates, dehydroepiandrosterone sulfate (a sulfoconjugated organic anion) and mitoxantrone, in the presence of GF120918 in mdr1a/1b (−/−) mice. Although *Bcrp1* gene and protein were expressed in mouse brain capillaries, the brain uptake of mitoxantrone and dehydroepiandrosterone sulfate did not differ between Bcrp1 (−/−) and wild-type mice. Furthermore, brain accumulation of dehydroepiandrosterone sulfate was increased in the presence of GF120918 in Bcrp1 (−/−), suggesting the possibility of an unknown GF120918-sensitive transporter at the blood-brain barrier that was not originally detected by

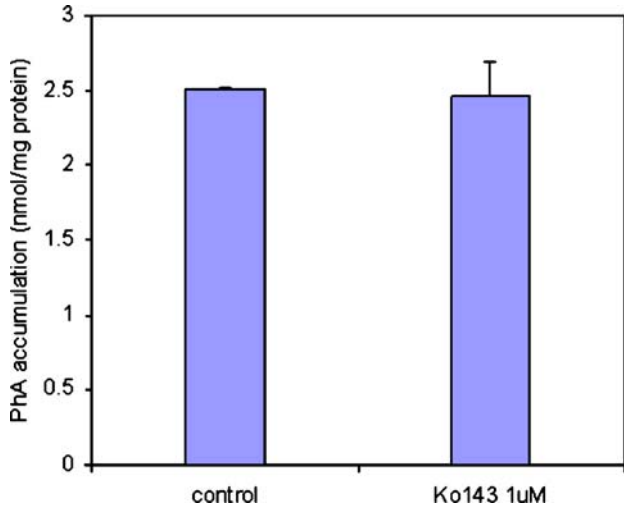

Fig. 16. Specificity of ABCG2 inhibitor, Ko143, in RBE4 monolayer cells. The accumulation of 10 uM pheophorbide A at 3 h by RBE4 cells was measured in the absence (control) or presence of ABCG2 inhibitor (1 μM Ko143) at 37°C. Results are expressed as mean ± SD of three separate experiments with each data point in an individual experiment representing quadruplicate measurements.

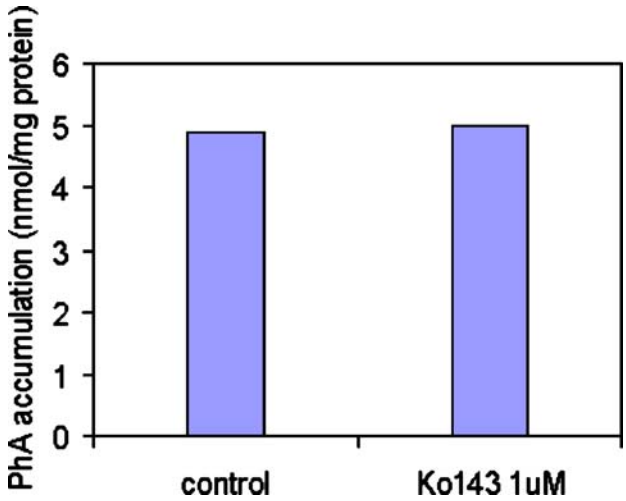

Fig. 18. Specificity of ABCG2 inhibitor, Ko143, in primary cultures of HBEC. The accumulation of 10 uM pheophorbide A at 3 h by HBEC was measured in the absence (control) or presence of ABCG2 inhibitor (1 μM Ko143) at 37°C. Results are expressed as a combination of two separate experiments with each data point in an individual experiment representing quadruplicate measurements.

Cisternino *et al.* (13). In addition, van Herwaarden *et al.* (12) reported that although murine Bcrp1 played a major role in the hepatobiliary and intestinal excretion of the food carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP), no significant increase in brain penetration of PhIP in Bcrp (−/−) mice was observed. These *in vivo* data in mice along with our *in vitro* studies in human and rat brain microvessel endothelial cells suggest that ABCG2 may not play a significant role at the blood-brain barrier, at least in the transport of these substrates.

CONCLUSION

In summary, low ABCG2 protein expression and undetectable activity are observed in human and rat brain microvessel endothelial cell culture systems. In addition, we characterize for the first time, the expression of ABCG2 in brain parenchyma, and report a lack of transport activity in cultured glial cells. It is important to indicate that other membrane efflux transporters, in particular, P-gp, are functionally expressed in the same cell culture systems (20,21,28,29,35,36). Furthermore, low ABCG2 expression and lack of ABCG2 activity in the brain cell culture systems is in agreement with data from recent *in situ* and *in vivo* studies suggesting that ABCG2 may play a minor role in the brain compared to P-gp (39). Interestingly, Aronica *et al.*(33) reported low ABCG2 protein expression in the cortex and white matter of normal human brain tissue and significantly increased protein expression in primary glial tumors from patients with refractory epilepsy. Whether an increase in ABCG2 expression actually correlates with an increase in activity in neuropathological conditions, including drug-resistant epilepsy, requires further investigation.

ACKNOWLEDGMENTS

This work is supported by grants to Reina Bendayan from the Canadian Institutes of Health Research (MOP-56976) and the Ontario HIV Treatment Network (OHTN). Gloria Lee is a recipient of an OHTN Studentship Award. Alexander Prat holds the Donald Paty Career Award from the Multiple Sclerosis Society of Canada as well as "Chercheur Boursier Junior" from the Fonds de la Recherche en Sante du Quebec. Karolina Wosik is a recipient of a fellowship from the Multiple Sclerosis Society of Canada. We would like to thank Dr. J. Henderson for the help and advice with the undertaking of fluorescence microscopy studies and Drs S. Bates and F. Roux for providing the MCF-7 and RBE4 cell culture systems, respectively.

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