Functional Expression of the Multidrug Resistance Protein 1 (MRP1) in Microglia.

Shannon Dallas¹, Xiaoping Zhu², Sylvain Baruchel³, Lyanne Schlichter² and Reina Bendayan¹

¹Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada; ²Division of Cellular and Molecular Biology, Toronto Western Research Institute, University Health Network, and Department of Physiology, University of Toronto, Toronto, Ontario, Canada; ³New Agent and Innovative Therapy Program, Division of Hematology/Oncology, The Hospital for Sick Children, Toronto, Ontario, Canada.
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Corresponding Author: Dr. Reina Bendayan  
Associate Professor  
Department of Pharmaceutical Sciences  
Faculty of Pharmacy, University of Toronto  
19 Russell St.  
Toronto, Ontario, M5S-2S2  
Canada  
Tel: 416-978-6979  
Fax: 416-978-8511  
email: r.bendayan@utoronto.ca

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Abbreviations List: ABC, ATP-binding cassette; bp, base pairs; BSO, L-buthionine-[S,R]-sulfoximine; EBSS, Earle’s balanced salt solution; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; HIV-1, human immunodeficiency virus type 1; MRP, multidrug resistance protein; hMRP1, human multidrug resistant protein 1; rMRP1, rat multidrug resistance protein 1; P-gp, P-glycoprotein, RT-PCR, reverse transcriptase-polymerase chain reaction.

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Abstract

Brain expression of the multidrug resistance proteins (MRP’s), a collection of membrane-associated ATP-dependent efflux transporters, is poorly understood. While several studies have examined the expression of these proteins within the brain barriers (i.e., the blood-brain barrier and choroid plexus), little information is available with respect to brain parenchyma cells such as microglia and astrocytes. Since microglia are the primary brain cells infected by the human immunodeficiency virus type 1 (HIV-1), MRP1 expression within microglia may contribute to lower brain accumulation of anti-HIV drugs. To examine the expression pattern of MRP1 within microglia, we performed reverse transcriptase-polymerase chain reaction analysis and Western blotting on a rat brain microglia cell line MLS-9, and in primary rat microglia cells. Both rMRP1 mRNA and protein were expressed in the cell line, as well as in primary microglia. We then characterized rMRP1-mediated transport properties in MLS-9 cells using [3H]vincristine, a known MRP1 substrate. Vincristine accumulation by monolayers of MLS-9 cells increased significantly in the presence of several well-established MRP1 inhibitors (MK571, genistein, sulfinpyrazone, probenecid and indomethacin), protease inhibitors, or the ATPase inhibitor, sodium azide. In addition, vincristine accumulation was significantly modulated by altering the intracellular concentration of the reduced form of glutathione (GSH), further suggesting the involvement of rMRP1-mediated transport. These results provide strong evidence that the MRP1 protein is both expressed and functional in microglia cells. They also suggest that brain parenchyma can act as a “second” barrier to drug permeability and regulate brain distribution/accumulation of various xenobiotics including protease inhibitors.
Multidrug resistance has long been considered to involve one specific ATP-dependent membrane efflux pump, P-glycoprotein (P-gp). However, over the last decade it has become apparent that cells that do not express P-gp can also be resistant to a variety of structurally unrelated drugs, which suggested the existence of other membrane-bound efflux proteins. This was confirmed in 1992 when Cole and Deeley cloned the first multidrug resistance gene, the product of which is now commonly referred to as the multidrug resistance protein 1, or MRP1. Although P-gp and MRP1 have many functional similarities and are both members of the ATP-binding cassette (ABC) superfamily of proteins, they possess only a 15% amino acid homology (Cole and Deeley, 1998). Nonetheless, expression of either protein is associated with reduced drug accumulation within cells and with the development of drug resistance (Cole and Deeley, 1998).

At present, nine MRP proteins ranging in size from 1325 to 1545 amino acids have been identified in humans (hMRP1-9). Amino-acid comparisons indicate 30-56% homology between the various isozymes. Complementary DNA’s for rat homologs of MRP1-6 have now been characterized (designated rMRP1-6) and exhibit 66-94% homology with their human counterparts. Most mammalian cells appear to express multiple forms of MRP, generally with one predominant isozyme (Borst et al., 1999).

Although MRP1 expression is most often associated with multidrug resistance to cancer chemotherapy agents, antiretroviral agents used in the treatment of human immunodeficiency virus-1 (HIV-1) infection may also interact with these proteins. Studies in MRP1 overexpressing cell lines (e.g. hMRP1-transfected LLC-PK1 kidney cell line; hMRP1 over-expressing CEM lymphocyte cell lines) and isolated capillaries from rat and pig brain indicate that the protease inhibitors saquinavir, ritonavir, nelfinavir and indinavir
can interact with MRP1 (Srinivas et al., 1998; Miller et al., 2000; Jones et al., 2001). Recently, Williams et al. (2002) provided direct evidence that saquinavir is a substrate for MRP1 and MRP2 in MDCKII overexpressing hMRP1 and hMRP2 cell lines, respectively. Furthermore, the ability of MRP1 to limit antiretroviral drug exposure in the clinic has been confirmed in HIV-infected patients using flow cytometry (Meaden et al., 2002). Significantly lower accumulation of saquinavir and ritonavir in peripheral blood mononuclear cells was seen in patients whose cells had greater MRP1 expression (Meaden et al., 2002).

HIV-1 infection within the central nervous system causes a number of neurological and psychiatric disorders in a large proportion of infected patients (Koutsilieri et al., 2001). Symptoms range from mild cognitive impairment to frank AIDS dementia, a progressive neurological disorder characterized by motor and behavioral abnormalities. HIV-1 infection within the brain appears to be limited primarily to microglia, the immune cells of the brain (Bagasra et al., 1996; Epstein, 1998). Microglia act as a cellular reservoir for the virus and upon activation release a number of factors that lead to neurotoxicity (Epstein, 1998). Since suboptimal levels of anti-HIV-1 drugs likely contribute to the emergence of drug-resistant viral strains, effective eradication of the virus from the brain requires sufficient penetration of antiretroviral drugs into microglia. The presence of efflux transporters within the therapeutic target of HIV-1 in the brain (i.e., microglia) may be one mechanism by which antiretroviral resistance emerges in patients.

MRP1 was previously identified in multiple brain compartments of several animal species, including bovine blood-brain barrier endothelial cells, mouse blood-cerebrospinal barrier endothelial cells and rat astrocytes (Declèves et al., 2000; Wijnholds
et al., 2000; Zhang et al., 2000). We previously characterized the cellular/subcellular localization and functional expression of P-gp in microglia and microvessel endothelial cells from rat brain (Lee et al., 2001b; Bendayan et al., 2002). Recently, rMRP1 transcripts were identified in cultured rat microglia cells (Ballerini et al., 2002; Hirrlinger et al., 2002), however levels of rMRP1 protein were not determined and complete functional studies specific to the rMRP1 isoform were not performed. Thus, the objective of the present study was to confirm the expression of the rMRP1 protein and extensively characterize its transport properties in microglia.
Methods and Materials

Chemicals. [3H]vincristine (2.5 Ci/mmol), [3H]digoxin (55 mCi/mmol) and ritonavir were purchased from Moravek Chemicals (Brea, CA). Anti-actin (clone AC-40), L-buthionine-[S,R]-sulfoximine (BSO), digoxin, ethylenediaminetetraacetic acid (EDTA), indomethacin, genistein, probenecid, and sulfinpyrazone were purchased from Sigma-Aldrich (Oakville, ON, Canada). MK571 and indinavir were a kind gift from Merck Research Labs (West Point, PA). Saquinavir was kindly provided from Roche Products Inc. (Hertfordshire, UK). The monoclonal antibodies MRPr1, and M2III-6 were purchased from Kamiya Biomedical (Seattle, WA).

Cell Culture. The microglia cell line (MLS-9) was derived from neopallia of 2- or 3-day old Wistar rat pups, as described previously (Zhou et al., 1998; Cayabyab and Schlichter, 2002). Briefly, microglia were isolated by enzymatic dissociation of tissue, and cultured in endotoxin-free medium (Invitrogen, Burlington, ON, Canada) for 10-12 days without feeding. Essentially pure microglia cultures (>98% as determined by isolectin B4 labeling) obtained from shaking the 10-12 day old flasks were treated with colony stimulating factor-1 to increase proliferation. From these cultures microglia colonies arose after 2-6 wk that continued to grow in the absence of added growth factors. The MLS-9 cell line was established from one of these colonies. The cell line displays morphological and phenotypical attributes characteristic of microglia, including pinocytosis of dyes (DiI-acetylated LDL, or Lucifer Yellow) and staining for several microglia cell markers: isolectin B4 (100% of cells), OX-42 antibody (98%) and ED-1 antibody (99%). The cells were negative for characteristic markers of astrocytes (glial fibrillary acidic protein) and fibroblasts (fibronectin) (Zhou et al., 1998).
The MLS-9 cells (passages 4-32) were grown as monolayers on 75 cm² polystyrene tissue culture flasks or multiwell dishes (Sarstedt, St.-Leonard, QC, Canada) at 37°C, in 5% CO₂, 95% air and 95% humidity. Cells were grown to confluency (4-6 d following seeding) in Minimum Essential Medium (MEM; pH 7.2) containing 2 mM L-glutamine, 5.55 mM D-glucose, 5% fetal bovine serum, 5% horse serum and 0.5% penicillin/streptomycin. For passaging, subconfluent MLS-9 cells (~75% confluent) were released from the flask with a 15 mM sodium citrate solution containing 130 mM NaCl, 10 mM D-glucose and 10 mM HEPES, pH 7.4.

The human MRP1 overexpressing cell line (WT-MRP1) and parent cell line (VF-HeLa) were kindly provided by Dr. S.P.C. Cole (Kingston, ON, Canada). The WT-MRP1 is a HeLa cell line, which is stably transfected with a pRc/CMV vector containing the complete coding sequence of hMRP1. The cells were maintained in Dulbecco’s Modified Eagle Medium containing 4 mM L-glutamine, 25 mM glucose, 400 µg/ml G418 with 10% fetal bovine serum, (pH 7.1) and subcultured weekly using 0.25% trypsin.

A human P-gp overexpressing cell line (CH²C5), originally derived from a colchicine-resistant Chinese hamster ovary cell line, was a kind gift of Dr. V. Ling (Vancouver, BC, Canada). The cells were maintained in Minimum Essential Medium-alpha containing 2 mM L-glutamine, 5.55 mM D-glucose, 10% fetal bovine serum, with 0.5% penicillin/streptomycin (pH 7.2) and subcultured weekly using 0.25% trypsin. We have previously confirmed through Western blotting analysis overexpression of P-gp in this cell line, compared to MLS-9 (Lee et al., 2001b).

**RT-PCR Analysis.** RT-PCR was performed as previously described by our group (Lee et al., 2001b). Total RNA from MLS-9 cells was isolated using TRIZOL reagent.
We used a cDNA reaction mixture containing 0.5 µg of pd(N)₆ and 1 µg of RNA, 50 mM Tris-HCl buffer, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM d(NTP)s and 200 units of superscript II reverse transcriptase. Then 2 µl of cDNA was amplified by PCR using a GeneAmp PCR 2400 system (PerkinElmer, Mississauga, ON, Canada) in 20 µl of reaction mixture containing 1 Unit Platinum Taq polymerase, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM d(NTP)s, and 0.8 µM forward and reverse primers. The sequences of rMRP1 primers were: forward primer 5’-AGAAGCAGCGTGTGAGCCT-3’ and reverse primer 5’-GCCAATAGTTAGAAGCCAGTGC-3’. The RT-PCR program consisted of 35 cycles of 3 phases: denaturing (20 sec at 94°C), annealing (30 sec at 60°C), and extension (30 sec at 72°C). Amplified DNA products were resolved on a 1.5% agarose gel with 0.5 mg/ml ethidium bromide. The RT-PCR product was purified and sequenced (ACGT Corp., Toronto, ON, Canada). All reagents were from Invitrogen (Burlington, ON, Canada).

**Western Blotting.** Crude membrane proteins were obtained as follows: primary microglia, MLS-9 and WT-MRP1 cells were harvested by centrifugation (400xg), the pellet collected and cells lysed for 30 min at 4°C in a 250 mM sucrose buffer containing 1 mM EDTA and protease inhibitor cocktail (P8340; Sigma-Aldrich, Mississauga, ON, Canada). Adult rat kidney tissue was cut into small pieces and washed several times in PBS and homogenized using a polytron homogenizer (Kinematica, Switzerland). The resulting lysate was centrifuged at 400xg to remove any blood cells and debris and then incubated on ice with lysis buffer (as above) for 30 min. Lysates from cells and tissues were then homogenized in a Dounce homogenizer at 10,000 rpm for three cycles of 20 sec each. Homogenates were centrifuged at 3000xg to remove cellular debris, and the supernatant was further centrifuged at 100,000xg for 1 hr at 4°C. The resulting
membrane pellet was resuspended in a 10 mM Tris-HCl suspension buffer (pH 8.8) and frozen at –20°C until use.

Crude membrane samples (12.5-200 µg) were separated on a 10% polyacrylamide gel using a Bio-rad minigel system (Bio-Rad, Hercules, CA), and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. Protein transfer was verified using 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% Tween-20 (TBS-T) and 5% dry skim milk powder. Following three washes (5 min each) with TBS-T, the membranes were incubated at room temperature with either the monoclonal MRP1 antibody MRPr1 at a dilution of 1:1000 in 5% milk in TBS-T for 4hr for MRP1 detection, or the actin antibody AC-40 (1:500; 5% milk) for actin detection. Following a second wash, the membranes were then incubated for 2hr in the presence of either anti-mouse (1:5000) or anti-rat (1:5000) horseradish peroxidase-linked secondary antibodies (Sigma-Aldrich, Mississauga, ON, Canada) in 5% milk in TBS-T. Proteins were visualized using enhanced chemiluminescence according to the manufacturer’s instructions (Pierce, Rockford, IL). Densiometric analysis was performed on the immunoreactive bands using KODAK ID image analysis software version 2.0.1.

Expression of MRP2 was determined in a similar manner using the M2III-6 (1:2000) antibody and an anti-mouse horseradish peroxidase-linked secondary antibody (1:5000).

[^3H]Vincristine and[^3H]Digoxin Transport Studies. MLS-9, WT-MRP1, VF-HeLa and CHRC5 cells were plated onto multiwell plates and uptake/accumulation of radiolabeled vincristine (30 nM) or digoxin (100 nM) was measured as described previously by our laboratory (Lee et al., 2001b). Briefly, cells were washed once and pre-conditioned for 30 min at 37°C with Earle’s Balanced Salt Solution (EBSS),
containing 1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄, 138 mM NaCl, 1.0 mM Na₂HPO₄, 5.5 mM D-glucose and 20 mM HEPES (pH 7.4). Cells were then incubated for the desired time with transport medium containing [³H]vincristine (30 nM) or digoxin (100 nM) with or without an inhibitor. At completion of the uptake/accumulation period, the transport medium was removed by aspiration and uptake was terminated by adding ice-cold 0.16 M NaCl. Cells were solubilized for 30 min with 1 M NaOH and then transferred to scintillation vials containing of 2N HCl and scintillation cocktail (Beckman Coulter; Fuller, CA). Cellular incorporation of the radiolabeled probe was measured using liquid scintillation counting. The sample counts were corrected for “zero time” uptake in each individual experiment and data were standardized to the amount of cell protein (mg/ml) in each culture plate determined by the Bradford colorimetric method (Bio-rad, Mississauga, ON) with bovine serum albumin (Sigma-Aldrich, Mississauga, ON) as the standard.

Efflux of [³H]vincristine was assessed as described previously, but with a few modifications (Lee et al., 2001b). Following pre-incubation with EBSS-transport buffer, cells were incubated with 30nM [³H]vincristine for 1 hr. Following this initial incubation, the reaction was stopped by addition of 1 ml of ice cold 0.16 M NaCl. The amount of intracellular vincristine accumulated at this time was designated as the “initial value” at time zero. Cells were then incubated for the specified time using either transport buffer, or transport buffer with added inhibitor. At the appropriate time, the reaction was stopped with ice cold 0.16 M NaCl and the cells were solubilized. Intracellular radioactivity remaining in the cells was quantified as described above.

The energetics of rMRP1-mediated transport of vincristine were investigated in MLS-9 cells using the ATPase inhibitor, sodium azide. Accumulation studies were carried out
as described previously, with a few modifications. The MLS-9 cells were pre-incubated in either EBSS containing 5.5 mM D-glucose or EBSS containing equimolar amounts of mannitol. Accumulation of [³H]vincristine was then assessed in EBSS (control cells) or in transport buffer containing 5.5 mM mannitol and 10 mM sodium azide.

The modulatory effects of the reduced form of glutathione (GSH) on vincristine or digoxin transport were studied by either depleting or augmenting intracellular GSH concentrations. GSH was depleted using the GSH-synthesis inhibitor, BSO, which is a standard method for depleting cellular GSH (Anderson, 1998). Cells were incubated with 25 µM BSO for 24 hr at 37°C, 5% CO₂/95% air and 95% humidity. Accumulation studies were then conducted as described above.

Several approaches have been used in whole cell transport studies to increase intracellular GSH concentrations, including using GSH pro-drugs (i.e., N-acetylcysteine, GSH ethylester) or GSH precursors, such as cysteine or methionine (Anderson, 1998). Since the extent of de novo synthesis of GSH in MLS-9 cells is not known, direct use of GSH precursors was not considered. Furthermore, in macrophages, cells that are derived from the same precursors as microglia, adding GSH itself increases intracellular GSH slightly more than does GSH ethyl ester (Minhas and Thornalley, 1995), possibly because GSH is imported intact through a bi-directional GSH transporter. Therefore, for transport experiments involving augmentation of GSH, MLS-9 cells were incubated with EBSS medium containing [³H]vincristine (30 nM) and 5 mM GSH.

**HPLC Analysis.** Intracellular concentrations of GSH following GSH depletion (BSO-treated) or augmentation (GSH-treated) were determined using a slightly modified HPLC method previously described and validated by our group (Stempak et al., 2001). MLS-9 and CH²C5 cells were cultured to ~90% confluency in 75 cm² tissue culture
flasks as described above. Following a 24 hr incubation with either fresh medium (control) or medium containing 25 µM BSO (GSH depletion), cells were washed and incubated with EBSS transport buffer. Similarly, augmentation of intracellular GSH was determined in MLS-9 in the presence and absence of 5 mM GSH. At the conclusion of the incubation periods, cells were washed twice with Hanks Balanced Salt Solution (Invitrogen, Carlsbad, CA) and removed from the flask surface using 15 mM sodium citrate. Cells were counted using a hemocytometer, solubilized with 15% perchloric acid containing 2 mM EDTA and immediately assayed by HPLC.

Intracellular GSH was measured using a Shimatzu HPLC system that consisted of a SIL-6B autoinjector, LCAT pump, and SCL-6B system controller (Mantech, Guelph, ON, Canada). Isocratic elution was performed on a Prodigy C-18 ODS column (5 µm particles; 150 x 4.6 mm) using a mobile phase of 10 mM NaH₂PO₄•H₂O, 0.03 mM octane sulfonic acid and 2% acetonitrile (pH 2.7), at a flow rate of 1 ml/min. The detector was a porous graphite, dual-electrode electrochemical detector (model 5010; ESA, Bedford, MA), which was connected to a porous graphite guard cell (ESA model 5020). The potentials applied to the guard cell, E1 and E2 electrodes were +850, 300, and 800 mV, respectively. The R2 sensitivity (gain range) for E2 was set at 50-100 µA. GSH peak height values were corrected per 1 million cells and BSO- or GSH-treated cells were compared to control cells and expressed as a percent value. The intraday and interday variability of the assay is less than 10% (Stempak et al., 2001).

**Data Analysis.** [³H]Vincristine and [³H]digoxin accumulation and efflux values are expressed as pmol/mg protein and are presented as mean ± S.D. from a minimum of 3 separate experiments. In an individual experiment, each data point represents quadruplicate trials. Intracellular GSH values, as determined by HPLC, are expressed as
mean percent control ± S.D., based on comparison of GSH peak heights/10^6 cells for three separate experiments. Statistical analysis for unpaired experimental data was carried out using the unpaired Student’s t test. For multiple comparisons, the test of repeated measures analysis of variance (ANOVA) and/or the post hoc multiple-comparison Bonferroni t test was used. A value of p<0.05 was considered statistically significant.
Results

MRP1 mRNA and Protein Expression

RT-PCR analysis was used to assess the expression of rMRP1 mRNA in MLS-9 cells (Fig. 1A). Based on the existing partial sequence of rMRP1 available at the time, we designed RT-PCR primers to amplify a region that is conserved across mice, rats and humans. Subsequently, the presumed full-length sequence of rMRP1 was published (Yang et al., 2002), showing that the primers we used are in the middle of the rat gene. hMRP1 has an additional ~400 bp at the C-terminus. These primers worked well for RT-PCR of the MLS-9 cell line and primary cultured microglia, the cells from which the MLS-9 cell line was derived. They amplified a single band of the expected size (669 bp) in both cell types (Lanes 4 and 5), which were confirmed to be rMRP1 by dideoxy sequencing.

Protein expression of rMRP1 and hMRP1 were detected using Western blotting (Fig. 1B). The monoclonal antibody, MRPr1, which was raised against a bacterial fusion protein (aa 238-247) of hMRP1, recognized a band of ~190 kDa in primary rat microglia (Lane 1) and MLS-9 cells (Lane 2). This antibody does not cross react with P-gp or MRP isoforms 2-6 (Hipfner et al., 1998). The same single band was also detected in two positive control samples: WT-MRP1, a HeLa cell line that was stably transfected with hMRP1 (Lane 3) and rat kidney (Lane 4). Using the same amount of crude membrane protein in each lane (25 µg), primary rat microglia produced a stronger rMRP1 band than MLS-9 cells. It is possible that rMRP1 is down regulated in the cell line (see Discussion). A single band of ~43 kDa corresponding to actin was detected in each lane, which verified that an appropriate amount of protein was loaded for each sample.
Functional Studies

The chemotherapeutic agent vincristine has been used widely to characterize MRP1-mediated transport in vitro (Loe et al., 1996). Vincristine is, however, a substrate of several ATP-dependent efflux transporters including MRP1, MRP2 and P-gp (Seetharaman et al., 1998; Borst et al., 1999). As shown in Figure 1C, we confirmed that rMRP2 is not expressed in MLS-9 (Lane 2) applying Western blotting analysis. Using the MRP2 specific antibody M2III-6, a ~190 kDa band was detected in rat liver (Lane 1), a tissue well known for high MRP2 expression (Borst et al., 1999). This antibody recognizes a 202 amino acid epitope in the C-terminus of hMRP2 and rMRP2 proteins and does not cross react with P-gp, MRP1, MRP3 or MRP5 (Scheffer et al., 2000). Despite loading a large amount of protein (200 µg), no rMRP2 band was observed in MLS-9 cells.

Given that the MRP1-mediated transport properties of vincristine have been examined extensively, and several properties (i.e. GSH-dependence; use of MRP specific inhibitors) can distinguish P-gp from MRP1-mediated effects, we determined transport properties of radiolabeled vincristine as one measure of rMRP1 functional expression in MLS-9. Furthermore, we verified the specificity of the inhibitors and glutathione modulation by measuring digoxin transport (a “reference” P-gp substrate) in a P-gp overexpressing cell line.

Accumulation and Efflux. A time-dependent increase in vincristine accumulation by MLS-9 cell monolayers was observed at 37°C, with a plateau beginning to be reached by 4hr (Fig. 2). Vincristine accumulation was significantly increased (i.e., 64% at 2 hr) in the presence of the leukotriene D4 antagonist, MK571, a potent inhibitor of MRP1. Increasing concentrations of MK571 over a 150-fold concentration range (0.5–75 µM)
produced a dose-dependent increase in vincristine accumulation at 2 hr (Fig. 3), and a plateau was observed at 50 µM MK571.

Since MRP1 is an efflux transporter, time-dependent vincristine efflux was directly examined in the presence or absence of MK571 (Fig. 4). Following one hr pre-loading with vincristine, a time-dependent decrease was observed in the amount of vincristine remaining in the cells. Adding 5 µM MK571 at the start of the efflux studies significantly increased the intracellular drug concentration after 2 hr \((p<0.001)\), suggesting that decrease in vincristine efflux in MLS-9 in the presence of the inhibitor was most likely mediated by rMRP1.

**Energetics.** The energetic properties of vincristine transport by MRP1 are well characterized (Versantvoort et al., 1995; Loe et al., 1996). To confirm that rMRP1-mediated transport of vincristine was ATP-dependent, effects of the ATPase inhibitor, sodium azide were assessed in MLS-9 cells (Fig. 5). In the presence of 10 mM sodium azide, accumulation of vincristine was increased significantly \((p<0.001)\) at three time points: 30, 45 and 60 min.

Efficient MRP1-mediated efflux of vincristine across cell membranes also requires physiological concentrations (1–5 mM) of intracellular GSH (Loe et al., 1996). That is, lowering the intracellular GSH concentration decreases vincristine efflux, whereas increasing GSH increases drug efflux. Since this property is specific to MRP1-mediated transport, modulation by GSH can discriminate between transport of vincristine by P-gp and MRP1. Following depletion of MLS-9 intracellular GSH using BSO, accumulation of vincristine was significantly increased at several time points, e.g. by 52% at 2hr (Fig 6A). Conversely, adding 5 mM exogenous GSH significantly \((p<0.001)\) decreased vincristine accumulation in MLS-9 at 2 hr by 44%. No significant alteration in the
accumulation of digoxin, an established P-gp substrate, was observed in the P-gp overexpressing cell line CH⁰C⁵ following GSH depletion (Fig. 6B). To confirm that altered vincristine accumulation in BSO- or GSH-treated cells was accompanied by parallel changes in intracellular GSH levels, GSH was measured by HPLC analysis. Following 24-hr incubation with 25 µM BSO, the GSH concentration in MLS-9 and CH⁰C⁵ cells was decreased by > 94%. Conversely, addition of 5 mM exogenous GSH in MLS-9 cells increased GSH by 30% within the first 10 minutes of the incubation period.

**Specificity.** Several other well-established MRP1 inhibitors were tested for their effect on vincristine transport by MLS-9 cells (Fig. 7A). The flavanoid genistein (200 µM), the non-steroidal anti-inflammatory drug, indomethacin (50 µM) and the antihyperuricemic agents, probenecid (100 µM) and sulfinpyrazone (2 mM), significantly increased vincristine accumulation at 2 hr from the control value (2.2 ± 0.2 pmol/mg protein) to 3.9 ± 0.1, 3.07 ± 0.3, 3.1 ± 0.3 and 3.2 ± 0.5 pmol/mg protein, respectively. The inhibitors used also significantly enhanced vincristine accumulation in the hMRP1 overexpressing cell line WT-MRP1 by up to 355% as compared to control (Fig. 7B), while no effect was observed in VF-Hela, the hMRP1 deficient parental cell line (data not shown). In addition, the inhibitors did not cross-react with P-gp when their effect was evaluated on digoxin accumulation by CH⁰C⁵ cells (Fig. 8).

The antiretroviral protease inhibitor drugs indinavir, ritonavir, saquinavir, are known to interact with MRP1 (Srinivas et al., 1998; Miller et al., 2000; Jones et al., 2001; Williams et al., 2002). In this study, indinavir (50 µM), ritonavir (10 µM) and saquinavir (10 µM) increased vincristine accumulation by up to 76 and 400 % in MLS-9 (Fig. 9A) and in the hMRP1 overexpressing cell line WT-MRP1 (Fig. 9B), respectively.
Discussion

The MRP family of efflux transport proteins is known to confer multidrug resistance to a variety of structurally unrelated compounds (Borst et al., 1999). The expression of these proteins in the brain has recently been examined within the blood-brain and blood-cerebrospinal fluid barriers (Wijnholds et al., 2000; Zhang et al., 2000). Multiple MRP homologs are expressed within these barriers and appear to contribute to the low brain penetration of a variety of therapeutic compounds, including antiretroviral drugs used in the treatment of HIV-1 (Miller et al., 2000). The primary cells infected by HIV-1 in the brain are microglia (brain macrophages) (Bagasra et al., 1996; Epstein, 1998) but little is known about expression of MRP-family members in microglia. The presence of rMRP1 transcripts in cultured rat microglia was recently reported (Ballerini et al., 2002; Hirrlinger et al., 2002), however protein levels and functional activity of rMRP1 were not extensively examined. The present study investigates the expression of rMRP1 in a rat microglia cell line (MLS-9) at both the protein and functional levels.

We had previously found rMRP1 transcripts in primary rat microglia, but did not detect it in MLS-9 cells using primers that recognize a region in the 3’ end of the rat gene (Lee et al., 2001b). In the present study, we amplified a region in the middle of the gene from primary microglia and from MLS-9 cells, and obtained a single band for each cell type. Each product was the expected size, and sequencing confirmed they were both rMRP1. Western blotting using the monoclonal antibody, MRPr1, confirmed the expression of rMRP1 protein in primary rat microglia and the MLS-9 microglia cell line. This antibody recognizes hMRP1 and rMRP1 (Rao et al., 1999), and does not cross react with MRP2-6 (Hipfner et al., 1998). A more robust rMRP1 band was detected in primary rat microglia than in MLS-9 cells, possibly due to differences in protein processing or
down regulation of rMRP1 in the cell line. A similar discrepancy in rMRP1 expression between primary cells and their immortalized counterparts has been reported, with less rMRP1 protein levels in an immortalized rat brain endothelial cell line (RBE4) than in primary cultures of rat brain endothelial cells (Regina et al., 1998).

To characterize rMRP1 function in MLS-9 cells, we investigated the transport properties of vincristine, a well-established MRP1 substrate (Loe et al., 1996; Renes et al., 1999). However, vincristine is also a substrate for P-gp and MRP2. Lack of rMRP2 expression in MLS-9 was confirmed by Western blotting using M2III6, an MRP2 specific antibody that does not cross-react with either MRP1, MRP3, MRP5 or P-gp (Scheffer et al., 2000). However, since MLS-9 cells do express both rMRP1 and P-gp, it was important to characterize separately the transport mediated by P-gp versus rMRP1 (Lee et al., 2001b). To do this, we used inhibitors specific to the MRP family and studied a selective modulator of MRP1-transport energetics. Several potent MRP1 inhibitors have been identified, including MK571, a leukotriene D4 receptor antagonist that competitively inhibits the transport of various MRP1 substrates ($K_i = 0.6 \mu M$) including leukotriene C4 and glutathione disulphide (Leier et al., 1996). Accumulation of vincristine by MLS-9 cells was significantly increased, and its efflux reduced by 5 $\mu M$ MK571. Four other well-established MRP1 inhibitors (indomethacin, genistein, probenecid, sulfinpyrazone) also significantly increased the accumulation of vincristine by 42% or greater in MLS-9 cells. These results agree with recent findings by Ballerini et al. (2002) where 2 mM sulfinpyrazone and 1 mM probenecid increased the accumulation of 10 nM vincristine by greater than 40% in primary cultures of microglia. Genistein (400 $\mu M$) was the strongest inhibitor of vincristine transport (10 nM) in primary cultures of brain endothelial cells and an immortalized cell line derived from
these cells (RBE4), followed by 2 mM sulfinpyrazone and 1 mM probenecid (Regina et al., 1998). A similar pattern of inhibition emerged in MLS-9. In WT-MRP1, a hMRP1 transfected cell line that expresses much higher levels of MRP1 than MLS-9, these same inhibitors significantly increased vincristine accumulation up to 355%.

To verify the specificity of the inhibitors for MRP1 we examined the ability of the compounds to alter the accumulation of digoxin in vitro. Digoxin is a prototypical P-gp substrate that has been shown to interact with the transporter at both the in vitro and in vivo level and is often used as a “reference” compound for P-gp transport studies (Fromm et al., 1999; Stephens et al., 2002). At the concentrations used in the present study, none of the MRP1 inhibitors tested significantly altered the transport of digoxin in a P-gp overexpressing cell line (CHRC5). It is therefore unlikely that these inhibitors modulate P-gp-mediated vincristine transport in MLS-9.

Although P-gp and MRP1 have several functional similarities, the effect of GSH on MRP1 transport remains an identifiable difference between the two proteins. Efficient vincristine transport by MRP1 requires physiological intracellular concentrations (1-5 mM) of GSH (Loe et al., 1996). It is well established that vincristine and GSH are cotransported by MRP1 (Loe et al., 1998; Zaman et al., 1995). While the exact nature of this co-transport process remains unresolved, proposed models include bipartite and multipartite binding, since neither GSH nor vincristine can be transported alone (Leslie et al., 2001). Therefore, we depleted intracellular GSH concentrations in MLS-9 cells using BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase, the first cellular enzyme used in the biosynthesis of GSH. Overnight incubation of MLS-9 cells with 25 µM BSO decreased intracellular GSH levels by >94%, as determined by HPLC analysis. This was a greater decrease than previously reported for MRP1-over-expressing cells treated in a
similar manner, where intracellular GSH was depleted by 80-87% (Feller et al., 1995; Zaman et al., 1995). We used a highly sensitive and specific HPLC assay coupled to electrochemical detection, which we have recently shown simultaneously detects GSH and other non-protein thiols (Stempak et al., 2001). In BSO-treated MLS-9 cells, vincristine accumulation consistently increased at several time intervals, suggesting that GSH-dependent drug efflux was decreased. However, BSO did not alter digoxin accumulation by CH\textsuperscript{R}C5 cells, further confirming the specificity of GSH-dependence for MRP1.

Adding 5 mM GSH to the MLS-9 cells increased intracellular GSH by 30%, and decreased vincristine accumulation by 44%, presumably due to faster efflux. These results concur with studies by Loe et al. (1996) and Renes et al. (1999) where increased vincristine accumulation in the presence of 5 mM GSH was observed in membrane vesicles from hMRP1-transfected and hMRP1-overexpressing cells. Taken together, our results demonstrate functional activity of rMRP1 in MLS-9 cells.

Microglia cells are the primary targets of HIV-1 infection \textit{in vivo} (Epstein, 1998). We were therefore interested in examining the ability of these cells to interact with a number of antiretroviral drugs (i.e. protease inhibitors). Numerous \textit{in vitro} studies suggest that protease inhibitors interact with MRP1 (Srinivas et al., 1998; Jones et al., 2001). In hMRP1 overexpressing lymphocytes, protease inhibitors significantly increase the efflux of calcein, a known MRP1 substrate, at concentrations of 50 µM (Srinivas et al., 1998). Recently, direct evidence that saquinavir is a substrate for MRP1 has been shown in MDCKII overexpressing hMRP1 cells (Williams et al., 2002). In the present study the protease inhibitors, saquinavir, indinavir and ritonavir significantly increased the accumulation of vincristine in both the microglia cell line, and hMRP1
overexpressing cells. However, the concentration of indinavir required to achieve similar levels of vincristine inhibition was 5 times that of saquinavir and ritonavir. The ability of these three compounds to also alter P-gp mediated transport (Srinivas et al., 1998; Jones et al., 2001; Lee et al., 2001b), precludes any comparison of the relative contribution of rMRP1 versus P-gp-mediated transport of vincristine in MLS-9 cells. Future studies examining the direct transport properties of protease inhibitors in cultured microglia should help us understand the relative contribution of P-gp versus MRP1 in this brain compartment.

In summary, we have shown that rMRP1, which is expressed in cultured microglia, is functionally active in a cell line derived from these microglia. Combined with our previous results showing functional expression of P-gp in this cell line (Lee et al., 2001b), our present data show that microglia express multiple subfamilies of ABC transporters. The novel expression of drug-efflux transporters in the brain parenchyma warrants a reconsideration of brain barriers and drug transport. It appears that membranes of microglia can act as a second “barrier” to drug permeability (Lee et al., 2001a). Since microglia are the primary targets of HIV-1 infection in the brain, the expression of transporters in these cells may play a significant role in the antiretroviral drug response and in the development of drug resistance.
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Reprint Requests: Dr. Reina Bendayan
Associate Professor
Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Toronto
19 Russell St.
Toronto, Ontario, M5S-2S2
Canada
Tel: 416-978-6979
Fax: 416-978-8511
e-mail: r.bendayan@utoronto.ca
Figure Legends

**Fig.1.** Expression of rMRP1 in MLS-9 cells.  **A.** RT-PCR analysis of rMRP1.  *Lane 1,* negative control (no cDNA); *lane 2,* negative control (no RNA), *Lane 3,* MLS-9 microglia cell line; *lane 4,* primary microglia; *lane 5,* size marker (1 kb Plus DNA ladder, Invitrogen). **B.** Western Blot analysis of MRP1 in crude membrane fractions were prepared from cell lysates and tissue samples and then were separated on a 10% polyacrylamide gel containing 0.1% SDS. Proteins were transferred to PVDF membrane electrophoretically. The blot was incubated with MRP1 monoclonal antibody, MRPr1 (1:1000) and an anti-actin antibody (1:500).  *Lane 1,* 25 µg primary rat microglia; *Lane 2,* 25 µg MLS-9 microglia cell line; *Lane 3,* 12.5 µg hMRP1-transfected HeLa cells (WT-MRP1); *Lane 4,* 12.5 µg adult rat kidney.  **C.** Western blot analysis of rMRP2 in crude membrane fractions were prepared in a similar manner as MRP1. The blot was incubated with MRP2 monoclonal antibody M2III-6 (1:2000) and an anti-actin antibody (1:4000).  *Lane 1,* 200 µg rat liver; *Lane 2,* 200 µg MLS-9 microglia cell line.

**Fig.2.** Effect of MK571 on vincristine accumulation by MLS-9 cells.  Vincristine accumulation (30 nM) was measured at 37°C in the presence of the MRP1 inhibitor MK571 (5 µM) at 0.25, 0.5, 1, 2, 3, and 4 hr.  The results are expressed as mean ± S.D. of 3 separate experiments, with each data point in an individual experiment representing quadruplicate measurements.  ***$p<0.001,$ significantly different from control.

**Fig.3.** Dose-dependent effect of MK571 on vincristine accumulation by MLS-9 cells.  Vincristine accumulation (30 nM) was measured at 2 hr (37°C) in the presence of increasing concentrations of MK571 (0.5-75 µM).  The results are expressed as mean ± S.D. of 5 separate experiments, with each data point in an individual experiment representing quadruplicate measurements.  ***$p<0.001,$ significantly different from control.

**Fig.4.** Effect of MK571 on vincristine efflux by MLS-9 cells.  Vincristine efflux (30 nM) by MLS-9 was measured at 37°C in the presence of MK571 (5 µM) at 0.5, 1 and 2 hr.  Amount of radioactivity remaining intracellularly was measured and is expressed as percent of initial value (i.e. at time zero).  The results are expressed as mean ± S.D. of 3 separate experiments with each data point in an individual experiment representing quadruplicate measurements.  *$p<0.05,$ ***$p<0.001,$ significantly different from control.

**Fig.5.** Effect of sodium azide on vincristine accumulation by MLS-9 cells.  Vincristine accumulation (30 nM) was measured in the presence of the metabolic inhibitor sodium azide (10 mM) at 0.5, 0.75 and 1 hr.  The results are expressed as mean ± S.D. of 3 separate experiments, with each data point in an individual experiment representing quadruplicate measurements.  ***$p<0.001,$ significantly different from control.

**Fig.6.** Glutathione modulation of vincristine and digoxin accumulation by MLS-9 or CH5C5 cells.  **A.** Vincristine accumulation (30 nM) was measured following depletion of intracellular glutathione with 25 µM BSO (24 hr) at 0.5, 1 and 2hr at 37°C.  **B.** Accumulation of digoxin (100nM) was measured at 0.5, 1, and 2 hr in the P-gp overexpressing cell line CH5C5 following intracellular glutathione depletion using 25
µM BSO (24 hr). Results are expressed as mean ± S.D. of 3-5 separate experiments, with each data point in an individual experiment representing quadruplicate measurements. ***p<0.001, significantly different from control.

**Fig.7.** Effect of MRP1 inhibitors on vincristine accumulation by MLS-9 and WT-MRP1 cell lines.  
A. Accumulation of vincristine (30 nM) in MLS-9 at 2 hr was measured in the presence of 5 µM MK571, 200 µM genistein (GEN), 2 mM sulfinpyrazone (SULF), 50 µM indomethacin (INDO) or 100 µM probenecid (PROB) at 37°C.  
B. Accumulation of vincristine (30 nM) in WT-MRP1 at 2 hr was measured in the presence of 5 µM MK571, 200 µM genistein (GEN), 2 mM sulfinpyrazone (SULF), 50 µM indomethacin (INDO) or 100 µM probenecid (PROB) at 37°C. The results are expressed as mean ± S.D. of 5 separate experiments, with each data point in an individual experiment representing quadruplicate measurements. *** p<0.001, significantly different from control.

**Fig.8.** Effect of MRP1 inhibitors on Digoxin accumulation by CHRC5 cells.  
Accumulation of digoxin (100nM) in the presence of 5 µM MK571, 200 µM genistein (GEN), 2 mM sulfinpyrazone (SULF), 50 µM indomethacin (INDO) or 100 µM probenecid (PROB) at 1hr (37°C). The results are expressed as mean ± S.D. of 3 separate experiments, with each data point in an individual experiment representing quadruplicate measurements.

**Fig.9.** Effect of protease inhibitors on vincristine accumulation by MLS-9 and WT-MRP1 cell lines.  
A. Accumulation of vincristine (30 nM) in MLS-9 at 2 hr was determined at 37°C in the presence of 10 µM saquinavir (SAQ), 10 µM ritonavir (RIT), or 50 µM indinavir (IND).  
B. Accumulation of vincristine (30 nM) in WT-MRP1 at 2 hr was determined at 37°C in the presence of 10 µM saquinavir (SAQ), 10 µM ritonavir (RIT), or 50 µM indinavir (IND). The results are expressed as mean ± S.D. of 3 separate experiments, with each data point in an individual experiment representing quadruplicate measurements. *** p<0.001, significantly different from control.