

Functional Expression of P-glycoprotein in Rat Brain Microglia

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ABSTRACT

In the central nervous system, the primary targets of the human immunodeficiency virus-1 (HIV-1) are microglia, resulting in a disorder called HIV-1 dementia. P-glycoprotein (P-gp), a membrane-associated ATP-dependent efflux transporter, limits entry into the brain of numerous xenobiotics, including anti-HIV drugs (i.e., protease inhibitors). This project investigates the functional expression of P-gp in the endogenous immune cells of the brain, a parenchymal compartment not previously studied. We used a cell line (MLS-9) derived from rat microglia to study the transport of digoxin, a known P-gp substrate. Reverse transcriptase-polymerase chain reaction analysis detected mRNA for only *mdr1b* in MLS-9 cells, whereas both *mdr1a* and *mdr1b* mRNA were expressed in primary cultured microglia from which they were derived. Western blot analysis

with the C219 antibody detected a single band at ~170 to 180 kDa in MLS-9 cells, which is the size previously reported for P-gp. Immunocytochemical analysis with the monoclonal antibodies C219, MRK16, and MAB-448 labeled P-gp protein along the plasma membrane and nuclear envelope of MLS-9 cells. [³H]Digoxin accumulation by monolayers of MLS-9 cells was significantly enhanced in the presence of any of several P-gp inhibitors (verapamil, cyclosporin A, quinidine, PSC 833), protease inhibitors (i.e., saquinavir, indinavir, and ritonavir), and sodium azide, an ATPase inhibitor. These results provide the first evidence for the functional expression of P-gp in microglia and imply that entry of pharmacological agents, including protease inhibitors, may be prevented within the brain parenchyma, as well as at the blood-brain barrier.

Human immunodeficiency virus-1 (HIV-1) infection of the brain results in a disorder known as HIV-1 dementia in adults and HIV-1 encephalopathy in children (Price et al., 1988). This disorder is characterized by psychomotor slowing, impaired memory, mutism, and paraplegia, among other symptoms that eventually lead to disabling cognitive and motor dysfunction (Navia et al., 1986). It is estimated that approximately 15 to 30% of individuals with acquired immunodeficiency syndrome will eventually develop HIV-1 dementia, with an annual incidence of about 5% (Chaisson et al., 1998).

The primary targets of HIV-1 infection in the central nervous system (CNS) are macrophages and microglia (Price et al., 1988). Microglia, first described by del Rio Hortega (1932), are the primary immune cells of the brain. These cells exhibit two morphological extremes that represent resting and activated cells; a small, ramified form that predominates in the normal brain, and a larger, spherical form during infection, injury, or inflammation (Davis et al., 1994).

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Protease inhibitors are effective in reducing HIV-1 viral load in HIV-infected patients; however, their low brain permeability may permit the CNS to become a reservoir for HIV-1 (Chun et al., 1997; Hoetelmans et al., 1997). It is believed that poor brain permeation of xenobiotics, including anti-HIV drugs, results from the effect of an ATP-dependent, membrane-bound efflux pump called P-glycoprotein (P-gp) (Kim et al., 1998b). The 170-kDa glycoprotein is a member of the ATP-binding cassette superfamily of transporters and is a product of the multidrug resistance (MDR) gene (Higgins, 1993). P-gp is encoded by *MDR1* and *MDR3* in humans and by *mdr1a*, *mdr1b*, and *mdr2* in rodents. The protein encoded by *MDR1*, *mdr1a*, and *mdr1b* confers the MDR phenotype (Ueda et al., 1987), whereas the protein encoded by *MDR2* and *mdr2* is involved in hepatic phospholipid transport into the bile (Smit et al., 1993). Gene knockout studies have demonstrated that P-gp-deficient mice are viable, fertile, and healthy compared with wild type but exhibit significantly elevated drug levels (including protease inhibitors) in the brain (Schinkel et al., 1997; Kim et al., 1998b; Washington et al., 2000). These results suggest that one of the roles of P-gp is to prevent the accumulation of toxic xenobiotics from various organs, including the brain. The polarized localization of

ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; CNS, central nervous system; P-gp; P-glycoprotein, MDR, multidrug resistance; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered solution; EBSS, Earle's balanced saline solution; MRP, multidrug resistance-associated protein; bp, base pair(s).

P-gp in normal tissues of the body seems to corroborate this hypothesis. P-gp is found on the apical surface of intestinal and renal epithelia and the secretory glands of the endometrium in pregnant mice (Croop et al., 1989). In the brain, P-gp is expressed on the subapical side of the choroid plexus epithelia (Rao et al., 1999) and on the luminal surface of brain microvessels (Beaulieu et al., 1997). Recently, Golden and Pardridge (1999) observed the expression of P-gp on the abluminal side of the blood-brain barrier on neighboring astrocyte foot-processes.

At present, the functional expression of P-gp in microglia, the primary target and reservoir of HIV-1 in the brain, is unknown. The goal of this project was to investigate the functional expression of P-gp in brain parenchyma, by using a rat brain microglia cell line (MLS-9). This cell line has been well characterized and when grown to confluence exhibits several morphological and functional properties of activated microglia seen in vivo. That is, MLS-9 cells round up like phagocytic microglia, they express high levels of complement C3 receptors and lysosomes, and can produce large amounts of nitric oxide (Schlichter et al., 1996; Zhou et al., 1998).

Materials and Methods

Chemicals. [³H]Digoxin (19 Ci/mmol) was purchased from Mandel (Guelph, ON, Canada). Ritonavir was purchased from Moravak Biochemicals (Brea, CA). [¹⁴C]D-Mannitol (51.5 mCi/mmol) was purchased from PerkinElmer Life Science Products (Boston, MA). Quinidine, sodium azide, verapamil sulfapyrazone, probenecid, and indomethacin were purchased from Sigma-Aldrich (Oakville, ON, Canada). Cyclosporin A and PSC 833 were a generous gift from Novartis Pharma (Basel, Switzerland). Saquinavir was a gift from Roche Products Ltd. (Hertfordshire, UK) and indinavir was a gift from Merck Research Labs (West Point, PA). The murine monoclonal anti-P-gp antibodies C219, MRK16, and MAB-448 were purchased from ID Labs (London, ON, Canada), Kamiya Biomedical (Seattle, WA), and Chemicon International (Temecula, CA), respectively.

Cell Culture. A microglia cell line (MLS-9) was produced, as described previously (Zhou et al., 1998) from cultured microglia isolated from the neopallia of 2- or 3-day-old Wistar rats (Schlichter et al., 1996). In brief, cultures that were >98% pure microglia, as judged by labeling with isolectin B4 (Sigma, St. Louis, MO), were induced to proliferate by adding a growth factor, colony-stimulating factor-1. After several weeks colonies arose, some of which were harvested and continued to grow in the absence of the growth factor. From one of these colonies, the MLS-9 cell line was established. MLS-9 cells display characteristic markers of microglia, i.e., 100% stained with isolectin B4, 98% stained with OX-42 antibody, and 99% stained with ED-1 antibody, whereas none of them stained with antibodies against glial fibrillary acidic protein (astrocyte marker) or fibronectin (fibroblast protein) (Zhou et al., 1998).

The MLS-9 cells (passages 4–30) were grown as a monolayer on 75-cm² polystyrene tissue culture flasks or 24-well plates (Sarstedt, St. Leonard, QB, Canada) at 37°C, 5% CO₂, and 95% humidity. The cells were fed every 2 days with minimal essential medium, pH 7.2, containing L-glutamine, D-glucose, 5% fetal bovine serum, 5% horse serum, and 0.5% penicillin/streptomycin suspension (all obtained from Invitrogen, Carlsbad, CA). Confluent cultures were subcultured with a sodium citrate solution containing 130 mM NaCl, 15 mM sodium citrate, 10 mM glucose, and 10 mM HEPES, pH 7.4.

A P-gp-overexpressing Chinese hamster ovary cell line (CH^RC5; selected for colchicine resistance) and the parent line (AuxB1) were kindly provided by Dr. Victor Ling (Toronto and Vancouver, Canada). The parent line and the multidrug-resistant line were grown as monolayers on 75-cm² polystyrene tissue culture flasks or 24-well plates as previously described (Leung and Bendayan, 1999) at 37°C, 5% CO₂, and 95% humidity in α -minimal essential medium, pH 7.2, supplemented with 10% fetal bovine serum, and 0.5% penicillin/streptomycin suspension. Confluent cultures were subcultured with 0.05% trypsin-EDTA.

RT-PCR Analysis. Total RNA was isolated using TRIZOL reagent (Invitrogen Canada Inc., Burlington, ON, Canada) from primary cultured microglia and MLS-9 cells. It was subjected to DNase I digestion (0.1 U/ml, 15 min, 37°C; Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) to eliminate genomic contamination. For reverse transcription, a cDNA reaction mixture was made using 0.5 μ g of pd(N)6 and 1 μ g of RNA in a 20- μ l reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM dNTP, and 200 units of SuperScrip II reverse transcriptase (Invitrogen). The RT-PCR reaction was conducted with 1.5 mM MgCl₂, 0.8 μ M forward and reverse primers (Table 1), and 10% of the cDNA reaction mixture, by using a GeneAmp PCR 2400 system (PerkinElmer, Mississauga, ON, Canada). After 35 cycles of a 20-s denaturing phase at 94°C, a 30-s annealing phase at 55°C, and a 30-s extension phase at 72°C, the amplified DNA products were resolved on 1.5% agarose gels with 0.5 mg/ml ethidium bromide and their identities confirmed by sequencing (Table 1).

Western Blotting. Crude membranes from cultured MLS-9 cells were prepared by centrifuging the cell suspension at 400g at 4°C for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 10 mM Tris buffer, pH 7.1, containing 300 mM mannitol and 0.1% protease inhibitor cocktail (Sigma-Aldrich). The cell suspension was homogenized at 10,000 rpm for 10 s and the lysate centrifuged at 3000g at 4°C for 10 min. The supernatant was collected and centrifuged at 15,000g at 4°C for 30 min. The resultant pellet was resuspended in 10 mM Tris buffer, pH 8.8. Protein concentrations of the crude membrane preparations were determined with Bradford's protein assay (Bradford, 1976). Samples were stored at -20°C until further use. For immunoblotting, 20- and 50- μ g aliquots of crude proteins were mixed in Laemmli buffer and resolved on a 7.5% SDS-polyacrylamide gel without boiling the samples. The gel was then electrotransferred onto a nitrocellulose membrane. P-gp was identified by labeling with the monoclonal antibody (C219; 1/100 dilution) for 5 h at room temperature, followed by the

TABLE 1
RT-PCR conditions and primers used

Gene	GenBank Accession No.	Primer Sequence (5' to 3')	Annealing Temp. °C	Expected Product Size bp
β -Actin	GI191581	TGGAATCCTGTGGCATCCATGAAAC (FP) TAAAACGCAGCTCAGTAACAGTCCG (RP)	55	349
Rat mrpl	X96394	CTGGCTTGGTGTGAACTGAT (FP) AGCCTCTGGCTTGGCTCTAT (RP)	55	394
Rat mdrla	S66618	GGACAGAAACAGAGGATCGC (FP) CCCGTCTTGATCATGTGGCC (RP)	55	440
Rat mdrlb	M81855	GGACAGAAACAGAGGATCGC (FP) TCAGAGGCACCACTGTCTACT (RP)	55	355

FP, forward primer; RP, reverse primer.

secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse; 1/1000 dilution) for 2 h at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for 1 min. The drug-sensitive Chinese hamster ovary parent cell line (AuxB1) and the MDR cell line selected for colchicine resistance (CH^RC5) were used as P-gp negative and positive controls, respectively.

Morphology and Immunocytochemistry Studies. MLS-9 cells were examined by transmission electron microscopy. Monolayers of MLS-9 cells were fixed in situ for approximately 12 h at 4°C with 5 ml of 1% glutaraldehyde in 0.1 M phosphate-buffer solution (PBS), pH 7.4. Ultrathin sections were cut, stained, and examined with a Philips 410 electron microscope following standard methods of detachment, dehydration in graded ethanol, and embedding in Epon.

Immunocytochemical staining was performed as described previously (Bendayan, 1995). MLS-9 cells were fixed in 1% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 2 h at room temperature, washed in the same buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C. Thin sections were cut (80 nm in thickness) and mounted on a parlodion-carbon-coated nickel grid. First, the grids were pretreated with the oxidizing agent sodium metaperiodate (11% saturated) for 30 min at room temperature. Ovalbumin (1% in PBS was used to block nonspecific binding sites. The cells were then exposed to the P-gp mouse monoclonal antibody C219, which recognizes a conserved cytoplasmic region present in all classes of P-gp nucleotide binding domains, (1/10 dilution) for 2 h at 37°C. The grids were further incubated for 30 min in 10-nm protein A-colloidal gold at 1/6 dilution in PBS-containing 0.02% polyethylene glycol, pH 7.4. Finally, sections were stained by incubating for 10 min in 3% uranyl acetate and viewed by electron microscopy. Immunocytochemistry was also performed with P-gp mouse monoclonal antibodies MAB-448 (1/50 dilution) and MRK16 (1/500 dilution), which recognizes an extracellular P-gp epitope.

Functional Studies. All the functional studies were performed on 4- to 5-day-old confluent monolayers of MLS-9 cells grown on polystyrene 24-well plates at a cell density of $\sim 1 \times 10^6$ cells/well. These cells displayed no significant variations in morphology or drug accumulation at different passage numbers. Initially, the cells were washed and conditioned for 30 min with 0.5 ml of an Earle's balanced saline 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. The pH was adjusted to 7.4 by using Trizma base. Cells were then incubated for the desired time with a medium containing a radiolabeled P-gp probe (i.e., digoxin) with or without a P-gp inhibitor. At the end of each interval, the incubation medium was aspirated and the reaction was terminated by adding 2 ml of ice-cold 0.16 M NaCl. The cells were solubilized in 1 ml of 1 N NaOH for 30 min and transferred to scintillation vials containing 0.5 ml of 2 N HCl to neutralize the NaOH. Cellular [³H]digoxin accumulation was measured by a Beckman liquid scintillation counter with automated quench correction. All samples were corrected for "zero time" and background radioactivity. Correction was not required for the distribution of the radiolabeled probe in the extracellular space because the amount of [¹⁴C]D-mannitol was found to be negligible (<5%). Accumulation of the P-gp probe was standardized to the protein concentration (mg/ml) in each culture plate as determined by the Bradford method (Bio-Rad, Mississauga, ON, Canada) by using bovine serum albumin (Sigma-Aldrich) as the standard. Digoxin cellular accumulation was expressed in picomoles per milligram of protein.

To determine digoxin efflux by cultured MLS-9 cells, the cells were incubated for 1 h at 37°C with 0.1 μM [³H]digoxin dissolved in standard EBSS incubation medium. The medium was then aspirated and the cells were rapidly washed with ice-cold 0.16 N NaCl. The incubation medium alone or medium containing a P-gp inhibitor was added for the desired times and then the cells were washed with ice-cold 0.16 N NaCl and solubilized with 1 N NaOH for 30 min.

Efflux was calculated from the remaining cellular [³H]digoxin content.

The ATP-dependent mechanism of P-gp transport in cultured MLS-9 cells was investigated by measuring [³H]digoxin accumulation in the presence of an ATPase-inhibitor, sodium azide. Prior to adding the incubation medium containing digoxin, with or without sodium azide, the cells were conditioned for 30 min with 0.5 ml of EBSS, pH 7.4, or with 0.5 ml of EBSS containing 5.5 mM mannitol (replacing D-glucose) and 10 mM sodium azide, pH 7.4.

Data Analysis. Each set of experiments was repeated two to three times on cells from different passages. In an individual experiment, each data point represents quadruplicate trials. The results are reported as mean ± S.D. from a minimum of two separate experiments. To determine the significance of inhibition for unpaired experimental values, the Student's *t* test was used. For multiple comparisons, the test of repeated measures analysis of variance and/or the post hoc multiple-comparison Bonferroni *t* test was used. A value of *p* < 0.05 was considered significant.

Results

mRNA Expression

RT-PCR analysis was used to detect mRNA expression of P-gp members in MLS-9 cells and primary cultured rat microglia (Fig. 1). Using gene-specific primers for *mdr1a* and *mdr1b* (Table 1), we detected both *mdr1a* and *mdr1b* in primary microglia (lanes 1 and 4), whereas only the *mdr1b* mRNA was detected in MLS-9 cells (lane 5). No transcripts for *mdr1a* were detected in MLS-9 cells (lane 2), despite optimizing the RT-PCR conditions by varying the number of cycles, Mg²⁺ concentration, and annealing temperatures. The identity of each correct-sized band was verified by dideoxy sequencing. As expected, no bands were detected when the reverse transcriptase was omitted (lanes 3 and 6), and the internal control (β -actin, lane 8) produced a strong signal. RT-PCR analysis was also used to detect the mRNA expression of the multidrug resistance-associated protein (MRP) homolog 1. No *mrp-1* mRNA was detected in MLS-9

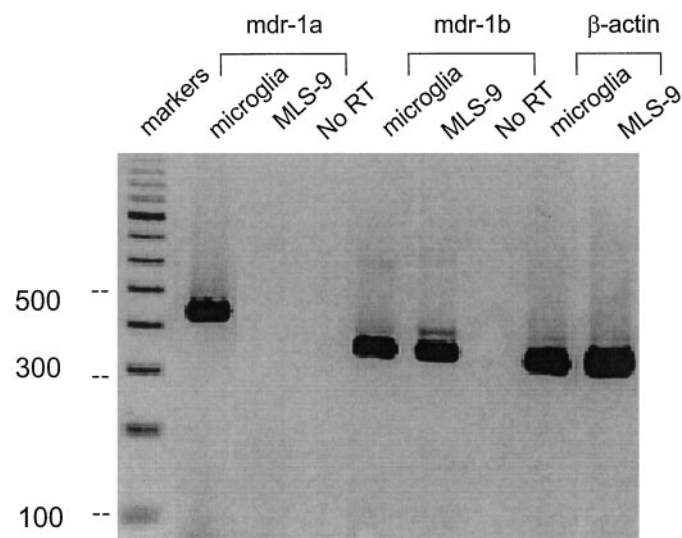


Fig. 1. RT-PCR analysis of P-gp in MLS-9 cells. An ethidium-bromide stained gel shows amplification of specific bands for *mdr1a* (~440 base pairs (bp)) from cultured microglia, and *mdr1b* (~355 bp) from microglia and MLS-9 cells. No bands were present when reverse transcriptase was omitted. As a control, ~350-bp β -actin bands were amplified from MLS-9 cells and primary cultured microglia.

cells, despite optimizing conditions and obtaining a clear band of the correct product in control cells (data not shown).

Protein Expression

Western blot analysis with the antibody C219 was used to detect P-gp protein in MLS-9 cells (Fig. 2). This antibody recognizes a common intracellular epitope of *mdr1a*, *mdr1b*, and *mdr2*. The CH^RC5 cell line, a P-gp-overexpressing line selected for colchicine resistance, was used as a positive control. The parent Chinese hamster ovary cell line AuxB1 was used as a negative control (Ling and Thompson, 1974). In the CH^RC5 cells, a robust band was detected at 170 to 180 kDa, a molecular weight previously reported for P-gp in other cells (Doige and Sharom, 1992; Regina et al., 1998). As expected, P-gp protein was not detected in the parent cell line AuxB1. In MLS-9 cells, the 170- to 180-kDa band was weaker but its intensity increased with increasing protein concentration. Because this antibody was apparently specific, it was then used for immunocytochemical analysis.

Cell Morphology and Immunocytochemistry

Figure 3A shows a transmission electron micrograph of confluent cultured MLS-9 cells grown as a monolayer on an impermeable substratum. Cells in these confluent monolayers are spherical, with short surface villi, and do not form tight junctions. A more detailed description of the morphology of MLS-9 cells cultured in our laboratory has been previously reported (Hong et al., 2000).

Immunocytochemical studies on cultured MLS-9 cells (Fig. 3) were conducted using protein A-gold immunolabeling with the P-gp monoclonal antibody C219. As seen in the micrographs, there was specific immunogold labeling with the C219 antibody along the nuclear envelope (Fig. 3B) and plasma membrane (Fig. 3C). Because the C219 antibody recognizes both class I (*mdr1a/b*) and class II (*mdr2*) P-gp isoforms, we also conducted this experiment with a P-gp monoclonal antibody that specifically recognizes class I P-gp, MRK16. Similar labeling of the membrane and nuclear envelope was observed with the MRK16 monoclonal antibody (data not shown). To further strengthen the results, immunocytochemistry studies were also undertaken with the MAB-448 antibody, which, like the C219 monoclonal antibody, recognizes all the MDR isoforms (data not shown).

Functional Studies

Accumulation and Efflux. To investigate the functional activity of P-gp, we measured the accumulation of the known

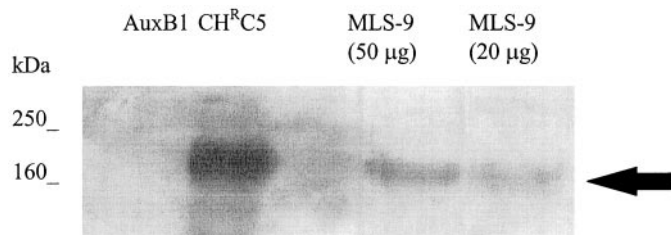


Fig. 2. Western blot analysis of P-gp in cultured MLS-9 cells. Crude membrane proteins (20 μ g) from AuxB1 cells (lane 1), CH^RC5 cells (lane 2), or 20 and 50 μ g from MLS-9 cells (lanes 3 and 4, respectively) were separated by electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to a nitrocellulose sheet. The protein of interest was detected using 1 μ g/ml monoclonal P-gp antibody C219, and the blot was developed as described under *Materials and Methods*.

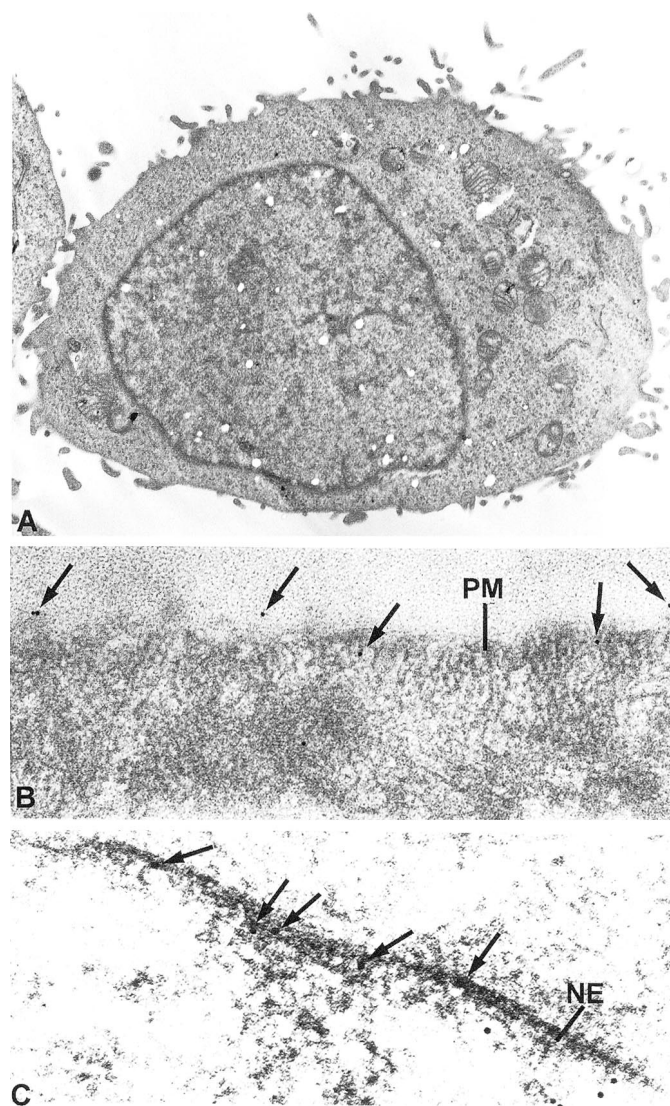


Fig. 3. Morphology of cultured MLS-9 cells. A, transmission electron micrograph of MLS-9 cells grown for 7 days into a confluent monolayer. The cells are round, with relatively smooth membranes, short villi, and large nuclei compared with the cell volume. No tight junctional complexes were seen between cells. Original magnification, 10,000 \times . B, immunogold labeling of P-gp at the plasma membrane. Immunocytochemistry studies on MLS-9 cells grown in monolayers were conducted by labeling the monoclonal P-gp antibody, C219, with 10 nm of protein A-colloidal gold. The gold particles (arrows), revealing P-gp antigenic sites, are located at the level of the plasma membrane (PM). Original magnification, 65,000 \times . C, immunogold labeling of P-glycoprotein at the nuclear envelope. This electron micrograph shows the immunolabeling of the monoclonal C219 antibody (arrows) at the level of the nuclear envelope (NE). Original magnification, 100,000 \times .

P-gp substrate digoxin (0.1 μ M) by MLS-9 cells grown as monolayers, in the presence or absence of a potent P-gp inhibitor, PSC 833, a cyclosporin A analog. The time course of digoxin accumulation at 37 $^{\circ}$ C (Fig. 4) shows increasing uptake until a plateau is reached by \sim 1 h under control conditions. At 1 h, digoxin uptake was significantly enhanced (by 286%) in the presence of 5 μ M PSC 833.

Digoxin efflux is expressed as the remaining [³H]digoxin content of cells after a 1-h preincubation with the substrate. Figure 5 shows that digoxin efflux is rapid and the remaining intracellular digoxin content is significantly enhanced at early times in the presence of 5 μ M PSC 833. These results,

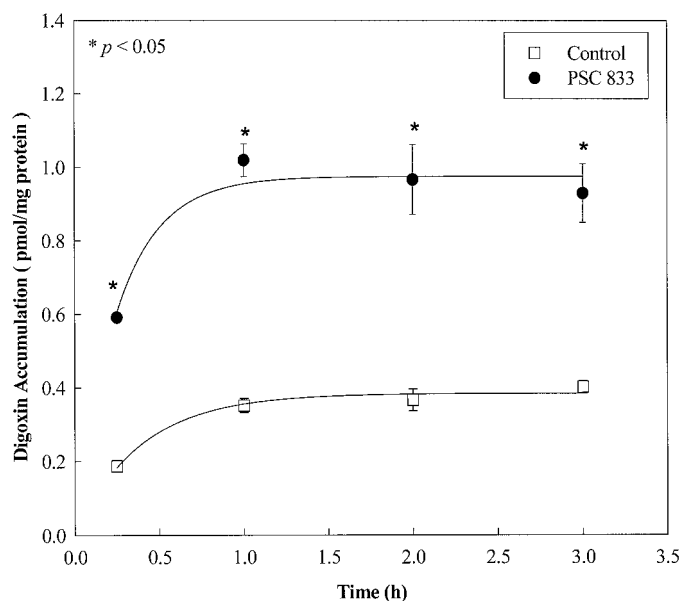


Fig. 4. Effect of PSC 833 on digoxin accumulation by MLS-9 cells grown in monolayers. Digoxin accumulation (0.1 μ M) was measured at 37°C in the presence of the potent P-gp inhibitor PSC 833 (5 μ M) at 0.25, 1, 2, and 3 h. The results are expressed as means \pm S.D. of two separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks indicate values that are significantly different from control.

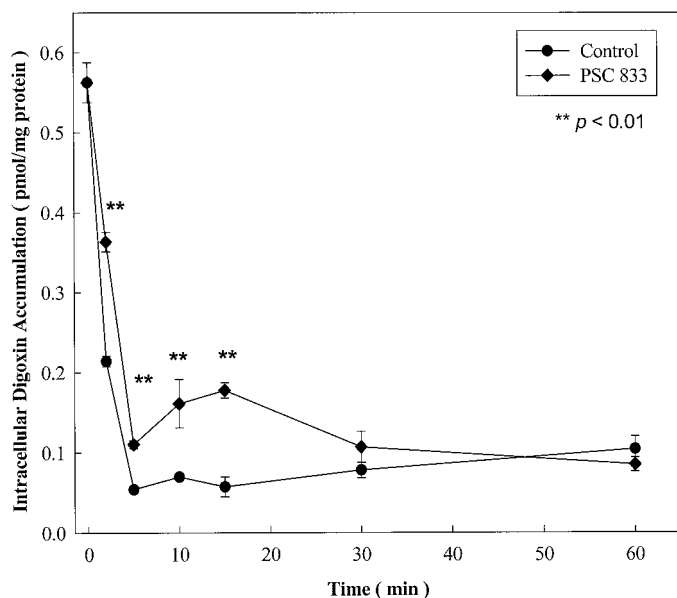


Fig. 5. Effect of PSC 833 on digoxin efflux by MLS-9 monolayer cells. P-gp-mediated efflux of digoxin (0.1 μ M) was measured at 37°C in the presence/absence of PSC 833 (5 μ M). The time course of digoxin efflux in the presence/absence of PSC 833 was expressed as the remaining intracellular digoxin content following 1-h incubation with the substrate. Data are expressed as means \pm S.D. of two separate experiments, and each data point represents quadruplicate measurements. Values marked with an asterisk are significantly different from control.

showing that digoxin efflux can be inhibited by PSC 833, suggest that P-gp mediates this process.

Specificity and Energetics. Previous studies have shown that protease inhibitors can inhibit P-gp-mediated transport of various drugs in tumor and intestinal cells (Washington et al., 1998; Profit et al., 1999). To investigate the inhibitory effect of protease inhibitors and various known

P-gp inhibitors, we measured their effect on the accumulation of digoxin by MLS-9 cells. The accumulation of 0.1 μ M digoxin at 1 h was significantly increased in the presence of 50 μ M of the protease inhibitors saquinavir (by 1.5-fold), indinavir (1.4-fold), ritonavir (1.4-fold), or various known P-gp inhibitors, such as 50 μ M verapamil (1.7-fold), 50 μ M quinidine (1.6-fold), 50 μ M cyclosporin A (1.8-fold), and 5 μ M PSC 833 (3.2-fold) (Fig. 6). PSC 833 was the most potent inhibitor and its effect on digoxin accumulation by the monolayer cells was dose-dependent (Fig. 7A). The protease inhibitors saquinavir and indinavir also enhanced digoxin accumulation in a dose-dependent manner (Fig. 7B). In contrast, digoxin accumulation was not significantly increased in the presence of various inhibitors of MRP, such as sulfinpyrazone (2 mM), indomethacin (50 μ M), or probenecid (1 mM) (Fig. 6). In addition, RT-PCR results show that the *mrp1* isoform is not expressed in MLS-9 cells (data not shown). Although it is currently not known whether the other *mrp* isoforms are expressed in MLS-9 cells, our results suggest that digoxin transport in microglia is not mediated at least by *mrp1*. Together, these results support the view that digoxin transport by MLS-9 cells is mediated by P-gp and not by *mrp1*.

To determine the specificity of the protease inhibitors and PSC 833 for P-gp, we measured digoxin accumulation in a well characterized P-gp-overexpressing cell line (CH^RC5) and its parent line (AuxB1), derived from Chinese hamster ovary

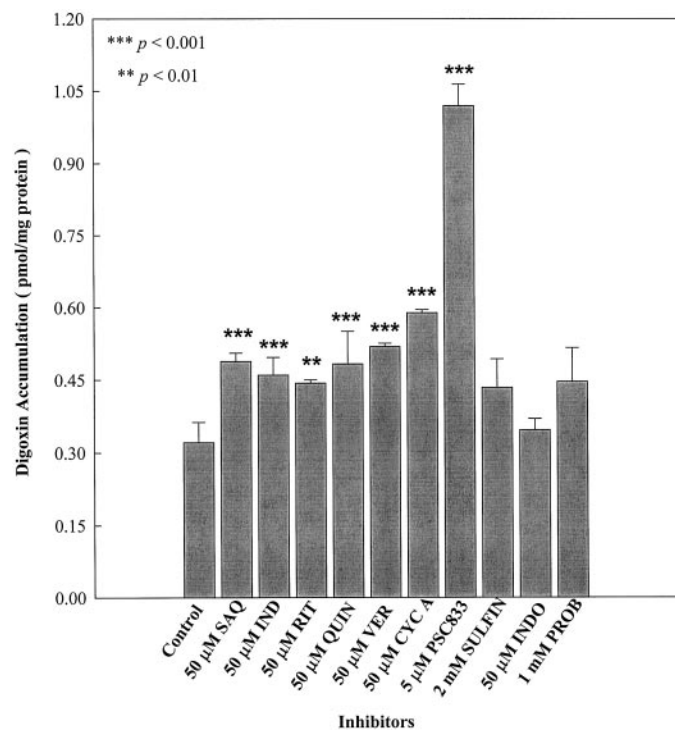


Fig. 6. Effect of various P-gp and MRP inhibitors on digoxin accumulation by MLS-9 cells grown as monolayers. The accumulation of digoxin (0.1 μ M) at 1 h was measured at 37°C in the presence of 50 μ M of the standard P-gp inhibitors verapamil (VER), quinidine (QUIN), cyclosporin A (CYC A), or 5 μ M PSC 833, or 50 μ M of the protease inhibitors saquinavir (SAQ), indinavir (IND), or ritonavir (RIT), or MRP inhibitors 2 mM sulfinpyrazone (SULFIN), 50 μ M indomethacin (INDO), or 1 mM probenecid (PROB). Results are expressed as means \pm S.D. of three separate experiments; each experimental point represents quadruplicate measurements. Asterisks represent values that are significantly different from control.

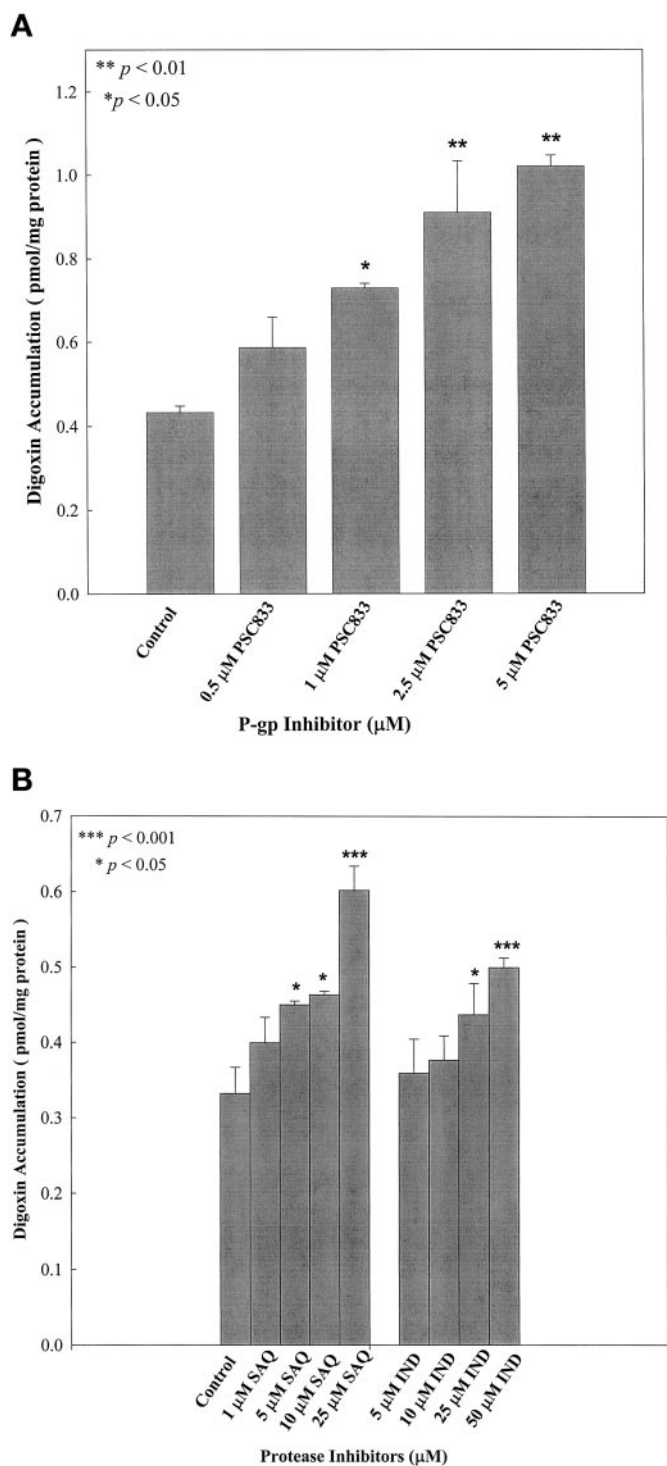


Fig. 7. Dose dependence of PSC 833 (A) and protease inhibitors saquinavir and indinavir (B) in inhibiting digoxin accumulation by MLS-9 cells. Digoxin accumulation (0.1 µM) was determined at 1 h, 37°C, in the presence of increasing concentrations of the potent P-gp inhibitor PSC 833 (0.5–5 µM) and the protease inhibitors saquinavir (1–25 µM) and indinavir (5–50 µM). Results are expressed as means ± S.D. of two separate experiments, and each data point represents quadruplicate measurements. Values marked with an asterisk are significantly different from control.

cells. In the CH^RC5 cell line, saquinavir (50 µM), indinavir (50 µM), cyclosporin A (50 µM), and PSC 833 (5 µM) significantly increased digoxin accumulation: by 1.8-, 1.6-, 2.3-, and 2.5-fold, respectively (Fig. 8A). These drugs had no sig-

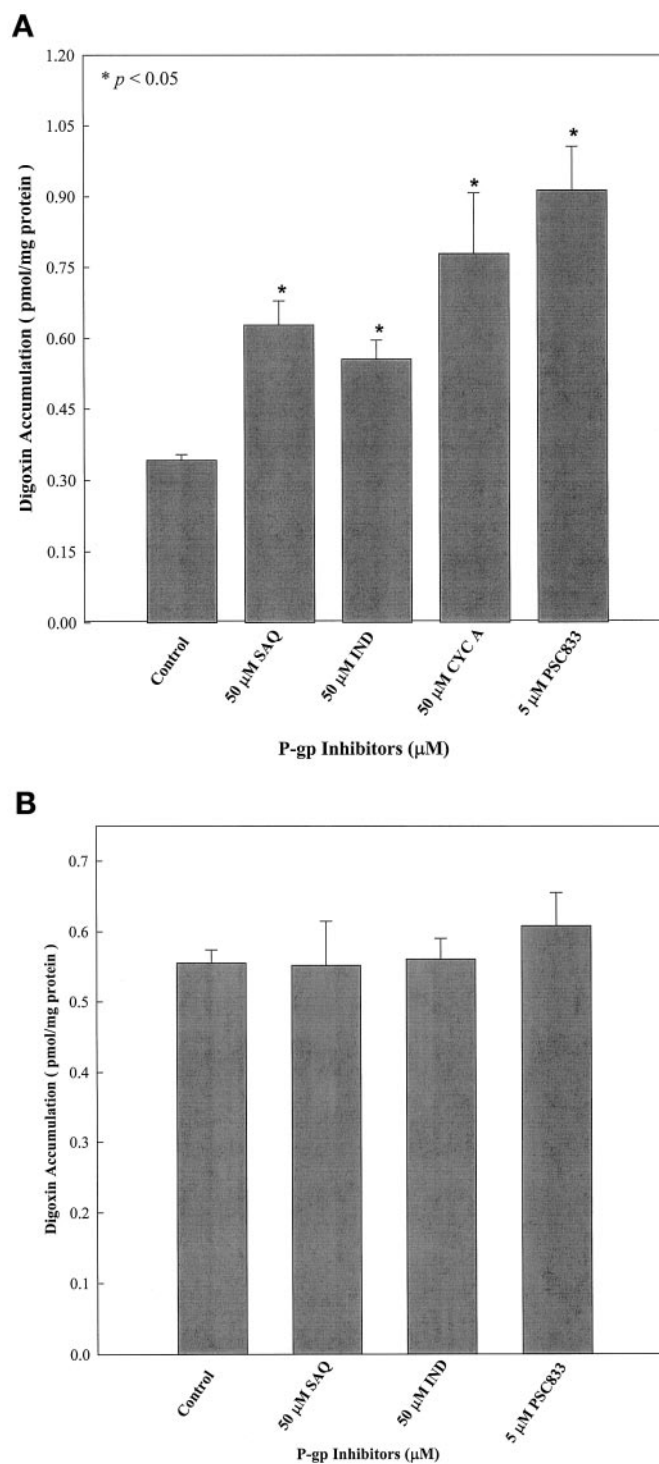


Fig. 8. Effect of PSC 833 and the protease inhibitors saquinavir and indinavir on digoxin accumulation by CH^RC5 (A) and AuxB1 (B) cells. Digoxin accumulation (0.1 µM) by the P-gp-overexpressing cell line CH^RC5 and the parent line AuxB1 was measured in the presence of PSC 833 (5 µM), cyclosporin A (50 µM), and 50 µM of the protease inhibitors, saquinavir or indinavir, at 1 h, 37°C. Results are expressed as means ± S.D. of three separate experiments, and each data point represents quadruplicate measurements. Asterisks indicate values that are significantly different from control.

nificant effect when tested in the parent line AuxB1 (Fig. 8B).

Because it is well established that P-gp transport is ATP-dependent (Higgins, 1993), we measured digoxin accumulation by MLS-9 cells in the presence of sodium azide, an

ATPase inhibitor. Digoxin accumulation was enhanced by ~1.5-fold in the presence of 10 mM sodium azide, which suggests that in the absence of a fully functional ATPase, digoxin efflux was inhibited (data not shown).

Discussion

A common theory of HIV-1-induced neuropathogenesis proposes that HIV-1 infection of microglia results in an amplified inflammatory pathological cascade of events involving microglia, astrocytes, and the secretion of inflammatory mediators. Activated microglia and astrocytes release cytokines and neurotoxins (i.e., interleukins-1 and -6, tumor necrosis factor- α , glutamate, platelet-activating factor, nitric oxide, arachidonic acid metabolites) that ultimately lead to the neuronal death or dysfunction that underlies HIV-1 dementia (Nath, 1999).

Although drug therapy with antiretrovirals, including protease inhibitors, reduces plasma viral loads in patients, the poor brain permeability of these drugs may have several consequences. It may result in ineffective treatment of HIV-1 dementia and lead to the emergence of resistant HIV-1 strains, resulting in a pharmacological sanctuary site for HIV-1 in the brain (Chun et al., 1997; Hoetelmans, 1997). Therefore, it is clinically important that antiretroviral drugs reach therapeutic concentrations in the brain, especially in microglia, the cells that harbor HIV-1. However, no information is available on the mechanism of antiretroviral drug transport in these cells. Recently, our laboratory has characterized a Na⁺-dependent nucleoside transporter (Hong et al., 2000) and a novel electrogenic zidovudine/H⁺-dependent transporter (Hong et al., 2001) in microglia, thus providing evidence that these cells express membrane transporters that may be important for drug transport. The purpose of this study was to investigate the functional expression of P-gp, a membrane-associated ATP-dependent transporter, in a rat microglia cell line (MLS-9).

Numerous studies of the blood-brain barrier have reported higher P-gp levels in isolated brain microvessels than in cultured endothelium (Barrand et al., 1995; Regina et al., 1998). RT-PCR analysis shows that the *mdr1a* P-gp-encoding gene is highly expressed in isolated rat brain capillaries, whereas both *mdr1a* and *mdr1b* are expressed in endothelial cell cultures derived from these capillaries (i.e., primary endothelial cultures and an immortalized cell line, RBE4), suggesting an up-regulation of the *mdr1b* gene in cultured cells (Barrand et al., 1995; Regina et al., 1998). This change in gene expression in culture may result from culture conditions and the absence of endogenous factors and signals that are present in vivo. Coculture systems (i.e., endothelial-astrocyte cocultures) may allow for greater cell differentiation and induce specific proteins that are not present in monoculture systems (Regina et al., 1998). In contrast to genes expressed in brain microvessels, in mouse and rat brain parenchyma, the *mdr1b* appears to be the major P-gp gene expressed (Regina et al., 1998). Declèves et al. (2000) also detected very low levels of *mdr1a* mRNA in primary cultures of rat astrocytes, whereas *mdr1b* was highly expressed in these parenchyma cells. Our RT-PCR results show that both P-gp encoding genes *mdr1a* and *mdr1b* are expressed in primary microglia cultures, whereas in MLS-9 cells only the *mdr1b* gene was detected. This difference suggests a down-regula-

tion of the *mdr1a* gene in the cell line. Together, our results provide the first evidence of P-gp expression in microglia, and that both P-gp encoding genes can be expressed in brain parenchyma.

Not only is the mRNA present but also Western blot analysis of the MLS-9 cells with the monoclonal P-gp antibody C219 detected a single band at 170 to 180 kDa, the size previously reported for P-gp (Doige and Sharom, 1992; Regina et al., 1998). A similar band was also observed in the P-gp-overexpressing cell line CH^RC5, further corroborating the antibody specificity. This antibody recognizes a highly conserved intracellular P-gp epitope (Okochi et al., 1997). Using the C219 antibody for immunocytochemical analysis, we showed the localization of P-gp along the plasma membrane and nuclear envelope of the MLS-9 cells, the first such study on microglia. Similar labeling patterns were observed with two other anti-Pgp monoclonal antibodies, MAB-448 and MRK16. MRK16 recognizes an extracellular epitope of the class I P-gp isoforms. In the CNS, P-gp protein has been seen mainly at the blood-cerebrospinal fluid barrier, the blood-brain barrier, and in astrocytes, and P-gp transcripts are present in primary cultures of astrocytes (Declèves et al., 2000). At the blood-cerebrospinal fluid barrier, P-gp is found on the subapical side of the choroid plexus epithelia (Rao et al., 1999). At the blood-brain barrier, its exact location along endothelial microvessels remains controversial. Immunohistochemistry and luminal membrane isolation studies have localized P-gp to the luminal surface of the brain endothelium (Sugawara et al., 1990; Beaulieu et al., 1997) but P-gp has also been identified at the abluminal surface of endothelial cells on neighboring astrocyte foot-processes (Golden and Pardridge, 1999). The presence of P-gp on the nuclear membrane of microglia cells is interesting. Baldini et al. (1995) also detected the presence of P-gp on nuclear membranes of some tumor cells and observed increased P-gp activity at the level of the plasma membrane and the nuclear membrane in some drug-resistant cells compared with MDR-sensitive cells. Others have found evidence that anthracyclines accumulate in the nucleus of MDR-sensitive cells, whereas they are undetectable in the nucleus of some drug-resistant cells (Keizer et al., 1989; Coley et al., 1993). These results suggest a multimodal involvement of P-gp-mediated drug resistance at the level of the plasma and nuclear membranes. Thus, P-gp expression on both surface and nuclear membranes may further serve as a protective mechanism, preventing the accumulation of xenobiotics and pharmacological agents in the nucleus. These results, together with our data, show that P-gp is expressed in various compartments of the brain: the blood-brain barrier, blood-cerebrospinal fluid barrier, and brain parenchyma.

Our functional studies show that digoxin accumulation by microglia is significantly enhanced and efflux reduced by the potent P-gp inhibitor PSC 833, a nonimmunosuppressive cyclosporin A analog. Furthermore, our data indicate that digoxin accumulation by microglia is energy-dependent. Although digoxin is a well known P-gp substrate that is transported efficiently in brain and kidney (Fromm et al., 1999), it is possible that digoxin transport in microglia is mediated by one of the MRPs, another family of ATP-dependent membrane-associated efflux transporters. However, our results show that digoxin accumulation by MLS-9 cells is not significantly altered by standard MRP inhibitors. Moreover, nei-

ther mrp1 mRNA nor protein was detected in MLS-9 cells (data not shown), so P-gp, not mrp1, appears to account for digoxin transport in MLS-9 cells. Further studies of the expression and activity of other mrp isoforms in MLS-9 cells will be needed to confirm the specificity of our functional studies.

Although P-gp-mediated transport of protease inhibitors has been investigated in various epithelial systems (Kim et al., 1998a,b; Profit et al., 1999), their transport properties have not previously been examined in the brain (i.e., the blood-brain barrier, astrocytes, microglia). We found that cellular accumulation of digoxin by MLS-9 cells was significantly enhanced, in a dose-dependent manner, by various known P-gp inhibitors, and by the protease inhibitors saquinavir, indinavir, and zidovudine. Further evidence of the P-gp specificity of these agents was that they increased digoxin accumulation in the P-gp-overexpressing cell line (CH^RC5) but not in the parent line (AuxB1). Recent *in vitro* and *in vivo* studies have demonstrated that protease inhibitors are substrates for, as well as inhibitors of, P-gp (Kim et al., 1998a; Profit et al., 1999; Washington et al., 2000). This transport can be directional, because renal and intestinal epithelia showed a specific ATP-dependent transport of saquinavir from the basolateral-to-apical side (Alsenz et al., 1998; Kim et al., 1998a). Perhaps most informative is that *in vivo* brain concentrations of protease inhibitors were elevated 4- to 36-fold in mice lacking the *mdr1a* gene (Kim et al., 1998b; Washington et al., 2000), and that in P-gp deficient mice, brain concentrations of the protease inhibitor nelfinavir were increased up to 37-fold by the potent cyclopropylidibenzosuberane P-gp inhibitor LY-335979 (Choo et al., 2000). These results, and our data, suggest that P-gp can contribute to the low accumulation of protease inhibitors in the brain.

In summary, our results provide the first evidence of the functional expression of P-gp in cultured rat brain microglia. This raises the possibility that the low CNS concentrations of a number of xenobiotics, including protease inhibitors, may result from P-gp activity in the brain parenchyma, as well as at the blood-brain barrier. Because microglia are the primary target of HIV-1 infection in the brain, P-g expression in these cells may play a significant role in antiretroviral drug response and resistance and may even permit HIV-1 to silently replicate in these cellular reservoirs without detection.

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