MDR1 P-glycoprotein Reduces Influx of Substrates
Without Affecting Membrane Potential

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Running title: MDR1 P-glycoprotein does not affect membrane potential

Abbreviations: Eₘₜ, transmembrane potential; ∆ψ, mitochondrial membrane potential; MDR, multidrug resistance; Pgp, P-glycoprotein; ⁹⁹mTc-Sestamibi, hexakis(2-methoxyisobutylisonitrile)[⁹⁹mTc]; TPB, tetraphenylborate
Abstract

Multidrug resistance (MDR1) P-glycoprotein (Pgp; ABCB1) decreases intracellular concentrations of structurally diverse drugs. While Pgp generally is thought to be an efflux transporter, the mechanism of action remains elusive. To determine if Pgp confers drug resistance through changes in transmembrane potential (E<sub>m</sub>) or ion conductance, we studied electrical currents and drug transport in Pgp-negative MCF-7 cells and MCF-7/MDR1 stable transfectants that were established and maintained without chemotherapeutic drugs. Although E<sub>m</sub> and total membrane conductance did not differ between MCF-7 and MCF-7/MDR1 cells, Pgp reduced unidirectional influx and steady-state cellular content of Tc-Sestamibi, a substrate for MDR1 Pgp, without affecting unidirectional efflux of substrate from cells. Depolarization of membrane potentials with various concentrations of extracellular K<sup>+</sup> in the presence of valinomycin did not inhibit the ability of Pgp to reduce intracellular concentration of Tc-Sestamibi, strongly suggesting that the drug transport activity of MDR1 Pgp is independent of changes in E<sub>m</sub> or total ion conductance.

Tetraphenylborate, a lipophilic anion, enhanced unidirectional influx of Tc-Sestamibi to a greater extent in MCF-7/MDR1 cells than control cells, suggesting that Pgp may, directly or indirectly, increase the positive dipole potential within the plasma membrane bilayer.

Overall, these data demonstrate that changes in E<sub>m</sub> or macroscopic conductance are not coupled to function of Pgp in multidrug resistance. The dominant effect of MDR1 Pgp in this system is reduction of drug influx, possibly through an increase in intramembranous dipole potential.
**Introduction**

*MDR1* Pgp, a member of the ATP-binding cassette family of membrane transporters, decreases intracellular concentrations of structurally diverse compounds, many of which are hydrophobic and cationic. Pgp has been proposed to function as an efflux transporter, although no experiment has demonstrated unequivocally that Pgp mediates transport of a substrate across a membrane bilayer against its electrochemical gradient.

Several models have been proposed to account for the remarkable variety of compounds that are recognized by this protein. Pgp may be an efflux transporter that recognizes substrates within the lipid bilayer (“hydrophobic vacuum cleaner”) and/or cytoplasm (1). Pgp has been hypothesized to be a pump with multiple binding sites for different drugs (2), a translocase for lipids (3), or a modifier of vesicular trafficking (4). Another model suggests that *MDR1* Pgp indirectly alters partitioning of substrates within cells through effects on \( E_m \), intracellular pH (\( pHi \)), and/or surface potentials and does not directly transport drugs (5). These disparate hypotheses may result from comparisons of data from transfected cells that have not been exposed to chemotherapeutic agents with cell lines in which expression of *MDR1* is induced or maintained through exposure to drugs in the MDR phenotype. Pathways of resistance other than Pgp may exist in drug-selected cell lines, potentially confounding identification of properties attributable solely to *MDR1*. In addition, many studies have used “Pgp-negative” control cells that later were shown to express low levels of endogenous Pgp, further complicating interpretations regarding function of the protein itself.

We investigated *MDR1* Pgp transport activity using MCF-7 breast adenocarcinoma cells, which do not express *MDR1* (6), and MCF-7/*MDR1* cells in which we established and
maintained overexpression of \textit{MDR1} using a bicistronic vector in the absence of MDR drugs. Effects of Pgp on \(E_m\) were measured directly by whole-cell patch clamping and transport activity of the protein was studied with Tc-Sestamibi, an organotechnetium cationic substrate for Pgp (7,8). In the absence of \textit{MDR1} Pgp, Tc-Sestamibi accumulates within the mitochondrial matrix of living cells in response to negative mitochondrial inner membrane (\(\Delta \psi\)) and plasma membrane (\(E_m\)) potentials while showing negligible non-specific binding to lipids and proteins (9-11). Tc-Sestamibi has no titratable proton, making accumulation within cells independent of \(pHi\) (8,12). Using this system, we determined effects of \textit{MDR1} Pgp on cellular content of substrate during steady-state and unidirectional influx or efflux conditions. As proposed over 25 years ago (13), we find that the dominant effect of Pgp is to establish a permeability barrier, limiting unidirectional influx of Tc-Sestamibi without affecting \(E_m\).

\textbf{Experimental Procedures}

\textit{Materials and Buffers} – Stock solutions of GF120918 (a substituted isoquinoliny1 acridonecarboxamide; gift of Glaxo-Welcome) (14), LY335979 (a difluorocyclopropyl dibenzosuberane; gift of Eli Lilly and Co.) (15), tetraphenylborate, and valinomycin were prepared in dimethyl sulfoxide (DMSO). \(^{99m}\text{Tc}\)-Sestamibi was prepared as described (Cardiolite, Du Pont Medical Products Division, Billerica, MA) (12). \(^3\text{H}\)-daunomycin (1.9 Ci/mmol) was obtained from DuPont NEN. G418 was from Life Technologies (Rockville, MD). Trace metal grade nitric acid was from Fisher Scientific (Pittsburgh, PA). All other reagents were from Sigma-Aldrich Co. (St. Louis, MO).
Control solution for transport experiments was a modified Earle’s balanced salt solution (MEBSS) containing (mM): 145 Na⁺, 5.4 K⁺, 1.2 Ca²⁺, 0.8 Mg²⁺, 152 Cl⁻, 0.8 H₂PO₄⁻, 0.8 SO₄²⁻, 5.6 dextrose, 4.0 HEPES, and 1% calf serum, pH 7.4 (12). Calf serum was omitted for all electrophysiology experiments. A 142 mM K+/20 mM Cl⁻ solution was prepared by equimolar replacement of potassium methanesulfonate for NaCl (16).

**Cell culture, MDR1 plasmid, and transfection** – MCF-7 cells were cultured in DMEM (GIBCO, Grand Island, NY), 10% fetal bovine serum, and 0.1% penicillin/streptomycin in a 5% CO₂ incubator at 37°C. pGEM3Zf(-)Xba-MDR1.1 was purchased from the ATCC and sequenced to confirm wild-type identity. MDR1 was excised with XbaI and subcloned into pBluescript SK (Stratagene, La Jolla, CA). The cDNA for MDR1 was then removed with NotI and BamHI and ligated into corresponding sites of pIRESneo (Clontech, Palo Alto, CA). Cells were transfected with MDR1 or vector using Fugene 6 (Roche, Nutley, NJ). Clones of transfected cells were isolated and maintained in medium containing 1 mg/ml G418. Drug-sensitive (Pgp-) KB 3-1 and MDR (Pgp+) KB 8-5 human epidermoid carcinoma cells were grown and maintained as described (17).

**Western blots** – Pgp was detected in enriched membrane fractions of cells with mAb C219 (Signet, Dedham, MA) as described previously by our laboratory (17).

**Immunofluorescence microscopy** – Cells were processed for immunofluorescence microscopy with mAb C219 as described for detection of Pgp in KB 8-5 and CHO cells (18).

**Cytotoxicity Assay** – 72-hour cytotoxicity assays with daunomycin and paclitaxel were performed using sulforhodamine B (19). Data are expressed as per cent growth relative to cells treated with vehicle only.
Electrophysiology – Whole-cell current-clamp or voltage-clamp experiments were carried out at room temperature using an Axopatch 200B amplifier (20). Micropipettes were pulled from thin-walled glass (WPI Inc., New Haven, CT) on a horizontal puller (Sutter Instrument, Co., Novato, CA). Two different K$_{\text{INT}}$ solutions were used in pipettes: 1) 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.35 with KOH; or 2) 140 mM KCl, 10 mM Na-HEPES, 1 mM K-EGTA, free Ca$^{2+}$ 10$^{-8}$-10$^{-9}$ M, and 2 mM MgATP, pH 7.35 with KOH. The bath solution was serum-free MEBSS without or with drugs as indicated. PClamp 6.0 software and a DigiData 1200 converter were used to generate command pulses and collect data. Data were filtered at 5 kHz. Off-line analysis was performed using ClampFit and Microsoft Excel programs. Current-voltage relationships were generated from steady-state currents at 300 msec. Data are presented as mean ± SEM (standard error of the mean).

Cellular accumulation of $^{99m}$Tc-Sestamibi or $^3$H-daunomycin – Transport function and modulation of MDR1 Pgp under steady-state conditions were assayed with $^{99m}$Tc-Sestamibi (12). Transport assays with $^{99m}$Tc-Sestamibi were performed within three days of all electrophysiology experiments to confirm functional expression of Pgp in MCF-7/MDR1 cells. To determine unidirectional influx of $^{99m}$Tc-Sestamibi or $^3$H-daunomycin during short periods of incubation (≤ 90 sec), the protocol was modified as follows: 1) concentrations of $^{99m}$Tc-Sestamibi or $^3$H-daunomycin were 40 µCi/ml (5-10 pmol/mCi) or 0.2 µCi/ml (1.9 Ci/mmol), respectively; and 2) coverslips with cells were washed 4 times in ice-cold MEBSS. Cell associated tracer is expressed as fmol (mg P)$^{-1}$ (nM$_o$)$^{-1}$, where cell content of Tc-Sestamibi or daunomycin (fmol) is normalized to cell protein (mg P) and extracellular concentration of tracer (nM$_o$).
For efflux experiments, cells were first incubated for 30 min at 37°C in MEBSS containing 10 µCi/ml ⁹⁹ᵐTc-Sestamibi to reach steady-state accumulation of radiotracer (12). Coverslips were blotted briefly (~ 5 sec) to remove excess buffer, transferred to isotope-free MEBSS (37°C) for various times, and then washed in ice-cold MEBSS. To measure steady-state content of ⁹⁹ᵐTc-Sestamibi prior to efflux, incubation in isotope-free buffer was omitted. Accumulation of Tc-Sestamibi was quantified as above and expressed as a percentage of steady-state values for each cell line.

*Intracellular water space* – Volumes of intracellular water space were determined with 3-O⁻³H-methyl-D-glucose and phloretin (21). Data are expressed as µL intracellular water space/mg cell protein.

*Intracellular K⁺ concentration* – Cells were plated as described for accumulation of ⁹⁹ᵐTc-Sestamibi. Coverslips without cells were incubated in the same medium for use as controls, and values for these blank coverslips were subtracted from data for samples with cells. Cells were washed in phosphate-buffered saline prepared without potassium and extracted with 1% nitric acid for 30 minutes. Samples were microwaved to prepare for analysis, and each sample run included a 1% nitric acid solvent blank and an external standard. K⁺ content was determined by an inductively-coupled plasma atomic emission spectrometer (IRIS Advantage Duo-View System, Thermo Jarrell Ash Corp., Franklin, MA), using a method that scanned each sample three times and provided a data value with one sigma level of confidence. Additional details of the microwave protocol for sample preparation are available from the authors upon request. Data for µg of K⁺ were normalized to mg cell protein, and intracellular K⁺ concentration (K⁺ᵢ) in each cell line was calculated by dividing K⁺ content by intracellular water space.
Results

Characterization of MCF-7/MDR1 cells. Because cell lines derived from step-wise selection with MDR drugs may have multiple mechanisms of drug resistance (22), we stably transfected MCF-7 cells with MDR1 and established clonal cell lines that express Pgp without use of MDR drugs. Pgp was detected specifically by Western blotting in several different clones that expressed the protein at levels comparable to or greater than KB 8-5 cells, a cell line derived from selection in colchicine (Figure 1A) (23). A clone (3-4) with higher amounts of Pgp than KB 8-5 cells was selected for further characterization. In the remainder of the manuscript, this clonal cell line is referred to as MCF-7/MDR1 cells. Pgp localized predominantly to the plasma membrane in MCF-7/MDR1 cells, as determined by immunofluorescence microscopy (Figure 1B). No immunodetectable Pgp was present in control MCF-7 cells by Western blotting or immunofluorescence (Figure 1A and data not shown). Function of transfected MDR1 Pgp initially was characterized with Tc-Sestamibi. Compared with parental cells, steady-state accumulation of Tc-Sestamibi after 30 minutes of incubation was almost 70-fold less in MCF-7/MDR1 cells (55 ± 11 fmol (mg P)⁻¹ (nM_o)⁻¹ versus 0.8 ± 0.1 fmol (mg P)⁻¹ (nM_o)⁻¹, respectively). Content of radiotracer in MDR1 transfectants increased to control values when cells were incubated with a saturating dose of LY335979 (1 μM), a specific inhibitor of MDR1 Pgp (15) (Figure 1C). LY335979 did not enhance accumulation of Tc-Sestamibi in MCF-7 cells, providing further evidence that this cell line does not express MDR1 Pgp (6,24). Previously, our laboratory has shown that KB 8-5 cells accumulate approximately 50-fold less Tc-Sestamibi than Pgp-negative parental KB 3-1...
cells (17). Thus, these functional data are consistent with differences in relative expression of Pgp in MCF-7/MDR1 and KB 8-5 cells. Furthermore, to verify that transfected \textit{MDR1} conferred multidrug resistance to MCF-7/\textit{MDR1} cells, we performed 72-hour cytotoxicity assays with doxorubicin and paclitaxel, two validated substrates for Pgp (15,25). Compared with control cells, MCF-7/\textit{MDR1} cells also were approximately 100-fold resistant to doxorubicin and at least 50-fold resistant to paclitaxel as determined by 72-hour cytotoxicity assays (Figure 1D and E). Overall, these data demonstrate that functional \textit{MDR1} Pgp was expressed and localized correctly, conferring MDR to transfected MCF-7 cells. As determined by differences in accumulation of Tc-Sestamibi between MCF-7/\textit{MDR1} and control cells, function of Pgp was stable over at least seven months of continuous culture (data not shown). Therefore, these matched cell lines provided an appropriate system for biophysical analysis of \textit{MDR1} Pgp without confounding effects of prior exposure to MDR drugs.

\textbf{Effects of \textit{MDR1} Pgp on resting E_m and macroscopic conductance.} Previous studies suggest that \textit{MDR1} Pgp (26) and other ABC transporters (27) reduce E_m in cells. To determine directly if \textit{MDR1} Pgp alone affects resting E_m, we compared electrical properties of the cell lines by conventional whole-cell patch clamp. Using K_{INT} solution 1 (no ATP) in the pipette and MEBSS in the bath solution, E_m did not differ between cell lines, measuring $-36.4 \pm 6.0$ mV and $-32.9 \pm 3.7$ mV in control and MCF-7/\textit{MDR1} cells, respectively ($n = 7, 9; p > 0.25$) (Figure 2A). Because ATP-dependent K$^+$-channels have been shown to influence E_m of MCF-7 cells (28), we also determined E_m using a pipette solution (K_{INT} 2) that contained ATP. E_m of MCF-7 and MCF-7/\textit{MDR1} cells was $-42.8 \pm 6.6$ mV and $-47.6 \pm 3.3$ mV, respectively, using K_{INT} 2 ($n = 3, p > 0.48$). For both cell
lines, $E_m$ was more negative as measured with $K_{\text{INT}}$ 2, although differences were significant only for MCF-7/MDR1 cells ($p < 0.01$). However, $E_m$ did not differ between control cells and Pgp-transfectants under either experimental condition. In MEBSS buffer, an approximately two-log difference in $E_m$ would be required to account for the almost 70-fold reduction of Tc-Sestamibi in the Pgp-expressing line (Figure 1), assuming a constant $\Delta \psi$ in both cell lines. We also did not detect differences in macroscopic currents between MCF-7/MDR1 and control cells as measured with a pipette solution of either $K_{\text{INT}}$ 1 or $K_{\text{INT}}$ 2 (Figure 2B, 2C, and data not shown). Measurements of $E_m$ and macroscopic currents were stable for at least two minutes, implying that the patch clamp did not disrupt membrane integrity and allow leakage of ions or macromolecules from cells. In addition, no difference in $E_m$ was measured between Pgp(-) KB 3-1 cells and Pgp(+) KB 8-5 cells (data not shown) for which colchicine (an MDR drug) was used in the latter to select and maintain expression of MDR1 Pgp.

If MDR1 Pgp were to confer MDR through alterations in $E_m$, specific inhibitors of Pgp should hyperpolarize $E_m$ only in cells that express Pgp. To test this possibility, whole-cell patch clamping was performed with saturating doses of either LY335979 (1 $\mu$M) or GF120918 (300 nM), another potent inhibitor of MDR1 Pgp (14). While these inhibitors blocked drug transport by MDR1 Pgp within seconds after addition to cells (see below), neither $E_m$ nor macroscopic conductance was affected in either cell line during two minutes of monitoring (data not shown). Overall, the data directly demonstrate that expression of functional MDR1 Pgp without selection in MDR drugs does not alter basal $E_m$ or membrane conductance.
MDR1 Pgp impacts accumulation of Tc-Sestamibi even in the presence of depolarized membrane potentials. To investigate further the relationship of Pgp to ∆ψ and E_m, we sought to quantify accumulation of Tc-Sestamibi under conditions in which membrane potentials were altered by variation of extracellular K⁺ concentration (K⁺_o) in the presence of valinomycin. Because expression of a truncated form of MDR1 Pgp in yeast has been reported to alter K⁺_i under selected conditions (29), we determined K⁺_i in both parental and MCF-7/MDR1 cells. K⁺_i was not affected by expression of MDR1 Pgp: water spaces were 3.3 ± 0.2 and 3.3 ± 0.4 µl/mg protein (n = 12), and calculated K⁺_i values were 140 ± 7 mM and 144 ± 12 mM (n = 4) for parental and MCF-7/MDR1 cells, respectively.

We performed transport assays with the K⁺ ionophore valinomycin added to standard buffer (5.4 mM K⁺_o) (Figure 3A). Because intramitochondrial and cytosolic K⁺ concentrations are approximately equal (30), these conditions were predicted to depolarize ∆ψ toward zero and hyperpolarize E_m toward the K⁺ reversal potential. In MCF-7 cells, radiotracer content decreased from 43.2 ± 4.0 fmol (mg P)^−1 (nM_o)^−1 to 2.3 ± 0.6 fmol (mg P)^−1 (nM_o)^−1 in the absence or presence of valinomycin (1 µg/ml), respectively, consistent with depolarization of ∆ψ as the dominant determinant for reduction in accumulation of hydrophobic, cationic compounds in these cells (9,12,31). Steady-state accumulation of Tc-Sestamibi in MCF-7/MDR1 cells was 0.8 ± 0.1 fmol (mg P)^−1 (nM_o)^−1 and could not be detected above background when valinomycin was added to 5.4 mM K⁺ buffer (threshold of detection of ≈ 0.1 fmol (mg P)^−1 (nM_o)^−1). Addition of GF120918 (300 nM) or LY335979 (1 µM) increased net accumulation of Tc-Sestamibi in MCF-7/MDR1 cells to values observed in parental cells, while control cells
were unaffected (Figure 3A). These results indicate that *MDR1* Pgp did not reduce cell content of Tc-Sestamibi by decreasing steady-state $\Delta \psi$.

To depolarize both $E_m$ and $\Delta \psi$ to $\approx 0$ mV, we equilibrated $K^+_{i}$ and $K^+_{o}$ by incubating cells in 142 mM $K^+/20$ mM $Cl^-$ buffer containing valinomycin (1 $\mu$g/ml). $Cl^-$ was reduced to prevent high $KCl$ buffer-induced increases in cell volume mediated by $K^+/Cl^-$ co-transporters expressed in mammalian cells (32). Under isoelectric conditions, steady-state content of Tc-Sestamibi in MCF-7 cells was reduced to $0.92 \pm 0.07$ fmol (mg P)$^{-1}$ (nM$_o$)$^{-1}$, which is 2.5-fold less than that observed in buffer containing 5.4 mM $K^+$ (Figure 3B). By comparison, accumulation of Tc-Sestamibi in MCF-7/*MDR1* cells was reduced to background levels. Thus, *MDR1* Pgp either transported tracer out of cells against a significant concentration gradient to levels well below that expected for passive distribution into intracellular water space [calculated as 3.3 fmol (mg P)$^{-1}$ (nM$_o$)$^{-1}$] or produced a diffusion barrier that prevented equilibration of an inwardly directed concentration gradient for the tracer. When Pgp was inhibited with GF120918 (300 nM) or LY335979 (1 $\mu$M), accumulation of Tc-Sestamibi in MCF-7/*MDR1* cells increased to that observed in MCF-7 cells. Thus, while these data demonstrated that net content of Tc-Sestamibi was reduced in fully depolarized cells, *MDR1* Pgp lowered cell content of radiotracer to an amount less than that produced by reductions in membrane potentials alone.

**Effects of *MDR1* Pgp on unidirectional influx and efflux of substrate.** Steady-state reduction in cell content of Tc-Sestamibi produced by Pgp could be due to alterations in influx (permeability) and/or efflux (active transport). To determine the relative
contribution of each component, we first quantified cell-associated Tc-Sestamibi over the initial 90 seconds of exposure to radiotracer. Uptake of Tc-Sestamibi in control cells was linear throughout this period and did not reach steady-state until ≈ 30 minutes, indicating that these early time points represented unidirectional influx of radiotracer (Figure 4A). In MCF-7/MDRI cells, accumulation of radiotracer reached a plateau of 1.0 ± 0.04 fmol (mg P)^{-1} (nM_o)^{-1} at 60 seconds, a level maintained throughout 120 minutes of incubation. At the earliest time point (10 seconds), content of Tc-Sestamibi in MCF-7/MDRI cells was approximately 7-fold less than control. Furthermore, effects of MDRI Pgp were completely reversed within 10 seconds by GF120918 (300 nM) or LY335979 (1 μM) (Figure 4B and C), providing additional evidence that MDRI Pgp markedly reduces influx of Tc-Sestamibi. To determine if Pgp also limits unidirectional influx of a different substrate, we measured net content of daunomycin at time points between 10 and 90 seconds in these cells. Similar to Tc-Sestamibi, cell content of daunomycin in MCF-7/MDRI cells was less than control cells at all time points, although differences did not become statistically significant until 30 seconds of influx (Figure 4D). However, unlike Tc-Sestamibi, cell-associated daunomycin in the MDRI-transfectants did not reach a plateau during the initial 90 seconds of incubation, which may be due to partitioning of anthracyclines into lipid bilayers (33).

To determine if MDRI Pgp also affects unidirectional efflux of Tc-Sestamibi, cells were incubated with Tc-Sestamibi for 30 minutes to reach steady-state levels of radiotracer and then transferred to isotope-free buffer (zero trans conditions). During the initial 30 seconds of efflux, cell-associated Tc-Sestamibi was not affected by Pgp, as evidenced by an approximately 30% decrease in radiotracer in both cell lines (Figure
Furthermore, LY335979 (1 µM) did not alter 30-second unidirectional efflux of Tc-Sestamibi in either control or MCF-7/MDR1 cells. Per cent steady-state content of Tc-Sestamibi after 30 seconds of efflux in the absence or presence of LY335979 was 73.1% and 70.4% in control MCF-7 cells, respectively, and 69.3% and 71.2% in MCF-7/MDR1 transfectants, respectively. Comparable results were observed when parental cells were loaded with 40-fold less radiotracer than MCF-7/MDR1 cells to achieve similar amounts of cell-associated Tc-Sestamibi at the start of efflux (data not shown). When incubations in isotope-free buffer were extended to longer periods of time, significant differences between cell lines were not detected until 5 minutes and were maintained over the ensuing 30 minutes (Figure 5B). Control cells retained 67.1 ± 8.4% and 26.2 ± 4.7% of initial content of Tc-Sestamibi after 5 and 30 minutes of efflux, respectively, compared with 22.2 ± 2.4% and 7.9 ± 2.2% in MCF-7/MDR1 cells, respectively. During these longer incubations, LY335979 (1 µM) increased cell content of Tc-Sestamibi in MCF-7/MDR1 cells to levels observed in parental cells (Figure 5B). However, these extended incubation times yielded data reflecting combined effects of efflux and contaminating influx (re-entry) of substrate into cells and no longer isolated unidirectional efflux kinetics. Overall, these studies demonstrate that MDR1 Pgp has no significant effect on initial efflux of Tc-Sestamibi from MCF-7/MDR1 cells.

Although MDR1 Pgp did not affect resting $E_m$ of cells as measured by whole-cell patch clamp, we considered that Pgp could directly or indirectly alter the dipole potential within the plasma membrane, producing a more positive intramembranous potential and limiting influx of cationic substrates like Tc-Sestamibi. Accordingly, tetraphenylborate (TPB), a lipophilic anion that imposes a negative dipole potential within lipid bilayers...
should reduce the effective intramembranous potential and preferentially enhance accumulation of Tc-Sestamibi in MCF-7/MDR1 cells. Previously, we showed that TPB enhances accumulation of Tc-Sestamibi in cultured cells, although effects of MDRI Pgp were not determined (12). In the presence of TPB, content of Tc-Sestamibi after 30 seconds of influx increased in both cell lines in a concentration-dependent manner (Figure 6A). However, the fold change in accumulation of radiotracer with TPB was significantly higher in Pgp-expressing cells. Influx of Tc-Sestamibi increased by 50-fold and 360-fold in control and MCF-7/MDR1 cells, respectively, when 30 µM TPB was added to buffer (Figure 6B). At concentrations greater than 30 µM TPB, accumulation of radiotracer decreased in both cell lines, likely due to toxicity (data not shown). When Pgp was inhibited with LY335979 (1 µM), influx of Tc-Sestamibi in the presence of TPB did not differ between MCF-7/MDR1 and parental cells, further indicating that functional MDRI Pgp mediated the differential effects of TPB on accumulation of radiotracer.

Discussion

We have critically examined potential mechanisms of substrate transport by MDRI Pgp, using stringent reagents and tools to determine effects mediated solely by Pgp. Our results demonstrate that MDRI Pgp can confer MDR without affecting resting $E_m$ or macroscopic conductance of the plasma membrane in cells not previously exposed to MDR drugs. Conversely, under conditions that depolarized $E_m$ and $\Delta\psi$, MDRI Pgp reduced net cell content of Tc-Sestamibi to levels below those produced by external manipulation of membrane potentials alone, showing that transport of Tc-Sestamibi by Pgp is not dependent on changes in transmembrane potential. Furthermore, specific
inhibitors of *MDR1* Pgp blocked transport activity without altering $E_m$ or macroscopic conductance of Pgp-positive or negative cells.

*MDR1* Pgp has been associated with a variety of perturbations in ion conductance. For example, enhanced Na$^+$ currents were measured in cell lines selected for expression of Pgp through drug exposure (35,36), but changes in Na$^+$ conductance were not coupled to drug transport (36). Despite changes in Na$^+$ currents, $E_m$ did not differ between drug-sensitive and drug-selected MDR cells, although effects of *MDR1* potentially could have been masked by effects of selection in chemotherapeutic drugs. Prior studies with LR73 cells reported that the resting $E_m$ of $-46$ mV in these cells was reduced by approximately 20 mV in cells transfected with *MDR1*. These measurements were made fluorometrically with K$^+$/valinomycin null point titration (37,38). In the current study, $E_m$ in control MCF-7 cells was $-43$ mV, as determined by whole-cell patch clamping with ATP in the pipette solution (K$_{INT}$ solution 2) and not significantly different in *MDR1* transfectants. These data argue strongly against a hypothesis that Pgp only depolarizes cells that are above a threshold value for $E_m$. Furthermore, the magnitude of depolarization attributed to *MDR1* Pgp in previous studies cannot account for the large differences in accumulation of Tc-Sestamibi between control and MCF-7/*MDR1* cells.

Ion channel activities have been associated with expression of *MDR1* Pgp in response to various stimuli. Pgp has been reported to mediate ATP- and Na$^+$-dependent Cl$^-$/H$^+$ antiport in response to rapid changes in extracellular ion gradients, thus alkalinizing cells and contributing to MDR (39). *MDR1* Pgp also has been reported to function as, or regulate, a swelling-activated Cl$^-$ channel (40). Our study did not investigate ion conductances stimulated by changes in ion gradients or channel activity...
during hypo-osmotic stress, but our data indicate that these conditions are not necessary to activate drug transport. While our data do not exclude possible channel activity associated with \textit{MDR1} Pgp under selected conditions, the results show that Pgp does not change steady-state currents in MCF-7 cells and imply that such alterations are not essential for drug resistance.

The present data show that \textit{MDR1} Pgp reduces intracellular content of Tc-Sestamibi by limiting influx without affecting unidirectional efflux. Most studies of \textit{MDR1} Pgp have reported that the transporter both reduces influx and enhances efflux of substrates, although exceptions have been described in which Pgp affects only influx (41) or efflux (42) of compounds. As indicated by data from our efflux protocol, content of substrate in non-polarized MCF-7/\textit{MDR1} cells was reduced only at time points beyond the experimental conditions of unidirectional efflux. Differences between our data and previous studies could be due to prior determinations at time points beyond the period of unidirectional efflux; the presence of multiple mechanisms of MDR in drug-selected cells; use of substrates with varying amounts of partitioning into membranes or trapping in subcellular compartments; or disparities between effects of Pgp in non-polarized versus polarized cells. Nevertheless, our system shows that transfection of \textit{MDR1} functionally establishes a permeability barrier to influx of Tc-Sestamibi. The “membrane vacuum cleaner” model for Pgp in effect predicts the same behavior (43). However, our previously published data show an identical in-to-out content ratio for Tc-Sestamibi in Pgp-expressing cells when assayed over a seven-log range in extracellular tracer concentration (pM to 10 \(\mu\)M), both in the absence and presence of the MDR modulator quinidine (12). The lack of sigmoidal activation with no evidence of saturable behavior
at high substrate concentrations is attributed best to a process dominated by diffusion rather than enzyme kinetics (39). As a whole, these data favor a model whereby Pgp establishes a permeability barrier to influx of substrates.

The mechanism through which Pgp establishes a permeability barrier remains to be identified. TPB preferentially enhanced influx of Tc-Sestamibi in MCF-7/MDRI cells, suggesting that Pgp may act by increasing the net positive dipole potential within the plasma membrane. The dipole potential is manifest between the hydrophobic interior of a bilayer and water molecules immediately adjacent to lipid head groups (44). In bilayers of phosphatidylcholine, the dipole potential within the membrane is calculated to be approximately +280 mV (45), which impedes diffusion of hydrophobic cationic compounds such as Tc-Sestamibi. If MDRI Pgp increased the intramembranous dipole potential to more positive values, influx of Tc-Sestamibi or other positively charged, hydrophobic compounds would be retarded. TPB, a hydrophobic anion, is predicted to associate with phospholipid membranes near the polar head groups, thereby reducing intramembranous dipole potentials and enhancing kinetics of cation translocation (46). Ion pairing effects on membrane solubility of substrates also may contribute to effects of TPB.

MDRI Pgp potentially could affect intramembranous dipole potentials by altering the distribution of lipids, such as sphingomyelin (47) or glucosylceramide (48), between inner and outer leaflets of the plasma membrane. Pgp localizes to low-density membrane domains (18,49), in which the magnitude of the dipole potential is increased due to enrichment with cholesterol (50). Interestingly, while TPB behaves as a modulator of Pgp, tetraphenylphosphonium, a hydrophobic cation that is the counterpart of TPB, is a
substrate for the transporter (51). Verapamil, a classic inhibitor of MDRI Pgp, also has been shown to decrease the intramembranous dipole potential in lipid vesicles (52), which would directly enhance influx kinetics of hydrophobic cations like Tc-Sestamibi. Consistent with this model, effects of TPB on dipole potential would be expected to impact the kinetics of transmembrane permeation, but not the final steady-state (46). Accordingly, maximal effects of TPB on cell content of Tc-Sestamibi were identical between Pgp-expressing and non-expressing cells. Another mechanism related to this model is that altering the dipole potential indirectly inhibits Pgp transport by changing lipid-protein interactions on the intramembranous surface of the transporter. Previous work has shown that function of MDRI Pgp is affected significantly by changes at the lipid-protein interface (53,54).

In summary, we have shown that MDRI Pgp significantly decreased influx of a cationic, organotechnetium substrate without affecting resting Em. However, Pgp did not alter unidirectional efflux of Tc-Sestamibi. These data suggest that the dominant function of MDRI Pgp in non-polarized cells is to produce a permeability barrier for Tc-Sestamibi, perhaps by altering the dipole potential within plasma membranes. These results provide features that should be incorporated into any comprehensive model of the mechanism of action for this transporter.
Acknowledgements

We thank Rachel Lindvall for mass spectrometry determinations and Julie Dahlheimer for technical assistance. These studies were supported by grants from the NIH (HL03683) and DOE (ER61885).
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**Figure Legends**

**Figure 1.** Expression and function of *MDR1* Pgp in MCF-7 cells. (A) Total membrane proteins (20 μg) of MCF-7 and clonal cell lines transfected with *MDR1* Pgp were separated by SDS-PAGE and immunoblotted for Pgp with mAb C219. Lane 1, MCF-7 cells; Lane 2, clone 3-4; Lane 3 KB 8-5 cells; Lane 4, clone 2-2; Lane 5, clone 3-5. Based on molecular size markers, the arrow indicates 170 kDa. (B) Immunofluorescence microscopy of MCF-7/*MDR1* cells (colony 3-4 in panel A) with mAb C219 and secondary antibody labeled with FITC. (C) Content of Tc-Sestamibi in MCF-7 (closed bar) and MCF-7/*MDR1* (open bar) cells was determined after 30 minutes of incubation in MEBSS without or with LY335979 (1 μM) as described in Experimental Procedures. Data are representative of 2 independent experiments with n = 4 for each condition. Error bars denote SEM in this and subsequent figures. (D, E) MCF-7 (△) and MCF-7/*MDR1* (◇) cells were incubated with increasing concentrations of doxorubicin (D) or paclitaxel (E) and harvested after 72 hours for determinations of cell survival as described in Experimental Procedures. Data are per cent control for n = 3 at each concentration.

**Figure 2.** *MDR1* Pgp does not alter $E_m$ or macroscopic conductance. (A) Mean resting $E_m$ in MCF-7 and MCF-7/*MDR1* cells as measured by whole-cell patch clamping using $K_{\text{INT}}$ solution 1 (open bars) or $K_{\text{INT}}$ solution 2 (cross-hatched bars) in the pipette as described in Experimental Procedures and MEBSS as the bath solution. (B) Representative families of macroscopic currents in MCF-7 and MCF-7/*MDR1* cells elicited by voltage clamp steps from –80 to +80 mV in 20 mV increments. The pipette contained $K_{\text{INT}}$. 
solution 1. The solid line to the left of each trace denotes zero current level. (C)

Composite current-voltage plots of MCF-7 (■, ●) and MCF-7/MDR1 (□, O) cells with K_{INT} solution 1 (■, □) and K_{INT} solution 2 (●, O). ATP is present in K_{INT} solution 2.

**Figure 3.** Pgp transports Tc-Sestamibi under conditions that depolarize E_{m} and Δψ.

Content of Tc-Sestamibi in MCF-7 (closed bar) and MCF-7/MDR1 (open bar) cells after 30 minutes in MEBSS buffer containing valinomycin (1 µg/ml) and either 5.4 mM K^{+}/152 mM Cl^{-} (A) or 142 mM K^{+}/20 mM Cl^{-} (B). Assays were performed without or with saturating concentrations of GF120918 (300 nM) or LY335979 (1 µM). Data are representative of two independent experiments with n = 3 for each condition.

**Figure 4.** MDR1 Pgp limits unidirectional influx of substrates in MCF-7 (▲) and MCF-7/MDR1 (●) cells. (A) Cells were incubated with Tc-Sestamibi in MEBSS buffer for various times between 10 and 90 seconds as described in Experimental Procedures. The inset shows accumulation of Tc-Sestamibi throughout 120 minutes of incubation with radiotracer in MEBSS. (B and C) Effects of GF120918 (300 nM; B) and LY335979 (1 µM; C) on cell content of radiotracer also were determined during unidirectional influx at times between 10 and 90 seconds in MCF-7 (▲) and MCF-7/MDR1 (●) cells. (D) MCF-7 (▲) and MCF-7/MDR1 (●) cells were incubated with 0.2 µCi/ml ³H-daunomycin, and cell-associated radiotracer was determined at time points between 10 and 90 seconds as described in Experimental Procedures. Data are representative of three (A) or two (B and C) independent experiments with n = 3 at each point. Data for (D) are from a single experiment with n = 4 for each point.
**Figure 5.** Pgp does not affect initial efflux of Tc-Sestamibi. (A) MCF-7 (Δ) and MCF-7/MDR1 (◆) cells were loaded to steady-state content of radiotracer for 30 minutes and then transferred to isotope-free MEBSS. Content of Tc-Sestamibi was determined at one-second intervals from 3 to 30 seconds. Data are mean values of two samples each from independent experiments. (B) Efflux experiments were continued through 30 minutes in MEBSS only [MCF-7 (Δ) and MCF-7/MDR1 (◆) cells], or buffer containing 1 µM LY335979 [MCF-7/MDR1 cells (◊)]. Data are mean values from three experiments, with n = 9 samples for each data point.

**Figure 6.** Tetraphenylborate (TPB) preferentially enhances influx of Tc-Sestamibi in MCF-7/MDR1 cells. (A) Influx of Tc-Sestamibi in MCF-7 (▲, Δ) and MCF-7/MDR1 (◆, ◊) cells after 30 seconds in buffer containing TPB, without (▲, ◆) or with (Δ, ◊) LY335979 (1 µM). Note logarithmic scales. (B) Fold increase in cell content of Tc-Sestamibi in the presence of 30 µM TPB, relative to buffer with no TPB, without (closed bars) or with (open bars) 1 µM LY335979. Data are representative of three independent experiments with n = 3 for each data point.
Figure 1

A

1  2  3  4  5

B

C

Te-Sesamthi (μmol/mg P. fum. A. m.)

MEBSS  LY335979

D

% Control

E

% Control

[paclitaxel] nM

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Figure 2

A

![Graph showing EM (mV) for MCF-7 and MCF-7/MDRI](graph)

B

![Graph showing MCF-7 and MCF-7/MDRI](graph)
Figure 3

A

B
Figure 4A

Graph showing the relationship between time (sec) and Tc-Sestamibi (fmol/mg P-nM).
Figure 5

A

B

% Steady-State Content of Tc-Sestamibi

Efflux Time (sec)

% Steady-State Content of Tc-Sestamibi

Efflux Time (min)
Figure 6

A

![Graph A]

B

![Graph B]
MDR1 P-glycoprotein reduces influx of substrates without affecting membrane potential
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J. Biol. Chem. published online October 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105192200

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