# Characterization of Multidrug Resistance P-Glycoprotein Transport Function with an Organotechnetium Cation†

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*Received March 3, 1995; Revised Manuscript Received July 19, 1995*⊗

ABSTRACT: Multidrug resistance (MDR) in mammalian cells and tumors is associated with overexpression of an ~170 kDa integral membrane efflux transporter, the *MDR1* P-glycoprotein. Hexakis(2-methoxyisobutyl isonitrile)technetium(I) (Tc-SESTAMIBI), a $\gamma$-emitting lipophilic cationic metallopharmaceutical, has recently been shown to be a P-glycoprotein transport substrate. Exploiting the negligible lipid membrane adsorption properties of this organometallic substrate, we studied the transport kinetics, pharmacology, drug binding, and modulation of P-glycoprotein in cell preparations derived from a variety of species and selection strategies, including SW-1573, V79, Alex, and CHO drug-sensitive cells and in 77A, LZ-8, and Alex/A.5 MDR cells. Rapid cell accumulation ($t_{1/2} \approx 6$ min) of the agent to a steady state was observed which was inversely proportional to immunodetectable levels of P-glycoprotein. Many MDR cytotoxic agents inhibited P-glycoprotein-mediated Tc-SESTAMIBI efflux, thereby enhancing organometallic cation accumulation. Median effective concentrations ($EC_{50}$; $\mu$M) were as follows: vinblastine, 13; daunomycin, 55; idarubicin, 65; actinomycin D, 235; colchicine, minimal inhibition; adriamycin, no effect. P-glycoprotein modulators generally demonstrated significantly greater potency ($EC_{50}$; $\mu$M): SDZ PSC 833, 0.08; cyclosporin A, 1.3; verapamil, 4.1; quinidine, 6.4; prazosin, >300. Modulator-induced enhancement up to 100-fold was observed with Hill coefficients ≈1, consistent with simple Michaelis−Menten kinetics. Vanadate was an efficacious transport inhibitor, while agents usually not included in the MDR phenotype were without effect. Scatchard analysis showed quinidine to be a noncompetitive inhibitor of P-glycoprotein-mediated Tc-SESTAMIBI transport, indicating allosteric effector sites on P-glycoprotein. The lipid bilayer adsorbing agents tetraphenyl borate and phloretin induced large increases in final Tc-SESTAMIBI accumulation, showing maximal accumulations 2-fold greater than classic MDR modulators and Hill coefficients ≫ 2. In V79 and 77A cells, modulators of PKC activity altered Tc-SESTAMIBI accumulation, while there was no indication of modulation of P-glycoprotein-mediated Tc-SESTAMIBI transport by hypotonic buffer, extracellular ATP, $Cl^-$, or $K^+$ (membrane potential). While recognized and avidly transported by the P-glycoprotein at buffer concentrations as low as 7 pM, Tc-SESTAMIBI at up to 100 $\mu$M only minimally modulated the cytotoxic action of colchicine, doxorubicin, or vinblastine in MDR cells. In conclusion, transport analysis with Tc-SESTAMIBI is a sensitive assay for detecting functional expression of low levels of P-glycoprotein and for the quantitative characterization of transporter modulation and regulation. The biochemical data favor a high $K_m$, high capacity allosterically modulated translocation mechanism for P-glycoprotein-mediated transport of this organometallic cation. In addition, the known physicochemical properties of Tc-SESTAMIBI combined with effects of the membrane adsorbing agents indicate that lipid−protein interactions are critical for transport of this metallopharmaceutical and would suggest that this drug gains access to protein transport domains from the lipid phase with rapid time constants.

One mechanism of multidrug resistance (MDR)[1] in mammalian cells and tumors is overexpression of a small gene family that encodes an ~170 kDa integral membrane protein, the P-glycoprotein (Gros et al., 1986; Shen et al., 1986). A member of the ATP-binding cassette (ABC) transporter family, P-glycoprotein is expressed in the plasma membrane

† This work was supported by grants from the Department of Energy (ER61885), National Institutes of Health (HL42966), Radiological Society of North America, and the American Cancer Society (JFRA-420). J.M.C. is a recipient of a Sandoz Pharmaceuticals grant. D.P.-W. is an Established Investigator of the American Heart Association.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: CCCP, carbonyl cyanide-*m*-chloro phenylhydrazone; CHO-DUKX, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; $EC_{50}$, half-maximal effective concentration; H7, isoquinolinylsulfonyl methylpiperazine; IAP, iodoaryl azidoprazosin; LC50, half-maximal lethal concentration; MDR, multidrug resistance; MEBSS, modified Earle's balanced salt solution; PdBu, phorbol dibutyrate; SRB, sulforhodamine B; Tc-SESTAMIBI, hexakis(2-methoxyisobutyl isonitrile) technetium(I).

0006-2960/95/0434-12210$09.00/0 

of multidrug resistant cells and appears to function as an energy-dependent efflux pump for a broad spectrum of natural product and synthetic chemotherapeutic agents, including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxol (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Clinical studies have shown that elevated levels of *MDR1* mRNA and P-glycoprotein are associated with chemotherapeutic failure in many types of cancer (Goldstein et al., 1989; Verrelle et al., 1991). It is thought that, by decreasing the intracellular content of chemotherapeutic drugs, *MDR1* P-glycoprotein contributes to tumor chemoresistance.

Circumvention of multidrug resistance has become one major target for new approaches to cancer chemotherapy (Ford & Hait, 1990). An expanding array of drugs has been found to interact with P-glycoprotein and reverse the multidrug resistance phenotype *in vitro,* including calcium channel blockers, quinine derivatives, calmodulin antagonists, isoprenoids, tamoxifen, reserpine analogs, cyclosporins, triazinoaminopiperidines, acridonecarboxamides, and FKBP binding ligands (Ford & Hait, 1990; Boesch et al., 1991; Pierre et al., 1992; Hyafil et al., 1993; Gleason-Guzman et al., 1994). These compounds, termed MDR reversal agents or modulators, possess diverse chemical features, but tend to be hydrophobic and positively charged at physiological pH (Pearce et al., 1989; Ford & Hait, 1990). When cultured cells *in vitro* and tumor models *in vivo* are exposed to these compounds, the agents characteristically increase the concentrations of cytotoxic agents within the cells, presumably by competing for common binding sites on the MDR efflux transporter or altering affinities of recognition domains within the protein.

We recently reported that hexakis(2-methoxyisobutyl isonitrile)technetium(I) (Tc-SESTAMIBI), a $\gamma$-emitting organometallic cation sequestered by mitochondria (Piwnica-Worms et al., 1990), is recognized as a transport substrate by the P-glycoprotein expressed in multidrug resistant rodent fibroblasts and human tumor cells (Piwnica-Worms et al., 1993) and by recombinant human *MDR1* P-glycoprotein overexpressed in insect cells (Rao et al., 1994). This metallopharmaceutical lacks the usual structural moieties previously thought important for recognition by P-glycoprotein, such as basic nitrogen atoms, titratable protons, or phenyl groups (Pearce et al., 1989), but nonetheless appears to be recognized and efficiently extruded from cells expressing the transporter. Furthermore, tracer analysis in phosphatidylcholine vesicles (Chernoff et al., 1993) and intact cells (Piwnica-Worms et al., 1990), HPLC of tissue extracts (Carvalho et al., 1992), and Tc-99 NMR spectroscopy of whole cell preparations (Piwnica-Worms et al., 1994) have indicated that nonspecific lipid partitioning (adsorption) of the radioprobe into membrane bilayers is a negligible fraction of total cell- and vesicle-associated drug. Thus, while the drug can rapidly permeate lipid bilayers, it resides primarily in the aqueous phases.

We hypothesized that Tc-SESTAMIBI could enable quantitative characterization of the transport properties of P-glycoprotein with a model substrate lacking significant lipid adsorption to evaluate lipophilic cation permeation and protein−lipid bilayer interactions. The physicochemical properties of Tc-SESTAMIBI combined with the results of this study indicate that transport of this organometallic cation is consistent with an allosteric transport model and further indicate that lipid−protein interactions are critical for the substrate to gain access to transport domains.

## MATERIALS AND METHODS

*Solutions and Reagents.* Stock solutions of actinomycin D, adenosine, adriamycin, amiloride, AZT, bumetanide, cisplatin, CCCP, colchicine, daunomycin, dipyridamole, geneticin, glybenclamide, methotrexate, ouabain, phloretin, quinidine, tetraphenyl borate, verapamil, and vinblastine (Sigma Chemical Co., St. Louis, MO), phorbol dibutyrate (PdBu) and isoquinolinylsulfonyl methylpiperazine (H7; gift of Tom Roberts, Dana Farber Cancer Institute), idarubicin, and SDZ PSC 833 (gift of Sandoz Pharmaceuticals) were prepared with dimethyl sulfoxide (DMSO). Final DMSO concentration in experimental buffers was typically <1%, which has been found to have no effect on Tc-SESTAMIBI net uptake in cultured cells (Chiu et al., 1990). Cyclosporin A (Sandoz Pharmaceuticals) was added directly to the buffers at the concentrations indicated.

Control solution for transport experiments was a modified Earle's balanced salt solution (MEBSS) containing (mM): 145 $Na^+$, 5.4 $K^+$, 1.2 $Ca^{2+}$, 0.8 $Mg^{2+}$, 152 $Cl^-$, 0.8 $H_2PO_4^-$, 0.8 $SO_4^{2-}$, 5.6 dextrose, 4.0 HEPES, and 1% bovine calf serum (v/v), pH 7.4 ± 0.05. A 150 mM KCl or potassium-methanesulfonate solution was made by equimolar substitution of NaCl as described (Piwnica-Worms et al., 1983). Hypotonic buffer (220 mOsm) was made by reduction of NaCl. Sodium gluconate buffer was made by equimolar substitution of NaCl. $MnCl_2$, $NiCl_2$, and $ZnCl_2$ were added directly to the buffers, but in the case of $ZnCl_2$ buffer, $NaH_2PO_4$ was deleted and $MgCl_2$ replaced $MgSO_4$ to minimize formation of precipitates.

*Cell Culture.* Monolayers of Chinese hamster V79 lung fibroblasts and the adriamycin-selected 77A and LZ-8 derivative cell lines were grown as described (Howell et al., 1984). Briefly, cells were plated in 100-mm Petri dishes containing seven 25-mm glass coverslips on the bottom and grown to confluence in α-MEM (GIBCO, Grand Island, NY) supplemented with L-glutamine (2 mM), penicillin/streptomycin (0.1%), and fetal calf serum (10%) in the presence of 0, 0.1, and 8 μg/mL adriamycin, respectively. Human SW-1573 non-small cell lung carcinoma cells were grown in supplemented α-MEM without adriamycin (Arceci et al., 1993). Chinese hamster ovary cells (CHO-DUKX) carrying the PED-25 expression vector containing DHFR were grown as described (Kaufman et al., 1991).

*Preparation of $^{99m}$Tc-SESTAMIBI and $^{99}$Tc-SESTAMIBI.* Synthesis of the radiolabeled compound $^{99m}$Tc-SESTAMIBI was performed with a one-step kit formulation (Cardiolite, kindly provided by E. I. Du Pont, Medical Products Division, Billerica, MA) containing solid stannous chloride (0.075 mg) as a reducing agent and MIBI as the $Cu(MIBI)_4BF_4$ salt. To the vial was added 2 mL of normal saline containing technetium (5−10 mCi of $^{99m}TcO_4^-$, 4−25 pmol/mCi, from a standard Mo/Tc generator), and the resultant solution was heated (100 °C) for 15 min in a water bath and then cooled. Product was purified chromatographically using 95% ethanol as the final eluent as described (Piwnica-Worms et al., 1990). Radiochemical purity was found to be >97% by thin-layer chromatography (aluminum oxide plates; J. T. Baker, Phillipsburg, NJ) using ethanol (absolute) as the mobile phase. Radiotracer vehicle (ethanol) concentration in the experi-

mental buffers was <0.5%, previously shown to have no effect on $^{99m}$Tc-SESTAMIBI cell transport kinetics (Piwnica-Worms et al., 1990).

Carrier-added $^{99}$Tc-SESTAMIBI was prepared by dissolving, in 0.5 mL of 95% ethanol, 8−10 mg (10−12 μmol) of solid $^{99}$Tc-$(MIBI)_6$ chloride powder that was synthesized, purified, and characterized as described previously (Kronauge et al., 1991; Van Wyk et al., 1991).

*Cell Tracer Studies.* Coverslips with confluent cells were used for cell kinetic studies as described with minor modification (Piwnica-Worms et al., 1993). In general, tracer uptake and retention experiments were initiated by washing coverslips with cells in control buffer and then immersing in 60-mm glass Pyrex dishes containing 4 mL of loading solution consisting of MEBSS with 0.1−3.0 nM [Tc-SESTAMIBI] (1−10 μCi/mL). Cells on coverslips were removed at various times, rinsed three times in 25 mL of ice-cold (2 °C) isotope-free solution for 8 s each to clear extracellular spaces, and placed in 35-mm plastic Petri dishes. Cells were extracted for ≥30 min in 1% sodium dodecyl sulfate solution containing 10 mM sodium borate, after which aliquots were obtained for γ counting and for protein assay by the method of Lowry et al. (1951), using bovine serum albumin as the protein standard. Aliquots of the loading buffer and stock solutions were also obtained for normalizing cellular counts to the extracellular concentration of Tc-SESTAMIBI ($nM_o$). Cell extracts, stock solutions, and extracellular buffer samples were assayed for γ activity in a well-type sodium iodide γ counter (Minaxi 5000, Packard, Meriden, CT). Knowledge of the elution history of the Mo/Tc generator and activity of stock solutions allowed use of generator equilibrium equations to calculate the absolute concentration of total Tc-SESTAMIBI in the solutions (Lamson et al., 1975). Data are presented as fmol of Tc-SESTAMIBI (mg of protein)$^{-1}$ $(nM_o)^{-1}$.

Concentration−effect curves for each MDR reversal agent and other pharmacological compounds were generated from 15 min Tc-SESTAMIBI accumulation experiments in the absence or presence of various concentrations of test agent. Control experiments indicated that a steady-state accumulation of the radiolabel was achieved by 15 min with these cells (Piwnica-Worms et al., 1993).

For washout experiments, cells on coverslips were equilibrated in loading solution for 15 min, washed three times in ice-cold control buffer, and then incubated in isotope-free control buffer (37 °C). Preparations were removed at various times and assayed for retained cell-associated counts as described above.

For evaluation of the effects of phosphorylation state of P-glycoprotein on transport function, V79 cells were preincubated for 10 min or 24 h in 100 ng/mL PdBu or in 50 μM H7 prior to initiating 15-min radiotracer uptake assays as above in the continued presence of drug.

*Cell Survival and Modulation Studies.* Survival of parental and multidrug-resistant cell lines exposed to colchicine or adriamycin and the modulating effect of carrier-added $^{99}$Tc-SESTAMIBI were assayed in 96 well microtiter plates (Piwnica-Worms et al., 1993). Cells (4000−20 000) were plated with increasing concentrations of cytotoxic agents in the absence or presence of the indicated concentration of $^{99}$Tc-SESTAMIBI and incubated for 72 h at 37 °C in triplicate in three separate experiments. Multidrug-resistant cells were cultured in drug-free media for 72−96 h prior to cytotoxicity assays. Cell survival was assayed using sulforhodamine B (SRB) (Mazzanti et al., 1990). Cells were fixed in 10% trichloroacetic acid for 60 min at 4 °C, washed 5 times with tap water, and stained with 0.4% SRB in 1% acetic acid for 15 min at room temperature. Excess SRB was removed with four 1% acetic acid washes and the stain redissolved in 10 mM unbuffered Tris base. Quantitation was carried out using an ELISA plate reader at a wavelength of 550 nm. Survival was expressed as the percentage of surviving cells relative to growth in MEM media without cytotoxic agent. $LC_{50}$ determinations were obtained by interpolation from the cell survival curves.

*Photolabeling of P-Glycoprotein.* Plasma membrane-enriched fractions from parental (V79) and multidrug resistant (LZ-8) cultured cells were isolated with a high speed spin (100000*g*) after Dounce homogenization in 10 mM HEPES-buffered 0.25 M sucrose (pH 7.3) and removal of nuclei and unbroken cells with a low speed spin (600*g*). Fifty micrograms of membranes in a final volume of 50 μL was incubated with 2.5 nM [$^{125}$I]iodoaryl azidoprazosin (IAP; 2200 Ci/mmol) for 60 min at 25 °C in the dark (Greenberger et al., 1990; Piwnica-Worms et al., 1993). $^{99}$Tc-SESTAMIBI, prazosin, quinidine, cyclosporin A, and MgATP were included at the concentrations shown. The sample was irradiated with an UV lamp (UVP, Model UVL-56, 366 nm wavelength) for 10 min at 25 °C and fractionated on a 7% polyacrylamide gel. The gel was fixed with 10% acetic acid, rinsed in distilled water, dried, and exposed to XAR-5 film with an intensifying screen at −70 °C for 4−24 h.

*Western Blots.* P-glycoprotein was detected in enriched membrane preparations from drug-sensitive and multidrug-resistant cells by Western blotting, using an anti-P-glycoprotein monoclonal antibody (mAb) C219 (Signet Corp., Dedham, MA). Immune complexes were revealed with goat anti-mouse antibody coupled to alkaline phosphatase as described (Rao et al., 1994).

*Analysis.* All data points were determined in triplicate or quadruplicate from preparations obtained from the same culture. Pharmacological constants of drugs and modulators were estimated by computer fit (Delta Graph) of concentration−effect curves of Tc-SESTAMIBI transport inhibition using the equation:

$$C = C_{max}([D]^{\gamma}/EC_{50} + [D]^{\gamma}) \quad (1)$$

where $C$ is cell content of the radioprobe, $C_{max}$ is maximum cell content, [D] is drug concentration, $EC_{50}$ is the half-maximal effective concentration of the drug, and $\gamma$ is the Hill coefficient. Multiple comparisons were made by one-way analysis of variance (Glantz, 1987). Pairs were compared by the Student's $t$ test. Analysis of covariance was used to compare lines or curves. Values of $p \leq 0.05$ were considered significant.

## RESULTS

*Validation of Tc-SESTAMIBI as a P-Glycoprotein Transport Probe. (A) Cell Transport Kinetics.* To further validate Tc-SESTAMIBI accumulation as a probe of MDR P-glycoprotein transport function, net tracer uptake and reversal experiments were performed in cell lines expressing varying levels of P-glycoprotein. Expression of MDR P-glycoprotein in drug-sensitive human SW-1573 carcinoma cells was not

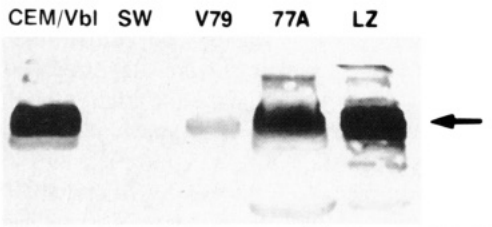


FIGURE 1: Expression of P-glycoprotein in human CEM/Vbl and SW-1573 cells and hamster V79, 77A, and LZ-8 cells as determined by Western blots of plasma membrane preparations with mAb C219. The arrow identifies 170 kDa. CEM/Vbl membranes serve as a positive control for the human SW-1573 immunoblot.

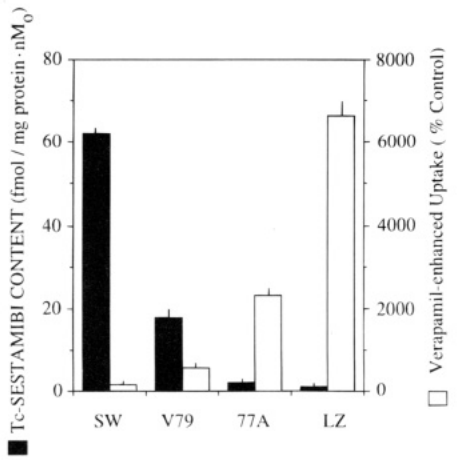


FIGURE 2: Characterization of Tc-SESTAMIBI transport by drug-sensitive and multidrug-resistant cells. Solid bars show 15 min net uptake [fmol (mg of protein)$^{-1}$ (nM$_0$)$^{-1}$] in control buffer of the organometallic cation in SW-1573, V79, 77A, and LZ-8 cells expressing no immunodetectable, low, intermediate, and high levels of P-glycoprotein, respectively. Open bars show net uptake in the presence of saturating concentrations of verapamil (100 − 300 $\mu$M) expressed as percent (%) of control. Each bar is the mean of 3−4 determinations; line above bar denotes SEM.

immunodetectable, whereas expression in drug-sensitive hamster V79 lung fibroblasts and the adriamycin-selected 77A and LZ-8 derivative lines was modestly low, intermediate, and very high, respectively (Figure 1). Tc-SESTAMIBI was accumulated by each cell line to a plateau within 15 min, and steady-state levels of the radiolabel could be maintained for up to 2 h as previously shown (Piwnica-Worms et al., 1993). Plateau levels of Tc-SESTAMIBI in each cell line were inversely related to the expression of P-glycoprotein (Figure 2), consistent with enhanced cellular extrusion of the imaging agent by the transporter. Prior experiments have excluded reduced influx as a mechanism contributing to the diminished accumulation of Tc-SESTAMIBI in the rodent multidrug-resistant cells (Piwnica-Worms et al., 1993). Addition of high concentrations of the MDR reversal agent verapamil (100−300 $\mu$M) to the buffer bathing the SW-1573, V79, 77A, and LZ-8 cells showed verapamil-induced enhancement of Tc-SESTAMIBI steady-state accumulation to 150%, 800%, 2500%, and 6700% of control, respectively, proportional to expression of P-glycoprotein (Figure 2). Maximal verapamil-induced content of Tc-SESTAMIBI was comparable in each cell line (see Table 1). Thus, the modulation data were most consistent with inhibition of P-glycoprotein-mediated extrusion of the radiopharmaceutical, thereby allowing Tc-SESTAMIBI to passively equilibrate with the unopposed negative membrane potentials of the plasma membrane and mitochondria.

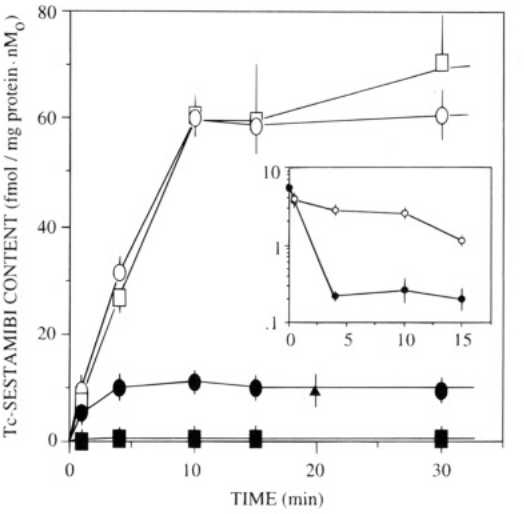


FIGURE 3: Kinetics of Tc-SESTAMIBI accumulation in V79 (●, ○, ▲) and LZ-8 (■, □) cells. Cells were incubated in buffer for various times in the absence of drug (●, ■) or in the presence of quinidine alone (10 $\mu$M, ○; 30 $\mu$M, □) or quinidine plus CCCP (100 and 5 $\mu$M, respectively, ▲). Inset: P-glycoprotein-mediated efflux of Tc-SESTAMIBI. V79 cells were equilibrated (15 min) in loading buffer containing $^{99m}$Tc-SESTAMIBI and then transferred to isotope-free control solution in the absence (●) or presence of 100 $\mu$M quinidine (○) for the times indicated. Cell-associated activity during washout is plotted. Each point represents the mean of 4 determinations; bars represent ±SEM. Note semilogarithmic scale of inset.

Quinidine, another well-characterized MDR reversal agent (Ford & Hait, 1990), also increased Tc-SESTAMIBI net accumulation in these cells. At the modestly high concentrations of 10−30 $\mu$M, quinidine showed no significant effect on the $t_{1/2}$ of accumulation, but increased Tc-SESTAMIBI cell content by 6-fold and 175-fold in V79 and LZ-8 cells, respectively (Figure 3). Confirmation that the organometallic cation was within subcellular aqueous compartments and not adsorbed onto membrane lipid was indicated by the effect of the respiratory uncoupler CCCP. Quinidine-induced enhancement of Tc-SESTAMIBI accumulation was entirely abrogated by CCCP (5 $\mu$M), confirming that blockade of P-glycoprotein allowed Tc-SESTAMIBI to concentrate within mitochondria (Piwnica-Worms et al., 1994).

To directly demonstrate inhibition of P-glycoprotein-mediated efflux of Tc-SESTAMIBI with quinidine, V79 cells were first loaded to plateau (15 min), washed, and then incubated in isotope-free control buffer in the absence and presence of 100 $\mu$M quinidine. Figure 3, inset, shows that, after 10 min of efflux, cells incubated in quinidine retained >10-fold more Tc-SESTAMIBI compared to control. Similar results were also obtained with LZ-8 cells previously loaded to plateau with the radioprobe, but using verapamil as the reversing agent; the $EC_{50}$ for this verapamil-inhibitable unidirectional efflux was 200 $\mu$M (data not shown).

Concentration-enhancement curves were generated for each cell line with quinidine. As shown in Figure 4, there was no significant enhancing effect of quinidine in SW-1573 cells, but there was a concentration-dependent increase in steady-state content of the radiolabel for each cell line expressing immunodetectable levels of P-glycoprotein. Computer fit of the data revealed an $EC_{50}$ for quinidine in V79, 77A, and LZ-8 cells of 6 $\mu$M, 45 $\mu$M, and 147 $\mu$M, respectively. Computer-generated maximal accumulation values were 55, 65, and 90 fmol (mg of protein)$^{-1}$ (nM$_0$)$^{-1}$,

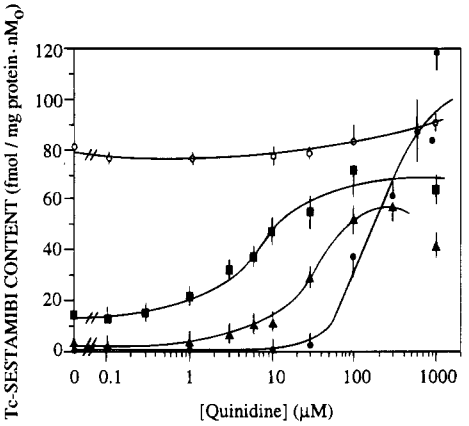


FIGURE 4: Effect of quinidine on Tc-SESTAMIBI accumulation. SW-1573 (○), V79 (■), 77A (▲), and LZ-8 (●) cells were incubated for 15 min in buffer containing Tc-SESTAMIBI and the indicated concentrations of quinidine. Accumulation in the absence of drug is represented by symbols on the ordinate. Each point represents the mean of 3−4 determinations; bars represent ±SEM.

respectively. Hill coefficients were 0.98, 0.94, and 1.5, respectively, consistent with simple Michaelis−Menten kinetics and a single site of drug binding for each agent. Thus, in a manner similar to verapamil and cyclosporin A (Piwnica-Worms et al., 1993), quinidine produced a proportional shift in the reversal curves to higher $EC_{50}$ values in the increasingly drug-resistant 77A and LZ-8 cells.

*(B) Steady-State Pharmacological Analysis.* Using hamster V79, 77A, and LZ-8, hamster PED-25, and human SW-1573 cells, pharmacological surveys of the potency of a wide variety of known MDR reversal agents and cytotoxic compounds to enhance steady-state Tc-SESTAMIBI accumulation were performed (Table 1). V79 and 77A cells were chosen to evaluate most drugs by this assay because of the following factors: (1) relative ease of detecting reliable base-line tracer content in these cells compared to the extremely low levels of Tc-SESTAMIBI observed in highly drug-resistant cells, (2) statistically more robust reversal curves which could be generated at pharmacologically relevant concentrations as a consequence of the generally lower $EC_{50}$ values found in these cells (see above), and (3) clinical relevance of the low to modest levels of P-glycoprotein expressed in these cells. The relative rank order of potency (based on $EC_{50}$ values) of several known reversal agents for enhancing Tc-SESTAMIBI steady-state accumulation (inhibition of Tc-SESTAMIBI efflux) in V79 cells was SDZ PSC 833 > cyclosporin A > verapamil > quinidine ≫ prazosin (Table 1). In 77A cells the potency was cyclosporin A > dipyridamole > quinidine > verapamil. The relative potency of several cytotoxic compounds included in the MDR phenotype to alter Tc-SESTAMIBI transport in V79 cells was vinblastine > daunomycin > actinomycin D ≫ colchicine ≃ doxorubicin. In PED-25 cells, Tc-SESTAMIBI also detected robust functional reversal curves in these low P-glycoprotein expressing cells with daunomycin slightly more potent than idarubicin (Table 1). Maximal drug-induced tracer content for all these compounds was comparable [60−90 fmol (mg of protein)$^{-1}$ $(nM_o)^{-1}$], suggesting that the P-glycoprotein-independent intracellular tracer compartments (mitochondrial and plasma membrane potential-dependent subcellular volumes) were comparable. Nearly all the computer-generated $\gamma$ values (Hill coefficients) tended to cluster around one, consistent with a single site of drug binding for each agent. However, cooperative interactions of multiple binding sites ($\gamma > 1$) were suggested with a few drugs (e.g., dipyridamole) and in the highly MDR cells. Vanadate, a general inhibitor of P-type ATPases (Horio et al., 1988; Sarkadi et al., 1992), was an efficacious inhibitor of P-glycoprotein-mediated efflux of Tc-SESTAMIBI at high concentrations. The high concentrations required for inhibition may reflect poor permeation of this anion into intracellular spaces in this whole cell assay. Of note, colchicine and doxorubicin (adriamycin), two cytotoxic agents in the MDR phenotype (Ford & Hait, 1990), had minimal and no significant enhancing (reversal) effect, respectively, at concentrations up to 300 μM (see Discussion). As anticipated, methotrexate and cisplatin, cytotoxic agents not included in the MDR phenotype (Ford & Hait, 1990), showed no effect on steady-state content of Tc-SESTAMIBI.

Further validation of the properties of the radioprobe to functionally assay P-glycoprotein transport activity was also obtained. Several plasma membrane cation transport inhibitors including ouabain, amiloride, and bumetanide had no effect on steady-state content of Tc-SESTAMIBI in V79 cells (Table 1). Glybenclamide (100 μM), a ligand of the sulfonylurea receptor, a recently cloned ABC transporter (Aguilar-Bryan et al., 1995), and also an ATP-sensitive $K^+$ channel blocker (Fosset et al., 1988), and adenosine (100 μM) had no enhancing effect (Table 1). The antiviral AZT (0.1−100 μM), previously implied to be a P-glycoprotein substrate (Antonelli et al., 1992), did not alter Tc-SESTAMIBI steady-state content nor washout kinetics in 77A and V79 cells, respectively. Geneticin, a cloning selection agent (Southern & Berg, 1982), was without enhancing effect and actually decreased net accumulation at the relatively low nontoxic concentration of 0.1 mg/mL, suggesting a mechanism involving enhanced efflux of Tc-SESTAMIBI. A previous study demonstrated that selected divalent cationic metals are modestly capable of inhibiting P-glycoprotein-mediated verapamil transport. In V79 cells, $Mn^{2+}$, $Ni^{2+}$, and $Zn^{2+}$ cations (1 mM), when added directly to the loading buffer as their corresponding dichloride salts, did not significantly effect Tc-SESTAMIBI net accumulation ($p > 0.4$) or transport kinetics (data not shown). There was no statistically significant effect of divalent cations on verapamil (30 μM)-mediated enhancement of Tc-SESTAMIBI uptake ($p > 0.4$), although we noted a trend for the divalent cations to produce slightly greater verapamil-induced tracer levels. However, net accumulation levels and verapamil-induced increases of Tc-SESTAMIBI in V79 cells were markedly reduced by $Ca^{2+}$-free buffer ($p < 0.05$; data not shown).

*Characterizing P-Glycoprotein Transport Function with Tc-SESTAMIBI. (A) Noncompetitive Inhibition of Transport by Quinidine.* To characterize P-glycoprotein-mediated transport of this organometallic cation in more detail, further steady-state analysis was undertaken of Tc-SESTAMIBI accumulation in V79 cells as a function of extracellular concentration of the radioprobe. Assay conditions were established from 7 pM to 10 μM [Tc-SESTAMIBI], the higher concentrations requiring carrier-added ($^{99}$Tc) synthesis of the complex (Kronauge et al., 1990). Figure 5 shows Scatchard plots of steady-state Tc-SESTAMIBI cell content in the absence and in the presence of a maximal inhibitory concentration of quinidine (100 μM). Tc-SESTAMIBI accumulation in the absence of quinidine was constant at

Table 1: Effect of MDR Modulators, Cytotoxic Agents, and Other Drugs on Tc-SESTAMIBI Cellular Accumulation[a]

| | V79 | | | PED-25 | | | 77A | | | LZ-8 | | | SW-1573 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| drug | $EC_{50}$ | $C_{max}$ | $\gamma$ | $EC_{50}$ | $C_{max}$ | $\gamma$ | $EC_{50}$ | $C_{max}$ | $\gamma$ | $EC_{50}$ | $C_{max}$ | $\gamma$ | $EC_{50}$ | $C_{max}$ | $\gamma$ |
| SDZ PSC 833 | 0.08 | 56 | 0.8 | | | | | | | | | | | | |
| cyclosporin A | 1.3 | 80 | 1.2 | | | | 2.6 | 69 | 1.4 | 74 | 75 | 1.0* | | | |
| verapamil | 4.1 | 89 | 1.1 | | | | 120 | 90 | 1.0* | 211 | 98 | 1.7 | NS | | |
| TPB | 4.7 | 213 | 2.9 | | | | | | | | | | | | |
| quinidine | 6.4 | 55 | 1.0 | | | | 45 | 65 | 0.9 | 147 | 90 | 1.5 | NS | | |
| vinblastine | 13 | 72 | 1.1 | | | | | | | | | | | | |
| dipyridamole | | | | | | | 16 | 85 | 2.1 | | | | | | |
| daunomycin | 55 | 55 | 1.1 | 50 | 112 | 1.0 | | | | | | | | | |
| idarubicin | | | | 65 | 100 | 0.8 | | | | | | | | | |
| actinomycin D | 235 | 122 | 0.9 | | | | | | | | | | | | |
| prazosin | >300 | 70 | 1.0* | | | | | | | | | | | | |
| phloretin | | | | | | | 329 | 181 | 4.5 | | | | | | |
| vanadate | 2900 | 186 | 0.9 | | | | | | | | | | | | |
| colchicine | MIN | | | | | | | | | | | | | | |
| doxorubicin | NS | | | | | | | | | | | | | | |
| amiloride | NS | | | | | | | | | | | | | | |
| bumetanide | NS | | | | | | | | | | | | | | |
| ouabain | NS | | | | | | | | | | | | | | |
| AZT | NS | | | | | | NS | | | | | | | | |
| geneticin | INHIB | | | | | | | | | | | | | | |
| glybenclamide | | | | | | | NS | | | | | | | | |
| adenosine | | | | | | | NS | | | | | | | | |
| methotrexate | | | | | | | | | | NS | | | | | |
| cisplatin | | | | | | | | | | NS | | | | | |

[a] Pharmacological characterization (eq 1) of concentration–effect curves for drug-induced enhancement of Tc-SESTAMIBI in various cell lines. Cells were assayed in quadruplicate for 15-min Tc-SESTAMIBI accumulation in the absence and presence of drug at various concentrations ($10^{-8}$–$10^{-3}$ M). $EC_{50}$, half-maximal effective concentration ($\mu$M); $C_{max}$, maximum cell content of Tc-SESTAMIBI [fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$]; $\gamma$, Hill coefficient; NS, no significant enhancing effect; MIN, minimal enhancing effect; INHIB, inhibitory effect on Tc-SESTAMIBI cell content. * denotes where $\gamma$ was preset to 1 for computer curve fitting to data.

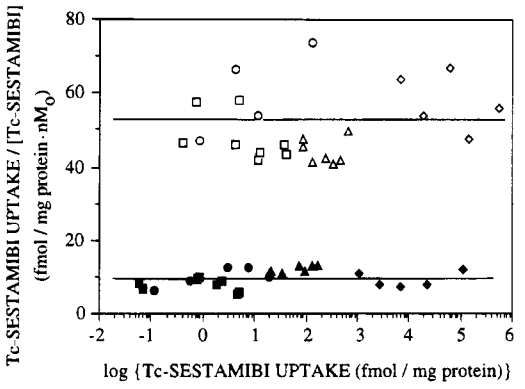


FIGURE 5: Allosteric binding of Tc-SESTAMIBI and quinidine to P-glycoprotein expressed in V79 cells. Summary Scatchard plot (uptake/free vs uptake) for Tc-SESTAMIBI in the absence of drug (solid symbols) and in the presence of 100 $\mu$M quinidine (open symbols) from four independent cultures is shown. Points represent the mean of duplicate or triplicate determinations. Each paired symbol shape represents a separate culture. Solid lines represent mean of all data points: 9.2 fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$ in control buffer, and 51.8 fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$ in the presence 100 $\mu$M quinidine.

9.2 ± 0.3 fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$, and in the presence of 100 $\mu$M quinidine, constant at 51.8 ± 6.0 fmol (mg protein)$^{-1}$ ($nM_o$)$^{-1}$ ($n$ = 73 preparations), regardless of the extracellular concentration of Tc-SESTAMIBI. Similarly, at concentrations of Tc-SESTAMIBI between 8 pM and 0.9 nM, and in the presence of 5 $\mu$M quinidine, Tc-SESTAMIBI accumulation was constant at 39.3 ± 2.5 fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$ ($n$ = 24). Thus, there was no evidence of transport saturation at any concentration, despite organometallic cation concentrations ranging almost 7 orders of magitude. The flat Scatchard plots implied an extremely high apparent $K_m$ for Tc-SESTAMIBI (≫10 $\mu$M) and a large capacity for P-glycoprotein-mediated efflux transport of Tc-SESTAMIBI. Because addition of quinidine yielded an identically high apparent $K_m$ (parallel slope), but different $V_{max}$, the data indicated that quinidine behaved as a noncompetitive (allosteric) inhibitor of P-glycoprotein-mediated Tc-SESTAMIBI transport under these steady-state conditions.

*(B) Agents That Alter the Lipid Bilayer Enhance Tc-SESTAMIBI Cell Accumulation.* Tetraphenyl borate (TPB), a lipophilic anion, and phloretin, a lipophilic neutral dipolar agent, are known to adsorb to lipid bilayers and enhance transmembrane transport kinetics of lipophilic cations (Flewelling & Hubbell, 1986). TPB and phloretin have been previously shown to increase Tc-SESTAMIBI accumulation in primary cultured chick heart cells by enhancing mitochondrial sequestration of the radiotracer (Piwnica-Worms et al., 1991). Both TPB and phloretin enhanced accumulation of the metallopharmaceutical (Table 1). Previous control experiments have shown that this cannot be attributable to enhanced lipid binding of Tc-SESTAMIBI (Piwnica-Worms et al., 1991). In V79 cells, the effect of TPB was not additive to the P-glycoprotein reversing action of verapamil [15-min Tc-SESTAMIBI accumulation (fmol (mg of protein)$^{-1}$ ($nM_o$)$^{-1}$): control, 12.2 ± 1.1; +ver (30 $\mu$M), 71.4 ± 2.2; +TPB (10 $\mu$M), 176.2 ± 19.7; +ver + TPB, 164.6 ± 6.3; $n$ = 4 each]. However, drug-induced maximal tracer uptakes for TPB or phloretin alone were >2-fold higher than that found with standard MDR reversal agents alone, and the Hill coefficients for TPB and phloretin were ≫2, pharmacologically distinguishing their sites of interaction from modulating agents usually included in the MDR phenotype.

*(C) Regulation of P-Glycoprotein Transport Probed with Tc-SESTAMIBI. (1) Phosphorylation.* P-glycoprotein function has been reported to be regulated by protein kinase C (PKC)-mediated phosphorylation (Chambers et al., 1990;

Table 2: Effect of Various Buffers and Extracellular Agents on Tc-SESTAMIBI Accumulation in P-Glycoprotein Expressing 77A and V79 Cells[a]

| | | extracellular agent [fmol (mg of protein)$^{-1}$ nM$_o^{-1}$] | | | |
|---|---|---|---|---|---|
| buffer | cells | control | +ATP | +verapamil | +ATP + verapamil |
| MEBSS | 77A | 3.0 ± 0.8 | 2.4 ± 0.7 | 45.4 ± 1.0 | 44.0 ± 2.2 |
| high KCl | | 0.6 ± 0.04 | | 25.8 ± 3.6 | 25.3 ± 0.7 |
| high K, low Cl | | 1.1 ± 0.1 | | 29.1 ± 5.7 | 21.6 ± 2.5 |
| isotonic | 77A | 1.5 ± 0.1 | | | |
| hypotonic | | 1.6 ± 0.2 | | | |
| hypo/iso | | 1.9 ± 0.2 | | | |
| iso/iso | | 1.3 ± 0.1 | 14.0 ± 0.6 | | |
| hypo/hypo | | 0.9 ± 0.4 | 23.6 ± 1.8 | | |

| | | extracellular agent | | | |
|---|---|---|---|---|---|
| buffer | cells | control | +PdBu | +PdBu (24 h) | +H7 |
| MEBSS | V79 | 13.4 ± 1.5 | 10.1 ± 1.6 | 9.9 ± 0.9 | 18.4 ± 2.3 |

[a] Effect of various putative biological modifiers on Tc-SESTAMIBI accumulation in cells expressing P-glycoprotein. Top: Effect of extracellular ATP, $Cl^-$, and $K^+$ on Tc-SESTAMIBI steady-state accumulation in multidrug-resistant 77A cells. Cells were incubated with radiolabel for 15 min in control MEBSS buffer, 150 mM KCl buffer, or 150 mM K-methanesulfonate buffer in the absence or presence of ATP (5 mM) ± verapamil (50 μM) as indicated. Middle: Effect of buffer tonicity on Tc-SESTAMIBI steady-state accumulation in multidrug-resistant 77A cells. Cells were incubated for 15 min in control buffer (iso) or 220 mOsm buffer (hypo) containing radiolabel or preincubated for 15 min in control or hypotonic buffer alone before equilibrating for an additional 15 min in control or hypotonic buffer containing radiotracer in the absence or presence of verapamil (50 μM) as indicated. Bottom: Effect of protein kinase C modulation on Tc-SESTAMIBI steady-state accumulation in V79 cells. Cells were preincubated in control buffer containing PdBu (100 ng/mL) for 10 min or 24 h or in buffer containing H7 (50 μM) for 10 min, and then incubated for 15 min in the same buffer containing the radiotracer. Each value is the mean of 4 determinations ± SEM.

Bates et al., 1992; Ahmad et al., 1994). To examine the sensitivity of Tc-SESTAMIBI transport to PKC-induced alterations in P-glycoprotein function, we treated V79 cells with agents known to alter PKC activity (Table 2). The phorbol ester PdBu (100 ng/mL), a PKC activator, when added to the buffer either 10 min or 24 h prior to exposing cells to Tc-SESTAMIBI, resulted in a modest, but statistically significant decrease in steady-state Tc-SESTAMIBI content. Conversely, the PKC inhibitor H7 (50 μM) enhanced by 40% organometallic cation content. Thus, detectable with Tc-SESTAMIBI even in these low P-glycoprotein expressing cells, the data were consistent with activation of transporter function by PKC-mediated protein phosphorylation.

*(2) Volume-Sensitive Transport Activity.* Several studies have suggested that P-glycoprotein may operate as a bifunctional membrane protein interconvertible between transport activity and volume-sensitive $Cl^-$ channel activity (Gill et al., 1992; Valverde et al., 1992). One model has suggested that the functions are mutually exclusive, whereby if channel activity is operational, the protein cannot function as a transporter, and vice versa (Gill et al., 1992). We studied the effect of buffer tonicity and $Cl_o$ concentration on Tc-SESTAMIBI transport in 77A cells under conditions thought to activate $Cl^-$ channel activity (Altenberg et al., 1994a). As shown in Table 2, steady-state accumulations of Tc-SESTAMIBI during 15-min incubations in isotonic (295 mOsm) and hypotonic (220 mOsm) buffers were not significantly different. Because it has been suggested that the presence of P-glycoprotein transport substrates may lock the protein into a transport mode (Gill et al., 1992), thereby preventing conversion to channel activity, a 15-min preincubation period in hypotonic buffer free of Tc-SESTAMIBI was performed, followed by exposure to the transport substrate. Again, no statistically significant effect of hypotonic preincubation on subsequent steady-state content of organometallic cation was observed. The results were identical whether performed with $Cl^-$ or the impermeant anion, gluconate, as the dominant counterion in the extracellular buffer ($p$ = NS). Furthermore, hypotonic buffer did not interfere with verapamil (50 μM)-induced enhancement of Tc-SESTAMIBI accumulation (Table 2), nor produce any detectable shift in the $EC_{50}$ of verapamil-induced enhancement (data not shown). Because this organometallic cation distributes freely in the water space when unopposed by P-glycoprotein (Piwnica-Worms et al., 1994), the 39% increase in verapamil-induced Tc-SESTAMIBI content observed in hypotonic buffer could be directly accounted for by a 36% increase in cell volume (Piwnica-Worms et al., 1993). Thus, we observed no significant effect of $Cl^-$ gradients or manipulations thought to induce P-glycoprotein-mediated $Cl^-$ channel activity on Tc-SESTAMIBI net transport in these MDR cells.

*(3) Effect of External ATP.* It has been suggested that P-glycoprotein may also function as an ATP channel regulated by extracellular ATP ($ATP_o$) (Abraham et al., 1993), although the mechanistic details are unresolved. If $ATP_o$ were to activate channel activity, thereby inhibiting efflux transport activity analogous to the above model, then $ATP_o$ would be predicted to enhance Tc-SESTAMIBI accumulation. On the other hand, if the energy of the transmembrane gradient for polyanionic ATP (approximately −3 charge at physiologic pH) were harnessed to mediate drug extrusion by coupled ATP/drug (1:$n$; $n$ <3) cotransport, then reduction of the transmembrane potential or elevation of $ATP_o$ would be predicted thermodynamically to inhibit drug efflux and also result in enhanced steady-state content of drug. We therefore tested these models by evaluating the effects of $ATP_o$ and membrane depolarization on Tc-SESTAMIBI transport in 77A cells (Table 2). $ATP_o$ (1 μM−6 mM) had no significant effect on steady-state accumulation of the organometallic cation in normal K (5.4 mM) buffer. To additionally test the effect of the chloride gradient under these conditions, hypotonic (220 mOsm) sodium-gluconate buffer was used. As above, addition of 5 mM $ATP_o$ had no significant effect on Tc-SESTAMIBI net content (data not shown). Moreover, in the presence of verapamil (50 μM), $ATP_o$ (5 mM) showed no significant effect (Table 2). Depolarization of cells in high KCl (150 mM) buffer, thereby eliminating the inward driving force of the plasma membrane for concentrative accumulation of Tc-SESTAMIBI into the cytosol (but maintaining, at least in part, mitochondrial potential), reduced steady-state accumulation of the radioprobe as previously reported (Piwnica-Worms et al., 1990). Furthermore, as expected, high KCl buffer reduced the verapamil-enhanced net accumulation, again due to the loss of the inwardly directed plasma membrane potential. However, addition of $ATP_o$ (5 mM) to high KCl buffer, both in the absence and in the presence of verapamil, had no significant effect on respective steady-state contents of the organometallic cation ($p$ >0.5). Use

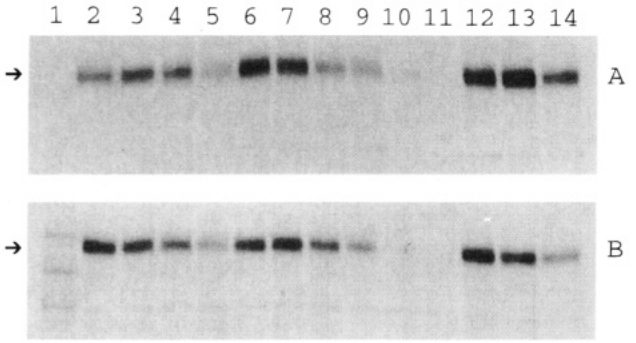


FIGURE 6: Effect of $^{99}$Tc-SESTAMIBI and ATP on [$^{125}$I]IAP photolabeling of P-glycoprotein. (A) Lane 1, V79, and lane 2, LZ-8, membranes photoaffinity labeled with IAP alone. P-glycoprotein labeling at 170 kDa is identified with arrow. LZ-8 membranes labeled with IAP in the presence of 0.25, 2.5, and 25 $\mu$M quinidine (lanes 3−5), prazosin (lanes 6−8), cyclosporin A (lanes 9−11), and carrier-added $^{99}$Tc-SESTAMIBI (lanes 12−14), respectively. (B) Identical to panel A except with added MgATP (5 mM).

of methanesulfonate, an impermeant chloride substitute (Piwnica-Worms et al., 1983), in high K buffer did not alter membrane depolarization-induced distribution of the cationic complex ($p > 0.25$), and furthermore, 5 mM $ATP_o$ was without effect, demonstrating again the lack of coupling of $ATP_o$ and the chloride gradient in net accumulation of Tc-SESTAMIBI. Thus, extracellular ATP, $Cl^-$, and $K^+$ gradients were without significant effect on P-glycoprotein-mediated Tc-SESTAMIBI transport in 77A cells.

*(D) IAP Photolabel Displacement by Tc-SESTAMIBI.* P-glycoprotein-mediated Tc-SESTAMIBI transport is cytosolic ATP-dependent (Piwnica-Worms et al., 1993), and therefore, it is likely that the energy of ATP hydrolysis induces favorable conformational states of the protein to mediate drug transport. Because Tc-SESTAMIBI is freely diffusible across membrane bilayers with low levels of nonspecific lipid partitioning (Chernoff et al., 1993), we hypothesized that the radioprobe could be used to detect any conformational changes that involve the IAP photolabel site(s). Membrane preparations from V79 and LZ-8 cells were therefore photolabeled in the presence and absence of MgATP (5 mM) and various concentrations of carrier-added $^{99}$Tc-SESTAMIBI. Quinidine, prazosin, and cyclosporin A served as control substrates. $^{99}$Tc-SESTAMIBI displaced IAP, showing a half-maximal effect at ~1000-fold molar excess, confirming specific binding of the organometallic cation to P-glycoprotein with affinity similar to those of quinidine and prazosin under these conditions (Figure 6). However, the presence or absence of ATP produced no significant differences in displacement potency for any of the substrates.

*(E) Cytotoxicity Modulation by Tc-SESTAMIBI.* To better understand the relationship between P-glycoprotein-mediated transport and cytotoxic modulation with Tc-SESTAMIBI, cell survival was determined in drug-sensitive V79 and Alex cells and the MDR derivative LZ-8 and Alex/A.5 cells, respectively, in the presence of carrier-added ($^{99}$Tc) quantities of Tc-SESTAMIBI and various concentrations of the cytotoxic agent colchicine. At high concentrations ($10^5$-fold higher than tracer levels), Tc-SESTAMIBI-mediated cell toxicity likely originates from disruption of mitochondrial function (Ritchie, 1984; Backus et al., 1993). We previously

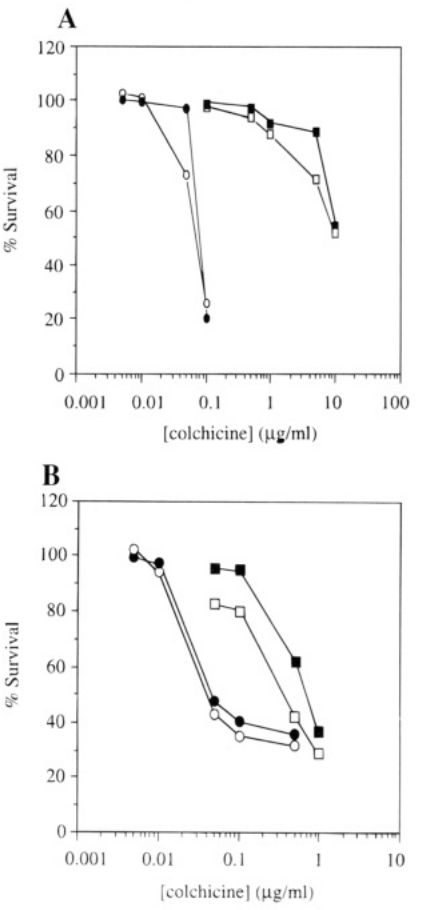


FIGURE 7: Cell survival and Tc-SESTAMIBI cytotoxic modulation studies. Survival of (A) parental V79 (○, ●) and multidrug-resistant LZ-8 cells (□, ■) and (B) parental Alex (○, ●) and multidrug-resistant Alex/A.5 cells (□, ■) in increasing concentrations of colchicine in the absence (●, ■) and presence (○, □) of 5 $\mu$M (V79, Alex) and 100 $\mu$M (LZ-8, Alex/A.5) carrier-added $^{99}$Tc-SESTAMIBI, respectively. Each point represents the mean of triplicate determinations.

established that LZ-8 and Alex/A.5 cells were 11- to 13-fold resistant to $^{99}$Tc-SESTAMIBI-mediated toxicity compared to parental V79 and Alex cells and that resistant and parental cell survival in $^{99}$Tc-SESTAMIBI alone exceeded 85% and 95% at concentrations of 100 and 5 $\mu$M, respectively (Piwnica-Worms et al., 1993). At these same sublethal test concentrations, respectively, $^{99}$Tc-SESTAMIBI was a relatively modest modulator of colchicine cytotoxicity in both cell lines (Figure 7), the MDR reversal effect being less than 2- to 3-fold overall (based on changes in $LC_{50}$). Similar lack of significant Tc-SESTAMIBI-mediated cytotoxic reversal at these concentrations was obtained with the agents doxorubicin and vinblastine. In addition, we examined the efficacy of the lipid-adsorbing agent TPB in modulating Tc-SESTAMIBI toxicity. TPB (10 $\mu$M) enhanced Tc-SESTAMIBI-mediated toxicity by 2- to 3-fold in V79 and Alex cells, probably by potentiating mitochondrial targeting of the organometallic cation, but did not show modulating effect in the highly resistant MDR cells.

## DISCUSSION

Tc-SESTAMIBI is a nonmetabolized metallopharmaceutical with a nontitratable delocalized monocationic charge used clinically for myocardial perfusion (Wackers et al., 1989) and tumor imaging (Hassan et al., 1989; Caner et al.,

1992). Cellular and subcellular biophysical analysis has shown that the agent is a high fidelity (Nernstian) probe of transmembrane potential (Piwnica-Worms et al., 1990; Chernoff et al., 1993), passive *influx* of this lipophilic cation being driven by the transmembrane potentials generated in living cells (Piwnica-Worms et al., 1990). Biochemical analysis and direct localization *in situ* by electron probe X-ray microanalysis have determined that the intracellular target for Tc-SESTAMIBI in living tissues is the mitochondrial inner matrix (Backus et al., 1993), the complex being reversibly sequestered within this organelle by the serial thermodynamic driving forces of the plasma membrane and mitochondrial inner membrane potentials. In humans, this likely accounts for the initial distribution of the agent into mitochondrial-rich tissues such as heart, kidney, liver, skeletal muscle, and tumors *in vivo*. We have recently shown that Tc-SESTAMIBI is also an *efflux* transport substrate recognized by rodent and human multidrug resistance (*MDR1*) P-glycoprotein (Piwnica-Worms et al., 1993; Rao et al., 1994). Thus, net cell content of the radioprobe is a function of passive potential-dependent influx and MDR P-glycoprotein-mediated extrusion. The relationship of the multidrug resistance-associated protein (MRP) (Cole et al., 1992) to Tc-SESTAMIBI transport remains to be evaluated.

In this study, we extended these initial observations by showing that P-glycoprotein expressing cells derived from a wide variety of species showed rapid accumulation ($t_{1/2}$ ~6 min) of Tc-SESTAMIBI to a steady state which was inversely proportional to expression levels of P-glycoprotein. Importantly, Tc-SESTAMIBI readily detected P-glycoprotein transport activity even in the "drug-sensitive", low P-glycoprotein-expressing V79 cells, functionally separating these cells from the drug-sensitive, but non-P-glycoprotein-expressing SW-1573 cells. We conclude that Tc-SESTAMIBI is a sensitive reporter of the functional expression of P-glycoprotein-mediated transport *per se*, not a reporter of whether a cell is drug-sensitive or multidrug-resistant. Consequently, expression of P-glycoprotein appeared to confer a range of measurable transport activities onto which conventional "drug-resistance" may represent a phenotypic threshold on a continuum.

Cytotoxic agents generally inhibited P-glycoprotein-mediated Tc-SESTAMIBI transport with significantly higher $EC_{50}$ values than P-glycoprotein modulators. Both the rank order and relative potency of these agents were comparable to those determined previously by various drug binding and transport assays (Horio et al., 1988; Yusa & Tsuruo, 1989; Ferry et al., 1992). Vanadate, a nonspecific inhibitor of ATPases (Sarkadi et al., 1992), was also an efficacious transport inhibitor, while drugs and agents not usually included in the MDR phenotype were without effect. These data establish that Tc-SESTAMIBI interacts with a common transport domain on P-glycoprotein shared by other substrates and further validate the potential use of Tc-SESTAMIBI as a $\gamma$-emitting imaging substrate for evaluating the functional expression and modulation of P-glycoprotein in cells and human tissues *in vivo* (Piwnica-Worms et al., 1993; Ballinger et al., 1995; Ciarmiello et al., 1995).

*Regulation of P-Glycoprotein-Mediated Tc-SESTAMIBI Transport.* Tc-SESTAMIBI was then used to probe the regulation of P-glycoprotein transport function under various steady-state and perturbed conditions in cell preparations: (1) In V79 cells, the phorbol ester PdBu enhanced, while the PKC inhibitor H7 decreased Tc-SESTAMIBI efflux transport. Thus, positive regulation of transporter function by PKC-mediated phosphorylation, as reported by others (Chambers et al., 1990; Bates et al., 1992; Ahmad et al., 1994), was detectable with Tc-SESTAMIBI, even in these low P-glycoprotein expressing cells. (2) A putative P-glycoprotein-mediated volume-sensitive $Cl^-$ channel activity has been reported (Gill et al., 1992; Valverde et al., 1992) which may be associated with other channel or transporter activity (Hardy et al., 1994; Roepe et al., 1994). In whole cell assays of V79 and 77A cell lines, we found no indication of hypotonic cell swelling-induced modulation of P-glycoprotein-mediated Tc-SESTAMIBI net transport. Furthermore, neither membrane potential *per se* ($K^+$ gradient) nor the $Cl^-$ gradient modulated P-glycoprotein-mediated organometallic cation transport. These results corroborate recent reports that hamster P-glycoprotein-mediated $Cl^-$ channel activity does not contribute to cell volume regulation nor abrogate drug transport (Altenberg et al., 1994a,b). (3) In 77A cells, extracellular ATP did not show any significant effect on P-glycoprotein-mediated Tc-SESTAMIBI transport, nor in LZ cell membranes did ATP alter the ability of Tc-SESTAMIBI to compete with IAP photolabeling of the protein. Thus, using this probe of P-glycoprotein transport function, these data show no modulation of P-glycoprotein-mediated Tc-SESTAMIBI transport by hypotonic buffer, extracellular ATP, $Cl^-$, or $K^+$ (membrane potential).

Detailed pharmacological and kinetic analysis from the present study showed that this organometallic cation was recognized and efflux transported by P-glycoprotein when present in the assay buffer at concentrations as low as 7 pM. This implied the existence of high avidity binding and translocation domains on the P-glycoprotein for Tc-SESTAMIBI. In addition, no evidence of saturating transport behavior as a function of probe concentration was observable, despite transport assays performed over a range of Tc-SESTAMIBI concentrations spanning 7 orders of magnitude from picomolar to 10 micromolar (see Figure 5), implying a high capacity system. These Tc-SESTAMIBI transport rates can be estimated. For example, at the maximum extracellular concentration of Tc-SESTAMIBI achievable for testing (10 $\mu$M), the nonsaturating data can be combined with our previous determinations of the unidirectional P-glycoprotein-mediated efflux rate of the agent [0.11 fmol (mg of total cell protein)$^{-1}$ ($nM_o$)$^{-1}$ (sec)$^{-1}$] (Piwnica-Worms et al., 1993) to yield a flux of 4 nmol (mg of total cell protein)$^{-1}$ (h) $^{-1}$, thereby establishing a lower limit to the transport capacity of P-glycoprotein for Tc-SESTAMIBI. This value exceeds estimates of maximal vinblastine (Horio et al., 1988; Ruetz & Gros, 1994a) and FK 506 transport rates (Saeki et al., 1993). Assuming 0.1% of total cell protein was P-glycoprotein in 77A cells, this Tc-SESTAMIBI transport rate is equivalent to 4 $\mu$mol (mg of MDR protein)$^{-1}$ (h) $^{-1}$, a value approaching maximal ATPase activity found in enriched membrane preparations derived from Sf9 cells overexpressing the recombinant protein (Sarkadi et al., 1992). Functionally, it appeared as if the overall P-glycoprotein transport process for this organometallic cation involved rapid vectoral translocation step(s) with high dissociation rate(s) producing an apparent $K_m \gg 10$ $\mu$M.

Detailed Scatchard analysis showed quinidine to be a noncompetitive inhibitor of P-glycoprotein-mediated Tc-

SESTAMIBI transport (parallel slopes; Figure 5). The classic model for this noncompetitive inhibition by quinidine would be each drug binding reversibly, randomly, and independently at two different sites. The protein−quinidine complex would, in effect, become catalytically inactive, or equivalently, transporter turnover would be rendered slow and inefficient, thereby preventing P-glycoprotein-mediated transmembrane efflux of Tc-SESTAMIBI. Furthermore, just as quinidine is a potent noncompetitive inhibitor of P-glycoprotein-mediated Tc-SESTAMIBI transport, so verapamil, vinblastine, and cyclosporin A are noncompetitive inhibitors of azidopine binding to P-glycoprotein (Tamai & Safa, 1991; Safa, 1992). It has also been shown that quinidine may be a competitive inhibitor of vinblastine transport by P-glycoprotein (Horio et al., 1991), while nicardipine and other 1,4-dihydropyridines allosterically modulate vinblastine binding (Ferry et al., 1992). In total, these transport and binding data indicate multiple allosterically coupled drug−acceptor sites on P-glycoprotein.

Interestingly, even at 10 $\mu$M, Tc-SESTAMIBI was a relatively poor modulator of colchicine cytotoxicity (Figure 7), consistent with the efficient P-glycoprotein-mediated efflux transport properties displayed by this metallopharmaceutical. Conversely, and at first surprising, $10^5$-fold molar excess doxorubicin and, to a lesser degree, colchicine were found to be poor inhibitors of Tc-SESTAMIBI transport (Table 1). However, this would be consistent with data that doxorubicin analogs, for example, are transported in a particularly efficient manner by P-glycoprotein (Sharom et al., 1993; Stein et al., 1994), thereby rendering them unable to interfere with organometallic cation translocation through the transporter. In this regard, classic MDR modulators as a group tended to have higher potencies for inhibition of Tc-SESTAMIBI transport compared to the cytotoxic agents included in the MDR phenotype. Thus, our data support observations indicating that efficiently transported substrates behave as poor cytotoxic modulators (Lee et al., 1994).

*Models of P-Glycoprotein Transport.* A number of mechanistic models have been proposed to characterize the detailed transport action of P-glycoprotein, including a translocating carrier pump, gated-pore carrier protein, membrane vacuum cleaner, bifunctional pump/ion channel, and membrane bilayer flippase (Ford & Hait, 1990; Higgins & Gottesman, 1992; Gottesman & Pastan, 1993). Prior studies addressing the merits of these models have used probes and drugs, generally analogs of known cytotoxic agents and modulators, which have high lipid partitioning properties or multiple pharmacological sites of actions (Ford & Hait, 1990) to characterize the transport function of P-glycoprotein. The results of several of these studies (Homolya et al., 1993; Stein et al., 1994), and the observation that the homologous murine *mdr2* P-glycoprotein is a membrane flippase of phospholipids (Ruetz & Gros, 1994b), have focused attention on the flippase model for *MDR1* P-glycoprotein transport function. By this mechanism, drug intercalates into one leaflet of the bilayer and gains access to a binding site which flips the drug to the other leaflet (Higgins & Gottesman, 1992).

P-glycoprotein-mediated transport of Tc-SESTAMIBI has implications for these mechanistic models of transporter function. In whole cell preparations equilibrated with Tc-SESTAMIBI, there is no evidence of lipid-induced chemical shift or line broadening of the metal complex when analyzed by Tc-99 NMR spectroscopy (Piwnica-Worms et al., 1994). These NMR spectroscopy data and previous phosphatidylcholine vesicle transport data with Tc-SESTAMIBI indicate that this organometallic substrate, although able to rapidly penetrate lipid bilayers, does not adsorb to the bilayer leaflets to any significant degree (Chernoff et al., 1993) nor with time constants resolvable by NMR (Piwnica-Worms et al., 1994). This indicates that the organometallic cation primarily resides in a cellular aqueous environment. If the flippase model pertains to Tc-SESTAMIBI transport, these data require that P-glycoprotein be very robust in its ability to rapidly intercept the drug during brief lipid phase translocation steps. In this regard, the effects of agents such as TPB and phloretin are interesting. Both are thought to adsorb onto the lipid bilayer and alter the intramembranous dipole moment so as to increase lipophilic cation fluxes across bilayers by as much as 3 orders of magnitude (Flewelling & Hubbell, 1986). We reasoned that if Tc-SESTAMIBI gained access to a P-glycoprotein transport domain from the aqueous phase and if these lipophilic adsorption agents merely enhanced Tc-SESTAMIBI flux through the membrane bilayer *per se*, then the agents should not produce any effect on cell content of the radioprobe; the robust efflux capacity of P-glycoprotein should readily handle the TPB-enhanced aqueous (cytosolic) load of Tc-SESTAMIBI under these tracer conditions (as in Figure 5, control). Conversely, if these lipid phase agents altered P-glycoprotein conformations, shielded recognition domains at the lipid−protein interface, or altered the presentation of Tc-SESTAMIBI within the bilayer (ion pairing) beyond the capacity of P-glycoprotein to recognize the compound, then these agents would serve to inhibit P-glycoprotein transport of Tc-SESTAMIBI. The data from this study showed that the membrane dipole agents clearly served to greatly enhance Tc-SESTAMIBI accumulation in a P-glycoprotein expressing cell line in a manner similar to previous results in a non-MDR cell preparation (Piwnica-Worms et al., 1991). Thus, alterations in lipid bilayer structure (or ion pairing effects) inhibited the efflux transport function of P-glycoprotein, thereby allowing the organometallic agent to bypass P-glycoprotein as it passed through the bilayer. In summary, lipid−protein interactions are important for P-glycoprotein-mediated transport of Tc-SESTAMIBI, and the data would generally favor a flippase model of transport over an aqueous lined pore model. Furthermore, previous NMR data with this organometallic cation and the pharmacological profiles suggest that lipid phase access of this substrate to allosterically coupled transport domains on P-glycoprotein must occur with extremely rapid time constants.

## ACKNOWLEDGMENT

We thank Lee Herman for computer analysis of tracer curves and Mary Chiu and Carolyn Crankshaw for technical assistance.

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BI950488U