ABSTRACT

The multidrug-resistant P-glycoprotein (Pgp), a Mr 170,000 plasma membrane protein encoded by the mammalian multidrug resistance gene (MDR1), appears to function as an energy-dependent efflux pump. Many of the drugs that interact with Pgp are lipophilic and cationic at physiological pH. We tested the hypothesis that the synthetic γ-emitting organotechnetium complex, hexakis(2-methoxyisobutylisonitrile)technetium(1) ([99mTc]SESTAMIBI), a lipophilic cationic radiopharmaceutical, could be a suitable Pgp transport substrate capable of functional imaging of the MDR phenotype. The cellular pharmacological profile of [99mTc]SESTAMIBI transport was examined in Chinese hamster V79 lung fibroblasts and the 77A and LZ derivative cell lines which express modestly low, intermediate, and very high levels of Pgp, respectively. Steady-state contents of [99mTc]SESTAMIBI in V79, 77A, and LZ cells were 10.0 ± 0.5 (SEM) (n = 9), 3.6 ± 0.5 (n = 8), and 0.4 ± 0.02 (n = 9) fmol/(mg protein)⁻¹ (μmol), respectively, consistent with enhanced extrusion of the imaging agent by Pgp-enriched cells. Maximal doses (>100 μM) of the multidrug-resistant reversal agents verapamil and cyclosporin A enhanced [99mTc]SESTAMIBI accumulation in V79, 77A, and LZ cells by approximately 10-, 25-, and 200-fold, respectively. The median effective concentration values for tracer accumulation in the presence of verapamil in V79, 77A, and LZ cells were 4, 100, and 200 μM, and those for cyclosporin A were 0.9, 3, and >25 μM, respectively. Pgp-mediated [99mTc]SESTAMIBI transport occurred against its electrochemical gradient and was found to be ATP dependent displaying an apparent Kₐ of 50 μM. Carrier-added [99mTc]SESTAMIBI was 11- to 13-fold less toxic in multidrug-resistant cell lines, and inhibited photolabeling of Pgp by [125I]iodoaryl azidoprazosin in a concentration-dependent manner; half-maximal displacement was observed at approximately 100- to 1000-fold molar excess of [99mTc]SESTAMIBI. Exploiting the favorable γ emission properties of 99mTc, functional expression of Pgp was successfully imaged in human tumor xenografts in nude mice with pharmacologically inert tracer quantities of [99mTc]SESTAMIBI. Functional imaging with these organotechnetium complexes may provide a novel mechanism to rapidly characterize Pgp expression in human tumors in vivo, target reversal agents in vivo, and ultimately provide a means to direct patients to specific cancer therapies.

INTRODUCTION

Overexpression of the mammalian multidrug resistance gene (MDR1) is responsible for resistance to a broad spectrum of diverse cytotoxic agents (1–3). Multidrug-resistant cell lines overexpressing Pgp are resistant to a structurally and functionally diverse group of chemotherapeutic compounds which include anthracyclines, Vinca alkaloids, and actinomycin D (1, 2, 4). Recent investigations have also focused on an expanding number of agents including calcium channel blockers, calmodulin antagonists, quinine derivatives, synthetic iso-
mg) (12). [*99mTc*]TcO2 (20–30 mCi; 5–10 pmol/mCi) in 1–2 ml saline (0.15 m NaCl) obtained from a commercial molybdenum/technetium generator (Du Pont Pharma, Billerica, MA) was added to the kit reaction vial, heated at 100°C for 15 min, and allowed to cool to room temperature, producing an almost quantitative yield of the [*99mTc*]MIBI, complex. Excess reducing agent and starting materials were separated from the radiolabeled component as follows: the contents of the reaction vial were loaded by syringe onto a reversed-phase C18-Sep-Pak cartridge (Waters Associates, Milford, MA) prewet with 3 ml 90% ethanol followed by 5 ml distilled water. Hydrophilic impurities were eluted from the cartridge by washing with 10 ml saline (0.15 m), and the desired [*99mTc*]TcSestamibi was collected by elution with ethanol/saline (2 ml; 9:1, v/v). Final total [*99mTc*]Tc activity in the 2-ml effluent (stock) was assayed in a standard dose calibrator (CRC-12, Capintec, Ramsey, NJ). Radiochemical purity was found to be greater than 97% by thin-layer chromatography (aluminum oxide plates; J. T. Baker, Phillipsburg, NJ) with the use of ethanol (absolute) as the mobile phase.

Carrier-added [*99mTc*]TcSestamibi was prepared from 8–10 mg (10–12 pmol) of solid [*99mTc*]MIBI, chloride powder dissolved in 0.5 ml of 95% ethanol solution as described (11, 12).

**Solutions and Reagents.** Control solution for transport experiments was MEBBS containing (mM): 145 Na+, 5.4 K+, 1.2 Ca2+, 0.8 Mg2+, 152 Cl−, 0.8 H2PO4−, 0.8 SO4−, 5.6 dextrose, 4.0 (2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1% bovine calf serum (v/v), pH 7.4 ± 0.05. A 130 mM potassium/20 mM chloride solution was made by equimolar substitution of potassium methanesulfonate for NaCl as described (12).

Verapamil, vinblastine, colchicine, daunorubicin, Adriamycin, quindine, CCCP, methotrexate, and valinomycin (Sigma Chemical Co., St. Louis, MO), but not cyclosporin A (Sandoz Pharmaceuticals), were dissolved in dimethyl sulfoxide before they were added to solutions. Final concentrations of dimethyl sulfoxide (drug carrier) and ethanol ([*99mTc*]TcSestamibi eluate) were typically <0.5%, which has been found to have no effect on [*99mTc*]TcSestamibi net uptake in cultured cells (12). IAP was obtained from New England Nuclear.

Cell Kinetic Studies. Cells on coverslips were incubated in glucose-free normal potassium or high potassium-low chloride MEBBS buffer containing 2.5, or 10 mM MeGlc (1 µCi/ml; 65 Ci/mmol) for 1 h at 37°C. Control experiments in cultured cells have shown complete equilibration of the nonmetabolizable hexose across cell membranes by 40 min (24). Cells were rinsed three times in 25-ml volumes of ice-cold buffer to clear extracellular MeGlc, extracted in 1% sodium dodecyl sulfate for 40 min (24). Cells were then lysed three times in 25-ml volumes of ice-cold buffer to clear extracellular MeGlc, extracted in 1% sodium dodecyl sulfate for protein determination as previously described, and assayed for *H activity by standard liquid scintillation techniques, along with a 200-µl sample of the extracellular solution. MeGlc uptake/mg protein increased linearly with extracellular [MeGlc] (r ≥ 0.99) and intersected the ordinate at a point not significantly different from the origin (P > 0.25).

**ATP Content.** Cellular ATP content established during a 15-min incubation in glucose-free MEBBS containing 1 γ/ml bovine serum albumin and various sublethal concentrations of the uncoupler CCCP in the presence or absence of verapamil (10 µM) was assayed fluorometrically by a standard hexokinase reaction on cells that had simultaneously been loaded as above with [*99mTc*]TcSestamibi. After tracer loading and rinsing in ice-cold isotope-free buffer, preparations were extracted in perchloric acid, neutralized with potassium carbonate, immediately assayed for *γ* activity, and frozen (−20°C) as previously described (12). Cell protein was determined by Lowry assay. Previously frozen extracts and washes were thawed within 5 days of the experiment and assayed for ATP content fluorometrically (SFM 25; Kontron Instruments, Zurich, Switzerland). ATP is expressed as nmol-(mg protein)−1.

**Photolabeling of Pgp.** Plasma membrane-enriched fractions from parental (V79 and CEM) and multidrug-resistant (LZ and CEM/VBL) cultured cells were isolated with a high speed spin (100,000 × g) after lysis homogenization in 4 mM 2-(hydroxyethyl)-1-piperazineethanesulfonic acid buffer 0.25 M sucrose (pH 7.3) and removal of nuclei with a low speed spin (600 × g). Fifty µg of membranes in a final volume of 50 µl were incubated with 2.5 nm [125I]iodoarylazidoprazosin for 60 min at 25°C in the dark (25, 26). [*99mTc*]TcSestamibi or verapamil were included at the concentrations shown. The sample was irradiated with an UV lamp (UVP, Model UVL-56, 366 nm wavelength) for 20 min at 25°C and fractionated on a 10% polyacrylamide gel. The gel was fixed with 10% acetic acid, rinsed in 2% glycerol, dried, and exposed to XAR-5 film with an intensifying screen at −70°C for 12–18 h.

**Western Blot Analysis.** Pgp was detected in enriched membrane preparations from parental, drug-sensitive and multidrug-resistant cells and tumors by Western blot with the use of a 1:150 dilution of the anti-Pgp mouse monoclonal antibody C219 (Centocor Corp.). Membrane preparations were fractionated on 7% sodium dodecyl sulfate-polyacrylamide gel, and transferred to nitrocel lulose where immune complexes were revealed with goat anti-mouse antibody coupled to alkaline phosphatase and developed as described (27).

**Sцинтиграфия.** All studies were approved by institutional animal welfare committees. Athymic BALB/c-nu/nu mice were inoculated s.c. with 106 drug-sensitive KB cells in the right flank and drug-resistant KB-8-5 cells (28) in the left flank, and tumors were allowed to grow to approximately 1.5 g. Animals were anesthetized with sodium pentobarbital i.p. (50 mg/kg) and positioned over a gamma camera (GE Starcam; LEAP collimator). A bolus of [*99mTc*]TcSestamibi or verapamil was included at the concentrations shown. The sample was irradiated with an UV lamp (UVP, Model UVL-56, 366 nm wavelength) for 20 min at 25°C and fractionated on a 10% polyacrylamide gel. The gel was fixed with 10% acetic acid, rinsed in 2% glycerol, dried, and exposed to XAR-5 film with an intensifying screen at −70°C for 12–48 h.

**Scintigraphy.** All studies were approved by institutional animal welfare committees. Athymic BALB/c-nu/nu mice were inoculated s.c. with 106 drug-sensitive KB cells in the right flank and drug-resistant KB-8-5 cells (28) in the left flank, and tumors were allowed to grow to approximately 1.5 g. Animals were anesthetized with sodium pentobarbital i.p. (50 mg/kg) and positioned over a gamma camera (GE Starcam; LEAP collimator). A bolus of [*99mTc*]TcSestamibi (0.25 mCi) (to characterize Pgp phenotype) mixed with [*99mTc*]thallous chloride (0.25 mCi) (to document perfusion) (29) was then injected via a tail vein. Sequential planar images were collected for 60-s intervals for 1 h. Energy discrimination was provided by 20% windows centered over the 140-KEV photopeak of [*99mTc*]Tc and the 68–92 KEV emission spectra of [*201Tl*]. Each image was corrected on line for camera nonuniformity with a 300-Million counts flood and stored at a digital resolution of 64 × 64. Images were corrected for emission spillover into each window and radioactive decay. No attenuation or scatter correction was used. Accumulation of tracers into internal organs such as heart, liver, kidneys, and bladder was masked to highlight the s.c. tumors in false color images.

Following imaging, mice were sacrificed by lethal pentobarbital injection, and tumors and major organs were removed, trimmed of connective tissue, and weighed in tared vials. Tissues were then assayed for [*99mTc*]Tc and [*201Tl*]Tc activity in a well-type gamma counter as above, using 20% energy discrimination windows, and then data were corrected for emission spillover and radioactive decay.
RESULTS

[99mTc]SESTAMIBI Transport Assays. Chinese hamster V79 lung fibroblasts and the Adriamycin-selected 77A and LZ derivative cell lines express modestly low, intermediate, and very high levels of the Pgp, respectively (Fig. 1). V79, 77A, and LZ cells in monolayer culture exposed to tracer [99mTc]SESTAMIBI accumulated the radiolabel to a plateau within 15 min (Fig. 2). Steady-state contents of [99mTc]SESTAMIBI in V79, 77A, and LZ cells were 10.0 ± 0.5 (n = 9), 3.6 ± 0.5 (n = 8), and 0.4 ± 0.02 (n = 9) fmol/(mg protein)⁻¹·(nmol)⁻¹, respectively, consistent with either reduced influx or enhanced extrusion of the imaging agent by Pgp-enriched cells.

To ascertain if [99mTc]SESTAMIBI cell content could be increased by inhibition of Pgp, the effect of several known multidrug resistance reversing agents were determined over a wide range of concentrations. As shown in Fig. 3, verapamil and cyclosporin A were each found to be potent enhancing agents of [99mTc]SESTAMIBI net uptake. Maximal doses (>100 μM) of these reversing agents enhanced [99mTc]SESTAMIBI accumulation in V79, 77A, and LZ cells by approximately 10-, 25-, and 200-fold, respectively. The EC50 values for [99mTc]SESTAMIBI accumulation in the presence of verapamil in V79, 77A, and LZ cells were 4, 100, and 200 μM, respectively. The reversal curves were proportionally shifted to higher concentrations in the increasingly drug-resistant 77A and LZ cells. Cytotoxic drugs included in the multidrug-resistant phenotype such as vinblastine, colchicine, and daunorubicin, as well as the reversing agent quinidine (30), also enhanced [99mTc]SESTAMIBI net uptake in a concentration-dependent manner. By comparison, methotrexate, a cytototoxic agent to which drug-resistant cells remain sensitive (5, 30), produced no enhancement of [99mTc]SESTAMIBI (data not shown).

Further evidence for Pgp-mediated [99mTc]SESTAMIBI transport was provided by inhibition of efflux with verapamil, a Pgp-reversing agent. V79 cells were loaded to steady state with the tracer and then incubated in isotope-free buffer in the presence or absence of verapamil. Initial uptake rates in V79 cells were indistinguishable (Fig. 5: 0.105 ± 0.01 versus 0.113 ± 0.01 fmol/(mg protein)⁻¹·(nmol)⁻¹·(s)⁻¹; p > 0.5). In the presence or absence of verapamil, initial uptake rates in V79 cells were also identical. However, in the absence of verapamil, there was early evidence of Pgp-mediated efflux of [99mTc]SESTAMIBI in 77A cells manifested by flattening of the accumulation curve within 15–20 s. Thus, steady-state differences between drug-sensitive and drug-resistant cells were unlikely to be a result of differences in influx of the agent.

Energy-dependent Efflux. The energetic requirement for [99mTc]SESTAMIBI efflux was established by quantitative compartmental analysis in V79 cells which express modestly low levels of Pgp. Cells were incubated in 130 mM KCl, 20 mM Cl⁻, buffer plus valinomycin (1 μM), thereby depolarizing both Δψ and Eₘ of cells toward zero and eliminating the inward driving force for [99mTc]SESTAMIBI accumulation against its concentration gradient (12, 13). Under these conditions, a steady-state content of 2.2 ± 0.1 fmol/(mg protein)⁻¹·(nmol)⁻¹·(s)⁻¹ (n = 9) was obtained. While cell water was 8.7 ± 0.4 μl (mg protein)⁻¹ in control buffer, it was 7.4 ± 0.6 (n = 10) μl (mg protein)⁻¹ in high KCl buffer, yielding a [99mTc]SESTAMIBI in:out ratio of 0.30 ± 0.02. This was significantly less than the equilibrium ratio of 1.0 expected for passive diffusion and equilibration of [99mTc]SESTAMIBI with intracellular water spaces under these conditions. Exclusion of [99mTc]SESTAMIBI from the cytosol of V79 cells at near zero-potential conditions implied the presence of an energy-dependent efflux pump mechanism. Addition of a 100 μM concentration of the reversing agent quinidine increased the [99mTc]SESTAMIBI in:out ratio to 0.75 ± 0.14 (P < 0.05), consistent with inhibition of active transport by the Pgp. This observation is further highlighted by the low levels of agent accumulation in the Pgp-
TAMIBI level was achieved within 15 min and maintained for at least 45 min under these conditions. \[^{99m}Tc\]SESTAMIBI and ATP contents were concurrently measured in each preparation in the presence or absence of verapamil (10 \(\mu M\)). Eadie-Hofstee analysis of verapamil-enhanced \[^{99m}Tc\]SESTAMIBI accumulation as a function of ATP concentration [based on the cell water determination of 8.7 \(\mu l\) (mg protein\(^{-1}\))] was consistent with Michaelis-Menton kinetics displaying an apparent \(K_m\) of 50 \(\mu M\) for ATP-dependent transport (Fig. 6). These data establish a pharmacological and biophysical profile consistent

enriched 77A and LZ cells (Fig. 2), which point toward Pgp-mediated exclusion of \[^{99m}Tc\]SESTAMIBI from their cytosols (assuming similar cell water), even in the presence of the strong inward driving force of normal membrane potentials.

\[^{99m}Tc\]SESTAMIBI content was ATP dependent as demonstrated by analysis of verapamil-enhanced accumulation of the tracer in relation to cytosolic ATP concentration. Enhancement of \[^{99m}Tc\]SESTAMIBI accumulation by verapamil was used to represent the transport mediated by Pgp. A range of intracellular ATP values was established by exposing V79 cells to graded sublethal concentrations of the mitochondrial uncoupler CCCP (0.1 to 10 \(\mu M\)) for 15 min (12, 31). Control experiments confirmed that a steady-state \[^{99m}Tc\]SESTAMIBI accumulation in the absence of drug. Points, mean of 3–4 determinations; SEM did not exceed 15% of mean values.

![Fig. 3. Effect of multidrug resistance reversing agents on \[^{99m}Tc\]SESTAMIBI accumulation. Cells were incubated for 15 min in buffer containing \[^{99m}Tc\]SESTAMIBI and the indicated concentrations of verapamil (A) or cyclosporin A (B). Closed symbols, accumulation in the absence of drug. Points, mean of 3–4 determinations; SEM did not exceed 15% of mean values.](image)

![Fig. 4. Pgp-mediated efflux of \[^{99m}Tc\]SESTAMIBI. V79 cells were equilibrated (15 min) in loading buffer containing \[^{99m}Tc\]SESTAMIBI and then transferred to isotope-free solution in the absence or presence of 10 \(\mu M\) verapamil for the times indicated. Cell-associated activity during washout is plotted. Points, mean of 3–4 determinations; SEM did not exceed 15% of mean values. Note semilogarithmic scale.](image)

![Fig. 5. Initial uptake rates of \[^{99m}Tc\]SESTAMIBI in V79 and 77A cells in the absence (○, ■) and presence (□, △) of verapamil (50 \(\mu M\)). Each point represents a single determination. Solid lines, linear regressions of the data with the following slopes: V79, 0.110 fmol/(mg protein)\(^{-1}\) (\(\mu M\))\(^{-1}\) (sec\(^{-1}\)); V79 + verapamil, 0.108; 77A, 0.002; 77A + verapamil, 0.113, respectively.](image)
with the hypothesis that \[^{99m}Tc\]SESTAMIBI is a transport substrate recognized by Pgp.

Cytotoxicity. To functionally assay carrier-added \[^{99m}Tc\]SESTAMIBI toxicity in relation to Pgp expression, survival of both hamster and human parental and multidrug-resistant cells was determined in the presence of the agent. \[^{99m}Tc\]SESTAMIBI can produce acute cellular toxicity, probably mediated by mitochondrial depolarization and uncoupling (13, 15), at concentrations exceeding 75 \(\mu\)M (>10^5-fold higher than the tracer concentrations used clinically). Carrier-added quantities of \[^{99m}Tc\]SESTAMIBI were synthesized and hamster V79 and LZ cells and the human Alexander hepatocellular carcinoma cell line (Alex) and a multidrug-resistant derivative (Alex/A.5) were cultured in increasing concentrations of the compound. As shown in Fig. 7, multidrug-resistant LZ and Alex/A.5 cells were 11- and 13-fold more resistant to \[^{99m}Tc\]SESTAMIBI compared to their drug-sensitive parental cell lines.

Inhibition of Photolabeling. The interaction of \[^{99m}Tc\]SESTAMIBI with Pgp was directly examined by displacement of the Pgp photoaffinity probe IAP from plasma membrane preparations derived from drug-resistant cells. Carrier-added \[^{99m}Tc\]SESTAMIBI was incubated in reaction buffer containing enriched membrane preparations from drug-resistant LZ cells or human leukemia CEM/VBL cells with the photoaffinity probe. Fig. 8 demonstrates that \[^{99m}Tc\]SESTAMIBI inhibited photolabeling of Pgp in a concentration-dependent manner. Half-maximal effect was observed at approximately 100- to 1000-fold molar excess \[^{99m}Tc\]SESTAMIBI, similar to other Pgp modulators (8, 26), including verapamil (Fig. 8).

Functional Imaging of Pgp. The use of \(\gamma\)-emitting \[^{99m}Tc\]SESTAMIBI to image Pgp function in human tumors in vivo was demonstrated with a nude mouse tumor model. Bilateral tumor xenographs were produced in opposite flanks of Athymic BALB/c-nu/nu mice with drug-sensitive KB human carcinoma cells and \(mdr1\) mRNA expressing multidrug-resistant KB-8-5 cells (28). Studies in vitro have reported that KB-8-5 cells are 3.2-fold resistant to Adriamycin compared to parental KB cells (28). A relative resistance of 2-2.5 for KB-8-5 tumors in vivo was documented for this study by growth analysis as follows. Tumor size (mg) at 2 weeks in BALB/c-nu/nu mice treated at Adriamycin doses of 8 and 12 mg/kg, respectively (percentage of control): KB, 25 and 13%; KB-8-5, 51 and 32%. Thus, low levels of \(mdr1\) mRNA expression were sufficient to confer resistance both in vitro and in vivo. Further evidence supporting maintenance of the MDR phenotype by KB-8-5 cells in vivo was provided by demonstrating that MDR reversal agents (e.g., cyclosporin A) increased activity of Adriamycin in KB-8-5, but not in parental KB tumors.¹

Simultaneous images of tumor Pgp function with \[^{99m}Tc\]SESTAMIBI and tumor perfusion with \([^{201}Tl]\)thallous chloride (29) were generated in this mouse tumor model with dual-isotope planar scintigraphy. Images from a representative mouse are shown in Fig. 9. The Pgp-enriched KB-8-5 tumor accumulated less \[^{99m}Tc\]SESTAMIBI compared to the parental tumor in the opposite flank (Fig. 9A, arrow), producing a readily visualized difference. Tumor perfusion, however, as reflected by \([^{201}Tl]\) imaging was similar for both tumors (Fig. 9B, arrows). Control studies in vitro with drug-resistant LZ cells confirmed that \([^{201}Tl]\) was not a Pgp transport substrate [net \([^{201}Tl]\) accu-

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¹ R. A. Kramer, unpublished observations.
mulation at 30 min [fmol/(mg protein)]⁻¹ (nMₖₒ)⁻¹: control, 135.4 ± 15.9; + 50 μM verapamil, 138.6 ± 4.1 (n = 4); P not significant).

The imaging results were confirmed with quantitative biodistribution analysis of the tracers in the tumors and major organs. The s.c. implanted tumors were modestly perfused as demonstrated by 60-min tumor/liver ratios for ²⁰¹Tl (per g tissue) of 0.17 ± 0.03 (n = 8). Perfusion was not significantly different between Pgp-enriched and parental tumors (²⁰¹Tl/g tumor: P not significant), and therefore delivery of diffusible tracers was similar. In accord with the scintigraphic images, analysis of paired tumor xenographs in four animals demonstrated [⁹⁹mTc]SESTAMIBI/²⁰¹Tl ratios 35 ± 4% lower in Pgp-enriched tumors compared to parental tumors implanted in the opposite flank. These biodistribution data confirmed enhanced Pgp-mediated transport of [⁹⁹mTc]SESTAMIBI out of multidrug-resistant tumors in vivo.

**DISCUSSION**

Pgp expression in human tumors has been characterized by use of immunohistochemical techniques, RNA expression analysis, and flow cytometry of fluorescent substrates (32–38). However, characterization of tumor specimens from patients with these techniques requires serial tissue biopsies, and the usefulness of these approaches has been limited by the labor intensity of current protocols, the invasiveness of
biopsies, sampling errors from heterogeneous tumors, and the sensitivity and specificity required of RNA and antibody probes (1, 39). Because an in vivo screen would ease detection of the Pgp and aid development of new drugs targeted to inhibit the Pgp, the goal of our study was to establish a scintigraphic substrate for the MDR1 gene product that could be used as a noninvasive probe of tissue expression.

Characterization of [99mTc]SESTAMIBI Interaction with Pgp.

Several lines of evidence supported the hypothesis that [99mTc]SESTAMIBI was a transport substrate recognized by Pgp: (a) drug-sensitivity and multidrug-resistant cells demonstrated steady-state accumulation of the agent in reverse rank order of their expression of Pgp; (b) known multidrug-resistant-reversing drugs such as verapamil and cyclosporin A enhanced [99mTc]SESTAMIBI levels in a concentration-dependent manner with EC50 values comparable to published values (5); (c) unidirectional efflux of [99mTc]SESTAMIBI was inhibited by the Pgp modulator verapamil; (d) initial uptake rates were identical in drug-sensitive V79 and drug-resistant 77A cells when Pgp was inhibited with verapamil. This further indicated that the lower net uptake in Pgp-enriched cells was a result of enhanced extrusion of the agent, not a difference in influx; (e) analysis of in/out ratios established that the freely diffusible [99mTc]SESTAMIBI could be excluded from the cytosol against its concentration gradient. Thermodynamically this required the presence of an active transporter such as Pgp to mediate drug efflux; (f) verapamil-enhanced accumulation of [99mTc]SESTAMIBI was ATP dependent, displaying Michaelis-Menten kinetics with an apparent Km of 50 μM, similar to that for the ATP dependence of Pgp-mediated vinblastine transport (7); (g) multidrug-resistant cells were 11- to 13-fold more resistant to the toxic effects of carrier-added [99mTc]SESTAMIBI compared to parental cells; and (h) [99mTc]SESTAMIBI inhibited photobleaching of Pgp by 123I]IAAP.

[99mTc]SESTAMIBI is a membrane potential-dependent probe similar to tetraphenylphosphonium (12, 13, 24). The opposing interaction of membrane potential-dependent influx and Pgp-mediated efflux of [99mTc]SESTAMIBI could be demonstrated in Pgp-enriched cells. For example, initial uptake rates of [99mTc]SESTAMIBI in drug-sensitive V79 and multidrug-resistant 77A cells were identical in the presence of verapamil, indicating that the plasma membrane potentials were similar in these cells, and therefore could not account for the transport differences in each cell line. However, the robust capacity of Pgp to extrude [99mTc]SESTAMIBI from 77A cells in the absence of verapamil was evident by flattening of the uptake curves after only 15 s. Given the disparate conclusions reported for the effects of Pgp expression on drug influx (5), these data highlight the potential for underestimation of true initial uptake rates of drugs in control buffer with multidrug-resistant cells, with the use of techniques using delayed sampling times.

The maximal accumulation levels of [99mTc]SESTAMIBI during Pgp inhibition approached similar levels in all hamster cell lines. This indicated that in the absence of Pgp-mediated efflux, the volume of distribution for [99mTc]SESTAMIBI (reflecting the sum driving forces of mitochondrial and plasma membrane potentials acting in series on inner matrix and cytosolic spaces) was not significantly different between drug-sensitive and multidrug-resistant cells. Thus, the differences observed in the absence of reversal agents were best attributed to expression of Pgp in the plasma membranes.

Interestingly, the [99mTc]SESTAMIBI reversal curves were proportionally shifted to higher concentrations of reversal agent (higher EC50 values) in the increasingly drug-resistant 77A and LZ cells. Due to the high levels of Pgp expression, these data may reflect a decrease in actual cell content of the competing reversal agent in resistant cells at constant extracellular concentrations of each compound. Hypothetically, however, a proportionally higher affinity for [99mTc]SESTAMIBI or lower affinity for the reversing agents in the Pgp-enriched cell lines could not be excluded, perhaps mediated by posttranslational modification on Pgp or by Pgp-induced changes in cytosolic pH (40). The shifted inhibition curves also indicated that the optimal reversal concentration for each individual agent may in general depend on the level of Pgp expression (5).

Identification of [99mTc]SESTAMIBI as a transport substrate for Pgp further broadens the structural and functional criteria for putative recognition domains of the transporter. While [99mTc]SESTAMIBI is biophysically similar to other lipophilic cationic probes of membrane potential recently reported as Pgp substrates (41), this organoanetchnium complex is not planar and contains no basic nitrogen atoms, titratable protons, or phenyl groups (11, 24). The only functionality is the external methoxy groups symmetrically distributed over the molecular surface.

Utility of a γ-emitting Pgp Substrate. This study demonstrated the feasibility of scintigraphically imaging Pgp expression in vivo with [99mTc]SESTAMIBI and points to applications in functional imaging with this transport substrate. This radiopharmaceutical combines a proven safety profile in humans at tracer doses (pm) (16) with the advantages inherent to [99mTc], the most commonly used radioisotope in planar and single photon emission computed tomography medical imaging. [99mTc] provides a nearly ideal γ emission energy (140 KeV), short half-life (6.02 h), high photon flux, favorable dosimetry, low cost, and extremely high specific activity (107 Ci/mmol) and is readily available from generator systems (42). Advantage can also be taken of the rapid blood clearance (16), low levels of nonspecific binding (12, 13), and lack of metabolism of [99mTc]SESTAMIBI (11, 43), thereby allowing early imaging and a higher biologically relevant signal compared to other labeled compounds.

This study used 201Tl, the classic perfusion tracer (29), to document independently equivalent initial delivery of the diffusible tracers to drug-sensitive and drug-resistant tumors. Initial tissue distribution of 201Tl has been well documented to correlate with regional perfusion (29), while net cell uptake and retention are largely dependent on active transport by the Na/K-ATPase (44, 45). Several human tumors have been reported to retain 201Tl (45–49); however, our data indicate that 201Tl is not recognized as a transport substrate by Pgp. Therefore, this enables dual isotope imaging, whereby the ratio of [99mTc]-SESTAMIBI to 201Tl (or any other perfusion tracer) may be used to normalize for differences in initial delivery of [99mTc]SESTAMIBI to the tumors.

The ability to functionally assay in vivo the Pgp transporter noninvasively with a pharmacologically inert tracer substrate provides a significant new tool for advancing the clinical understanding of multidrug-resistant phenotype in cancer patients. This approach may ultimately be used to guide chemotherapeutic protocols, assist clinical trials or screening of new Pgp reversing agents, and direct tumor biopsies. In vitro testing of cytotoxic and reversing agents in multidrug-resistant cells may also be assisted by application of a simple radionuclide method. Scintigraphic identification of Pgp-enriched tissues in vivo necessarily requires detection of diminished accumulation of [99mTc]SESTAMIBI, a result of outward transport of this substrate. Remaining to be ascertained is the ability to detect heterogeneous tumor expression with this constraint. In addition, although there is rapid excretion of [99mTc]SESTAMIBI by liver, kidney, and bowel (16), perhaps reflecting a normal transport function of Pgp, the high levels of initial tracer uptake in these tissues will require development of optimal imaging times and judicious use of single photon emission computed tomography to visualize abdominal tumors. In this regard, we envision future research targeted toward development of agents of this class with high affinity binding, rather than transport properties, to enable direct imaging of high Pgp expression. The relatively simple chemical structure of these organoanetchnium complexes would allow facile chemical modification for this purpose. An in vivo functional...
IMAGING OF MULTIDRUG-RESISTANT Pgp

assay for Pgp should extend our understanding of this unique membrane transport protein in human physiology and cancer biology.

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Functional Imaging of Multidrug-resistant P-Glycoprotein with an Organotechnetium Complex

David Piwnica-Worms, Mary L. Chiu, Mark Budding, et al.


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