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Effects of *MDR1* and *MDR3* P-glycoproteins, MRP1, and BCRP/MXR/ABCP on the Transport of ^{99m}Tc-Tetrofosmin

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ABSTRACT. Multidrug resistance (*MDR1*) P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and breast cancer resistance protein (BCRP/MXR/ABCP) are members of the ATP-binding-cassette (ABC) superfamily of membrane transporters and are thought to function as energy-dependent efflux pumps of a variety of structurally diverse chemotherapeutic agents. We herein report the characterization of ^{99m}Tc-Tetrofosmin, a candidate radiopharmaceutical substrate of ABC transporters. ^{99m}Tc-Tetrofosmin showed high membrane potential-dependent accumulation in drug-sensitive KB 3–1 cells and low antagonist-reversible accumulation in MDR KB 8–5 and KB 8–5-11 cells in proportion to levels of *MDR1* Pgp expression. In KB 8–5 cells, EC_{50} values of the potent MDR antagonists *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), (2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979), and (3′-keto-Bmt′)-[Val^2]-cyclosporin A (PSC 833) were 40, 66, and 986 nM, respectively. Furthermore, only baculoviruses carrying human *MDR1*, but not *MDR3*, conferred both a decrease in accumulation of ^{99m}Tc-Tetrofosmin in host *Spodoptera frugiperda* (Sf9) cells and a GF120918-induced enhancement. Transport studies with a variety of stably transfected and drug-selected tumor cell lines were performed with ^{99m}Tc-Tetrofosmin and compared with ^{99m}Tc-Sestamibi, a previously validated MDR imaging agent. *MDR1* Pgp readily transported each agent. To a lesser extent, MRP1 also transported each agent, likely as co-transport substrates with GSH; neither agent was a substrate for the BCRP/MXR/ABCP half-transporter. In *mdr1a(−/−)* and *mdr1a/1b(−/−)* mice, ^{99m}Tc-Tetrofosmin showed ~3.5-fold greater brain uptake and retention compared with wild-type, with no net change in blood pharmacokinetics, consistent with transport *in vivo* by Pgp expressed at the capillary blood–brain barrier. Molecular imaging of the functional transport activity of ABC transporters *in vivo* with ^{99m}Tc-Tetrofosmin and related radiopharmaceuticals may enable non-invasive monitoring of chemotherapeutic and MDR gene therapy protocols. BIOCHEM PHARMACOL **60**;3:413–426, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. multidrug resistance; ABC transporters; P-glycoprotein; radiopharmaceuticals; technetium; ^{99m}Tc-Sestamibi; ^{99m}Tc-Tetrofosmin; MDR modulators; blood–brain barrier; cancer; gene therapy

Emergence of MDR† is a major obstacle to successful chemotherapy of cancer. Several of the best characterized mechanisms of MDR include transporter-mediated resistance conferred by increased expression of the M_r 170,000 transmembrane glycoprotein, Pgp, the product of the *MDR1* gene [1–5], and a related M_r 190,000 membrane glycoprotein, MRP1 [6, 7]. Pgp and MRP1 are members of the ABC superfamily of membrane transport proteins [4] and confer resistance to an overlapping array of structurally and functionally unrelated toxic xenobiotics, natural product drugs, and, in the case of MRP1, conjugated compounds [8, 9]. BCRP/MXR/ABCP, a recently cloned ABC "half-transporter," also has been reported to be associated with MDR, particularly resistance to mitoxantrone and anthracyclines in cultured cells [10–12]. Cells in culture exhibiting MDR derived by selection in cytotoxic drugs or transfection with these recombinant transporters generally show reduced net drug accumulation. Whereas Pgp- and MRP1-

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† *Abbreviations*: ABC, ATP-binding-cassette; BCRP/MXR/ABCP, breast cancer resistance protein; BSO, buthionine sulfoximine; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; $\Delta\Psi$, mitochondrial membrane potential; DMEM, Dulbecco's modified Eagle's medium; E_m, plasma membrane potential; mAb, monoclonal antibody; MDR, multidrug resistance; MEBSS, modified Earle's balanced salt solution; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; ^{99m}Tc-Sestamibi, hexakis(2-methoxyisobutyl isonitrile)^{99m}Tc(I); and ^{99m}Tc-Tetrofosmin, [1,2-bis{bis(2-ethoxyethyl)phosphino}ethane]$_2O_2{}^{99m}$Tc(V).

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mediated decreases in drug accumulation have usually been attributed to enhanced drug efflux [4, 5, 13], evidence exists for contributions from altered membrane permeability resulting in decreased drug influx [1, 14], altered intracellular distribution of drug [6, 9, 15], and modulation of programmed cell death pathways [16, 17].

MDR1 Pgp, MRP1, BCRP/MXR/ABCP, and other ABC transporters have been targets for cancer therapy on two fronts. First, reversal of multidrug resistance in tumor cells by nontoxic agents that block the transport activity of these ABC proteins has been an important target of pharmaceutical development [13, 18–22]. When co-administered with a cytotoxic agent, these antagonists, known as MDR modulators, enhance net accumulation of cytotoxic compounds within the tumor cells. Second, transgenic expression of the *MDR1* gene has been explored for hematopoietic cell protection in the context of cancer chemotherapy [23–25], wherein Pgp could protect hematopoietic progenitor cells from chemotherapy-induced myelotoxicity. Hematopoietic cells transduced via retroviral-mediated transfer of the *MDR1* gene have shown preferential survival after treatment of the animal with MDR drugs [25], and recent pilot clinical data support the approach [26]. For applications relevant to the use of new modulators as well as gene therapy in chemotherapeutic protocols, identification of transporter-mediated resistance could guide the choice of chemotherapeutic agents and provide important prognostic information for cancer patients. Thus, non-invasive imaging with a radiolabeled transport substrate serving as a surrogate marker of chemotherapeutic agents may identify those tumors and tissues in which ABC transporters are expressed and active. In this regard, gamma emitting compounds exemplified by ^{99m}Tc-Sestamibi, a widely available radiopharmaceutical, have been characterized as substrates for *MDR1* Pgp [27–31] and may enable scintigraphic analysis of MDR with imaging cameras commonly available in nuclear medicine facilities [32–40].

The targeted synthesis and validation of other single-photon radiopharmaceuticals [41–45] and positron emission tomography (PET) agents (reviewed in Ref. 46) for imaging MDR have been reported. Among these, a clinically available radiopharmaceutical known as ^{99m}Tc-Tetrofosmin (Fig. 1), a hydrophobic cationic compound, was designed originally as a radiopharmaceutical for myocardial perfusion imaging. Both ^{99m}Tc-Tetrofosmin and ^{99m}Tc-Sestamibi are extremely stable complexes, but ^{99m}Tc-Tetrofosmin offers possible formulation advantages over other ^{99m}Tc-complexes, such as room temperature reconstitution from a lyophilized kit [47]. Also, compared with ^{99m}Tc-Sestamibi, ^{99m}Tc-Tetrofosmin has been reported to clear from the liver more rapidly *in vivo* and shows a somewhat greater degree of renal elimination [48]. Whereas ^{99m}Tc-Tetrofosmin has been proposed to be transported by *MDR1* Pgp [49], the pharmacological sensitivity and specificity of the agent in response to newer, more potent MDR antagonists are unknown, and the impact of expression of other ABC transporter family members is unexplored. Thus, we undertook a full characterization of ^{99m}Tc-Tetrofosmin as a transport substrate of *MDR1* Pgp and related ABC transporters and evaluated the radiopharmaceutical as a reporter of the relative potency of newer MDR modulators that are now in clinical trials. These data provide a basis for further exploration of molecular imaging of the MDR phenotype with this and related radiopharmaceuticals in cancer and gene therapy *in vivo*.

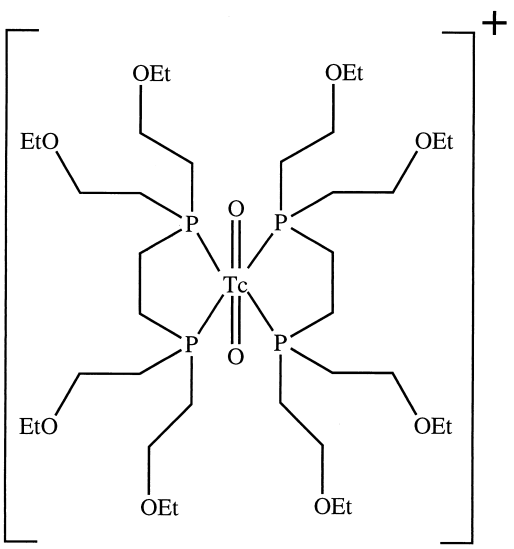

FIG. 1. Structure of Tc-Tetrofosmin.

MATERIALS AND METHODS

Solutions and Reagents

Stock solutions of *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918; gift of Glaxo-Wellcome), (2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979; gift of Eli Lilly & Co.), (3′-keto-Bmt′)-[Val^2]-cyclosporin A (PSC 833; gift of Mallinckrodt, Inc.), methotrexate, and cisplatin were prepared in DMSO. The final concentration of DMSO in experimental buffers was $< 0.5\%$, which has been found to have no effect on net uptake of ^{99m}Tc-complexes in cultured cells [50]. Cyclosporin A was purchased as the Cremophor formulation and was added directly to buffers in the concentrations indicated. All reagents except as indicated were obtained from the Sigma Chemical Co.

The control solution for transport experiments was a MEBSS containing 145 mM Na^+, 5.4 mM K^+, 1.2 mM Ca^{2+}, 0.8 mM Mg^{2+}, 152 mM Cl^-, 0.8 mM $H_2PO_4^-$, 0.8 mM SO_4^{2-}, 5.6 mM dextrose, 4.0 mM HEPES, and 1% bovine calf serum (v/v), pH 7.4 $\pm$ 0.05.

Cell Culture

Monolayers of human epidermoid carcinoma drug-sensitive KB 3–1 cells and the colchicine-selected KB 8–5 (Pgp^{+}) and KB 8–5-11 (Pgp^{++}) derivative cell lines were grown as previously described [27, 42, 51]. Briefly, cells were plated in 100-mm Petri dishes containing seven 25-mm glass coverslips on the bottom and grown to sub-confluence in DMEM (GIBCO) supplemented with L-glutamine (1%), penicillin/streptomycin (0.1%), and heat-inactivated fetal bovine serum (10%) in the presence of 0, 10, and 100 ng/mL of colchicine, respectively. Growth conditions for parental 8226 and drug-selected Dox 6 (Pgp^{+}) cells, CEM and drug-selected CEM-Vbl (Pgp^{++}) cells, NIH3T3 and stably transfected NIH3T3-MDR1 (Pgp^{++}; gift of Dr. Michael Gottesman), as well as NIH3T3-MRP1 ($MRP1^{++}$; gift of Dr. Gary Kruh) cells, and H69 and drug-selected H69AR ($MRP1^{++}$) have been described [6, 42, 52, 53]. Parental S1M1 and MCF-7 cells and their *BCRP/MXR/ABCP*-expressing derivative cells, S1M1–80 and MCF-7-AdVp3000, respectively (gift of Dr. Susan Bates), were maintained in RPMI in the absence or presence of 80 μM mitoxantrone or in IMEM (Richter's medium) in the absence or presence of 3000 ng/mL of Adriamycin® plus 5 μg/mL of verapamil, respectively, as described [11].

Wild-type baculovirus (*Autographa californica*, Invitrogen), recombinant baculoviruses containing human *MDR1* and *MDR3*, and the host insect cell line, *Spodoptera frugiperda* (Sf9), were grown and maintained as previously described [42, 54]. Sf9 cells were infected with wild-type or recombinant baculovirus at 5 plaque-forming units per cell and harvested using procedures described previously [54]. For transport assays, virus-infected Sf9 cells were cultured in 100-mm Petri dishes containing seven glass coverslips with approximately 1×10^7 cells per dish. Transport experiments were performed 44–48 hr post-infection, the optimal pre-lytic time to execute these cell transport studies [54].

Preparation of ^{99m}Tc-complexes

The radiopharmaceuticals ^{99m}Tc-Tetrofosmin (gift of Mallinckrodt, Inc.) and ^{99m}Tc-Sestamibi (Dupont Radiopharmaceuticals) were prepared from one-step commercial kit formulations by the addition of $[^{99m}Tc]TcO_4^-$ according to the manufacturer's recommendations [28, 47]. Separation of the radiolabeled ^{99m}Tc-Tetrofosmin from kit reagents was accomplished by diluting the preparation with 20 mL of water, loading it onto a pre-wet C_{18} Sep-Pak, and eluting the product with ethanol:saline (80:20). The eluate was diluted with normal saline to obtain an ethanol concentration of 10%. Quality control effected by reverse-phase HPLC [PRP-1 column (250 × 4.1 mm, 10 μm) and an acetonitrile (pH 5.3):5 mM KH_2PO_4 (pH 6.7) gradient (20→80% in 20 min) at a flow rate of 2.0 mL/min] showed > 95% radiochemical purity. Sep-Pak purification (>95%) and quality control of ^{99m}Tc-Sestamibi were performed as described [28, 50].

Cell Transport Studies

Coverslips with confluent cells were used for studies of cell transport and kinetics as previously described [28]. Cells on coverslips were removed from culture media and washed for 15–30 sec in MEBSS, then immersed in 60-mm glass Pyrex dishes containing 4 mL of loading solution consisting of MEBSS, drug or vehicle, and 5–20 pM Tc-complex (5–9 pmol/mCi; 1–2 μCi/mL). Coverslips with cells were removed at various times, rinsed three times in 25 mL of ice-cold isotope-free MEBSS for 8 sec each to clear extracellular spaces, and placed in 35-mm plastic Petri dishes. Cells were extracted in 1% SDS with 10 mM sodium borate before protein assay by the method of bicinchoninic acid analysis (BCA; Pierce Chemical Co.), using BSA as the protein standard. Aliquots of the loading buffer and stock solutions also were obtained for standardizing cellular data with the extracellular concentration of Tc-complex. For cells in suspension culture, transport studies were performed as described [52]. All cell extracts, stock solutions, and extracellular buffer samples were assayed for gamma activity in a well-type sodium iodide gamma counter (Cobra II, Packard). The absolute concentration of total Tc-complex in solution was determined from the activity of stock solutions and the specific activity of technetium, based on equilibrium equations for the Mo/Tc generator [55]. Data are reported as fmol Tc-complex (mg protein)$^{-1}$ $(nM_o)^{-1}$, as previously described [28], with $(nM_o)^{-1}$ representing total concentration of Tc-complex (as both ^{99m}Tc and ^{99}Tc) in the extracellular buffer.

Determination of Intracellular GSH Levels

Intracellular GSH was measured by suspending cell samples in 3% (w/v) 5-sulfosalicylic acid in water to induce lysis. After centrifugation (2000 *g* × 15 min) to clear precipitates, 200-μL aliquots of the supernatant were assayed for GSH with Ellman's reagent [56]. To deplete intracellular GSH, cells were pretreated with BSO (25 μM) added to the culture media for 24 hr prior to analysis and transport experiments.

Western Blots

Pgp and MRP1 were detected in crude, enriched membrane preparations from cell lines using western blotting with monoclonal antibodies C219 and QCRL1 (Signet Corp.), respectively [57]. Immune complexes were identified with sheep anti-mouse antibody (1:2000 dilution) coupled to horseradish peroxidase using the ECL western blotting detection system (Amersham Life Sciences).

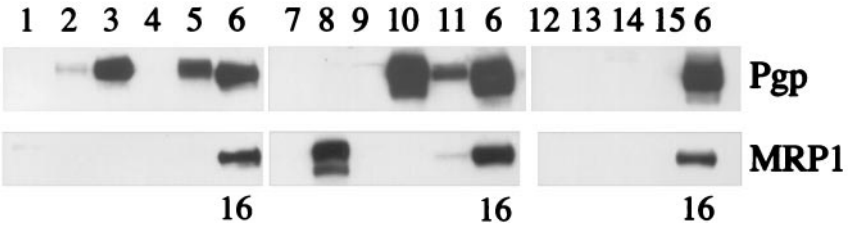

FIG. 2. Expression of Pgp and MRP1 in panels of human tumor and mammalian cells as determined by western blots of plasma membrane preparations with mAbs C219 (Pgp) and MRPr1 (MRP1). The top row identifies M_r 170,000 Pgp, and the bottom row identifies M_r 190,000 MRP1. Cells: (lane 1) KB 3–1; (lane 2) KB 8–5; (lane 3) KB 8–5-11; (lane 4) 8226; (lane 5) 8226/Dox 6; (lane 6 or 16) NIH3T3-MDR1 or NIH3T3-MRP1, respectively; (lane 7) H69; (lane 8) H69AR; (lane 9) CEM; (lane 10) CEM-Vbl; (lane 11) NIH3T3 cells transfected with MRP empty vector; (lane 12) MCF-7; (lane 13) MCF-7-AdVp3000; (lane 14) S1M1; (lane 15) S1M1–80.

Biodistribution Studies

Vertebrate animal procedures were approved by the appropriate institutional review committees. Distribution of ^{99m}Tc-Tetrofosmin in the blood and brain tissues of parental strain FVB mice (FVB/NTacfBR), FVB *mdr1a(−/−)* gene knockout mice (FVB/TacfBR-[KO]*mdr1a*N7), and FVB *mdr1a/1b(−/−)* gene knockout mice (FVB/TacfBR-[KO]*mdr1a*-[KO]*mdr1b*N7) (Taconic) was determined as previously described [41, 42]. For experiments using GF120918 in parental FVB mice, the drug was prepared in 0.5% hydroxypropylmethylcellulose/1% Tween-80/dH_2O vehicle and administered by oral gavage (250 mg/kg) 4 hr prior to injection of ^{99m}Tc-Tetrofosmin; control animals for these experiments received vehicle only. Approximately 4 hr after oral administration of modulator, peak serum levels of 500–600 ng/mL are achieved (unpublished data; [19]), a concentration of drug shown to be saturating for inhibition of *MDR1* P-glycoprotein-mediated transport activity *in vitro*. ^{99m}Tc-Tetrofosmin was diluted in saline:ethanol (90:10) to a final concentration of 20 μCi/mL. Mice were anesthetized by metofane inhalation and injected with 2 μCi (5–9 pmol/mCi) of radiotracer via bolus injection through a tail vein. Animals were killed by cervical dislocation at 5, 15, 30, 60, and 120 min post-injection (N = 2–4 each). Blood samples were obtained by cardiac puncture, and brain tissue was harvested rapidly. Gamma activity in organ samples was counted for 1 min, or until two standard deviations of sampling were below 0.5%. Radio-HPLC analysis of tissue extracts confirmed recovery of nearly 95% intact complex.* Data are expressed as percent of injected dose per gram of tissue [(tissue μCi) (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100].

Analysis

All data points for transport assays were determined from preparations obtained from the same culture. The EC_{50} values (half-maximal effective concentration of drug) for modulators were estimated by computer fit (Sigma Plot, Jandel Scientific) of concentration–effect curves of Tc-complex transport inhibition using the following sigmoid equation:

$$C = \{(C_{max} - C_{min})/[1 + (D/EC50)\gamma]\} + C_{min}$$

where C is the cell content of the Tc-complex, C_{max} is the maximum cell content of the Tc-complex, C_{min} is the minimum cell content of the Tc-complex, γ is the slope, D is the concentration of MDR modulator, and EC_{50} represents the half-maximal effective concentration [58].

From biodistribution time–activity curves, AUC was calculated using trapezoidal integration (KaleidaGraph, Synergy Software) and reported as tissue μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min. Data are reported generally as means ± SEM. Pairs were compared by Student's *t*-test. Values of $P \leq 0.05$ were considered significant.

RESULTS

Validation of Tc-Tetrofosmin as a Substrate for MDR1 Pgp: Transport Analysis in KB Cells

To validate the transport of ^{99m}Tc-Tetrofosmin by *MDR1* Pgp, cell accumulation of the tracer was determined in

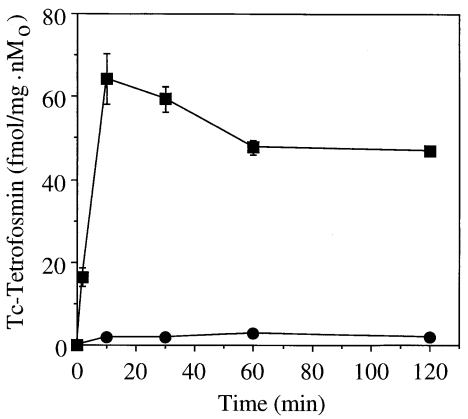

FIG. 3. Kinetics of Tc-Tetrofosmin accumulation in KB tumor cells (KB 3–1, ■; KB 8–5, ●). Cells were incubated in buffer for the indicated times. Each point represents the mean of 4 determinations; bars represent ± SEM when larger than the symbol.

* M. Marmion, Mallinckrodt, Inc., unpublished data. Cited with permission.

TABLE 1. Survey of Tc-Tetrofosmin accumulation in cells expressing ABC transporters

Parental/MDR cell (ABC)	Uptake [fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$]				Cell uptake ratios		
	Parental cells	MDR cells	MDR cells + Cyclo A (5 μM)	MDR cells + GF120918 (300 nM)	Parental/MDR uptake ratios	MDR + CA / MDR − ratios	MDR + GF / MDR − ratios
KB 3-1/KB 8-5 (*MDR1*)	56.8 ± 2.6	1.3 ± 0.06	41.0 ± 0.5	54.3 ± 3.3	43.7	31.5	41.8
8226/Dox 6 (*MDR1*)	138 ± 11	10.6 ± 0.8	54.4 ± 2.0	68.2 ± 0.7	13.0	5.1	6.4
CEM/CEM-Vb1300 (*MDR1*)	118 ± 7	10.3 ± 0.5	9.6 ± 0.5	3.6 ± 0.5	11.5	0.9	0.3
NIH3T3/NIH3T3-MDR1 (*mdr1* + *MDR1*)	16.4 ± 2.9	1.5 ± 0.4	2.9 ± 0.4	27.8 ± 1.5	10.9	1.9	18.5
NIH3T3/NIH3T3-MRP1 (*mdr1* + *MRP1*)	20.4 ± 1.3	4.3 ± 0.2	7.2 ± 0.4	4.9 ± 0.7	4.7	1.7	1.1
H69/H69AR (*MRP1*)	97.2 ± 11.8	13.8 ± 4.0	15.5 ± 0.4	8.6 ± 0.9	7.0	1.1	0.6
S1M1/S1M1-80 (*BCRP/MXR/ABCP*)	46.5 ± 0.6	86.7 ± 5.9	153 ± 11	78.4 ± 2.0	0.5	1.8	0.9
MCF-7/MCF-7-AdVp3000 (*BCRP/MXR/ABCP*)	21.3 ± 1.1	30.7 ± 2.7	34.4 ± 2.6	30.0 ± 1.9	0.7	1.1	1.0

Cell survey and pharmacological characterization of drug-induced enhancement of Tc-Tetrofosmin in various drug-sensitive and derivative MDR cell lines. the ABC gene conferring MDR is indicated within the parentheses under each cell pair. Thirty-minute Tc-Tetrofosmin accumulation in drug-sensitive cells or MDR cells in the presence or absence of the MDR modulators cyclosporin A (5 μM) or GF120918 (300 nM) was assayed. Cellular accumulation of each complex is expressed as [fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$]. Values represent the means ± SD of 3–8 determinations each. Cell uptake ratios between paired drug-sensitive (parental) and derivative (MDR) cells, or MDR cells in the presence or absence of the indicated modulator are calculated and shown in the two right-hand columns.

drug-sensitive KB 3–1 and MDR derivative cell lines. KB 3–1 cell lines expressed no immunodetectable *MDR1* Pgp, whereas KB 8–5 and KB 8–5-11 expressed moderate and high levels of Pgp, respectively (Fig. 2). Time–activity curves show large differences in accumulation of Tc-Tetrofosmin between KB 3–1 and KB 8–5 cells (Fig. 3). Cell-associated counts were normalized to total Tc-Tetrofosmin concentrations in the loading buffer and to protein content. Both KB 3–1 and KB 8–5 time–activity curves reached a plateau within 30 min of initial exposure to tracer amounts of Tc-Tetrofosmin in the uptake buffer. Steady-state levels of Tc-Tetrofosmin were 59.4 ± 3.0 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$ in KB 3–1 cells and 1.9 ± 0.06 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$ in KB 8–5 cells, approximately half the values of 104.6 ± 4.1 and 2.85 ± 0.1 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$, respectively, obtained for Tc-Sestamibi from the same cultured cells. The ratio of steady-state accumulation (30 min) of the radiopharmaceutical in KB 3–1 and KB 8–5 cells was used as a measure of functional *MDR1* Pgp, thereby correcting for differences in absolute cell content of each Tc-complex. The ratio of cell accumulation (KB 3–1/KB 8–5 cells) for Tc-Tetrofosmin was 43.7 (Table 1), comparable to the ratio for Tc-Sestamibi of 37.4 (Table 2). These data confirm that the sensitivity for detection of functional *MDR1* Pgp in KB cells using Tc-Tetrofosmin is similar to that of Tc-Sestamibi [44].

To further define the interactions of Tc-Tetrofosmin with cells, monolayers of confluent KB 3–1 cells were incubated with tracer amounts of the Tc-complex in buffer alone or buffer containing 130 mM K^+/20 mM Cl^- and 1 μg/mL of the potassium ionophore valinomycin. Under high K^+ plus valinomycin conditions, electrical potentials of the mitochondrial membrane ($\Delta\Psi$) and plasma membrane (E_m) are depolarized toward zero, eliminating the inward driving force for uptake of hydrophobic cations such as Tc-Tetrofosmin [50, 59]. The residual net accumulation of a radiopharmaceutical under isoelectric membrane potential is one measure of nonspecific adsorption of hydrophobic cationic complexes to lipid compartments within cells [50, 60]. Prior studies have established that Tc-Sestamibi is a Nernstian probe of membrane potential with minimal adsorptive binding to lipid bilayers [50, 61, 62], and thus, in the presence of high K^+ and valinomycin, residual uptake of Tc-Sestamibi maps intracellular water space [50, 61]. For Tc-Tetrofosmin, net uptake in KB 3–1 cells (30-min incubation) under isoelectric membrane potential conditions was 5.2 ± 0.3 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$ (N = 4), similar to Tc-Sestamibi assayed in cells from the same culture [4.7 ± 0.3 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$; N = 3]. By contrast, in KB 3–1 cells incubated in control buffer, accumulation of Tc-Tetrofosmin was less than predicted by membrane potential as defined by Tc-Sestamibi [56.8 ± 4.2 vs 104.6 ± 4.1 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$, respectively; N = 4].

Pharmacological Analysis in KB Cells

To further validate transport of Tc-Tetrofosmin mediated specifically by *MDR1* Pgp, three potent antagonists of *MDR1* Pgp function were tested in KB 8–5 cells. Inhibition

TABLE 2. Survey of Tc-Sestamibi accumulation in cells expressing ABC transporters

Parental/MDR cell (ABC)	Uptake [fmol (mg protein)$^{-1}$ (nM_o)$^{-1}$]				Cell uptake ratios		
	Parental cells	MDR cells	MDR cells + Cyclo A (5 μM)	MDR cells + GF120918 (300 nM)	Parental/MDR uptake ratios	MDR + CA / MDR − ratios	MDR + GF / MDR − ratios
KB 3-1/KB 8-5 (*MDR1*)	104.6 ± 4.1	2.8 ± 0.1	129 ± 8.5	104.0 ± 10.4	37.4	46.1	37.1
8226/Dox 6 (*MDRl*)	230 ± 13	9.9 ± 0.9	174 ± 7	171 ± 15	23.2	17.6	17.3
CEM/CEM-Vb1300 (*MDR1*)	216 ± 4	10.8 ± 1.1	39.4 ± 1.8	4.0 ± 1.0	20.0	3.6	0.4
NIH3T3/NIH3T3-MDR1 (*mdr1 + MDR1*)	119 ± 19	0.9 ± 0.2	18.9 ± 1.4	121 ± 8	132	21.0	134
NIH3T3/NIH3T3-MRP1 (*mdr1 + MRP1*)	59.1 ± 4.6	11.2 ± 4.0	13.0 ± 0.7	8.4 ± 1.4	5.3	1.2	0.6
H69/H69AR (*MRP1*)	127 ± 5	11.3 ± 0.7	17.4 ± 4.2	11.3 ± 0.5	11.2	1.5	1.0
S1M1/S1M1-80 (*BCRP/MXR/ABCP*)	136 ± 14	254 ± 11	331 ± 9	181 ± 5	0.5	1.3	0.7
MCF-7/MCF-7-AdVp3000 (*BCRP/MXR/ABCP*)	58.9 ± 11.9	91.5 ± 5.7	70.9 ± 4.7	126 ± 12	0.6	0.8	1.4

Cell survey and phamacological characterization of drug-induced enhancement of Tc-Sestamibi in various drug-sensitive and derivative MDR cell lines. See legend of Table 1 for details.

of a putative efflux transport of Tc-Tetrofosmin would be indicated by an increase in net accumulation of the tracer. Concentration–effect curves were generated for the modulators GF120918, a substituted isoquinolinyl acridonecarboxamide [19], LY335979, a difluorocyclopropyl dibenzosuberane [20], and PSC 833, a cyclic undecapeptide analogue of cyclosporin A [18], added directly to uptake buffers over a range of concentrations in three separate experiments (Fig. 4). Cells were incubated in the respective buffers for 30 min, and median effective concentrations (EC_{50}) of the modulators were estimated by computer fit of the curves. Data are expressed as percentages of maximal Tc-Tetrofosmin accumulation after treatment with the modulator. GF120918, LY335979, and PSC 833 all enhanced uptake of Tc-Tetrofosmin in KB 8–5 cells. The EC_{50} values for GF120918, LY335979, and PSC 833 were 40, 66, and 986 nM, respectively. For GF120918, tested at 300 nM, antagonist-induced enhancement was independent of extracellular concentrations of Tc-Tetrofosmin between 2 and 143 pM (data not shown).

Direct evidence that the modulator-induced enhanced uptake of Tc-Tetrofosmin was sequestered into mitochondria was provided by experiments with the respiratory uncoupler CCCP, a reagent that depolarizes mitochondrial membrane potential [63]. GF120918-induced enhancement of Tc-Tetrofosmin in KB 8–5 cells was abrogated significantly by CCCP {30-min Tc-Tetrofosmin accumulation [fmol (mg protein)$^{-1}$ (nM_o)$^{-1}$]: control, 1.3 ± 0.06; + CCCP (5 μM), 0.9 ± 0.01; + GF120918 (300 nM), 63.4 ± 16.8; + GF120918 + CCCP, 8.2 ± 0.1; N = 3–4 each}, similar to Tc-Sestamibi {30-min accumulation [fmol (mg protein)$^{-1}$ (nM_o)$^{-1}$]: control, 2.8 ± 0.1; + CCCP (5 μM), 0.3 ± 0.1; + GF120918 (300 nM), 104 ± 10.4; + GF120918 + CCCP, 11.8 ± 0.8; N = 3–4 each}. Control experiments showed that ATP content of KB-8–5 cells was not affected by GF120918 and was depleted approximately 30% by CCCP, not sufficient to reduce ATP below the apparent K_m for Pgp [27]. These CCCP data confirmed that blockade of Pgp enabled Tc-Tetrofosmin to permeate into mitochondria in a membrane potential-dependent manner

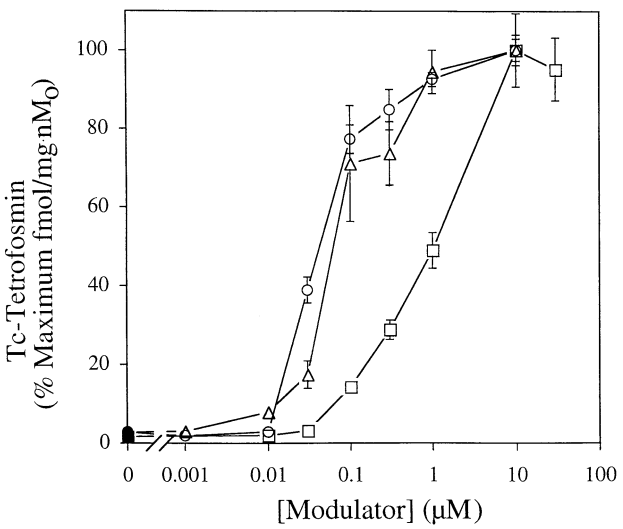

FIG. 4. Effects of modulators on Tc-Tetrofosmin cellular accumulation in cells with modest levels of *MDR1* Pgp. KB 8–5 cells were incubated for 30 min in buffer containing Tc-Tetrofosmin alone (solid symbols) or the indicated concentrations of each modulator (GF120918, ○; LY335979, △; PSC 833, □). Percent of maximum cellular content of Tc-Tetrofosmin is plotted. Each point represents the mean of 4 determinations; bars represent ± SEM when larger than the symbol.

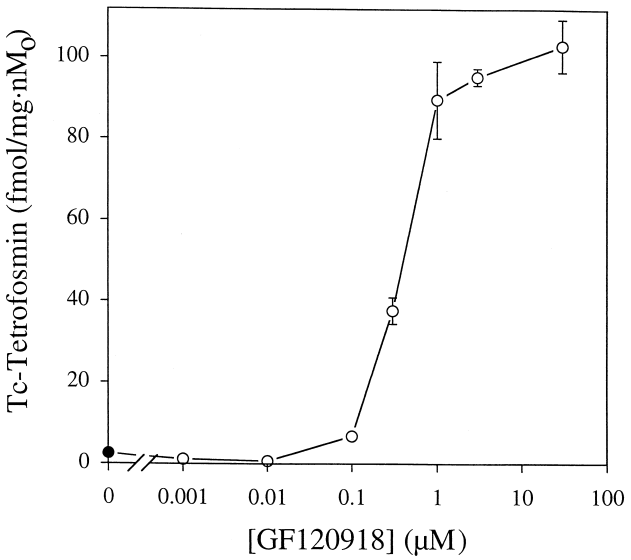

FIG. 5. Effects of GF120918 on Tc-Tetrofosmin cellular accumulation in highly drug-resistant cells. KB 8–5-11 cells were incubated for 30 min in buffer containing Tc-Tetrofosmin alone (●) or the indicated concentrations of GF120918 (○). Each point represents the mean of 4 determinations; bars represent ± SEM when larger than the symbol.

[59, 63, 64] and further provided evidence that Tc-Tetrofosmin was adsorbed only minimally onto membrane lipids and hydrophobic compartments. Note that the slightly greater accumulation values in the presence of both GF120918 and CCCP relative to CCCP alone may reflect, in the absence of functional Pgp, the effect of residual plasma membrane potential on passive influx of the tracers. In addition, modulator-induced tracer enhancement was remarkably temperature-sensitive. At 4°, KB 8–5 cells accumulated only 0.23 ± 0.05 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$ of Tc-Tetrofosmin in the presence of GF120918 (300 nM), consistent with a strong temperature dependence for the Tc-Tetrofosmin permeation and retention mechanism. The classic MDR modulator cyclosporin A (6 μg/mL; 5 μM) was used as a positive control in each modulator experiment and showed maximal enhancement of Tc-Tetrofosmin at this concentration [41.0 ± 0.5 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$]. Negative control experiments with methotrexate (0.1 to 100 μM) and cisplatin (0.1 to 500 μM) resulted in predictably low cellular accumulation of Tc-Tetrofosmin in MDR cells, not differing from control ($P > 0.5$), indicating a lack of effect of these non-Pgp drugs on Tc-Tetrofosmin transport.

In KB 8–5-11 cells, which express high levels of *MDR1* Pgp, the titration curve for GF120918, the most potent of the three modulators tested, was shifted rightward to higher concentrations (Fig. 5). In the KB 8–5-11 cell line, the EC_{50} for GF120918-induced Tc-Tetrofosmin enhancement was shifted to 385 nM, approximately 10-fold higher than in the modestly Pgp-expressing KB 8–5 cells.

Specificity of Tc-Tetrofosmin for MDR1 Compared with MDR3 Pgp in a Baculoviral Expression System

To begin to test the specificity of Tc-Tetrofosmin for *MDR1* Pgp, transport assays were performed in Sf9 cells infected with either wild-type or recombinant baculoviruses containing either the human *MDR1* or *MDR3* gene (Fig. 6). Sf9 cells infected with the wild-type baculovirus show no immunodetectable levels of Pgp, whereas Pgp is detected in Sf9 cells infected with baculoviruses containing either *MDR1* or *MDR3* [42]. As shown in the western blot, relative expression of *MDR1* Pgp was consistently greater than that of *MDR3* in the baculovirus system (Fig. 6, inset). Differences in expression of *MDR1* and *MDR3* Pgp also have been observed previously in yeast [65] and mammalian cells [66]. Intracellular accumulation of Tc-Tetrofosmin was assayed after a 30-min incubation period in control buffer containing the radiotracer using the same method as above. Sf9 cells infected with the wild-type baculovirus showed high Tc-Tetrofosmin accumulation [165.6 ± 3.7 fmol (mg protein)$^{-1}$ (nM$_o$)$^{-1}$], whereas Sf9 cells infected with the *MDR1*-containing baculovirus showed less than half the control level of Tc-Tetrofosmin accumulation. Furthermore, incubating the *MDR1*-infected Sf9 cells with GF120918 (300 nM) enhanced accumulation of Tc-Tetrofosmin back to control levels. Sf9 cells infected with the *MDR3*-containing baculovirus showed only a 24% decrease in Tc-Tetrofosmin accumulation. In contrast to *MDR1*-infected cells, GF120918 had no significant effect on the accumulation of Tc-Tetrofosmin in Sf9 cells infected with

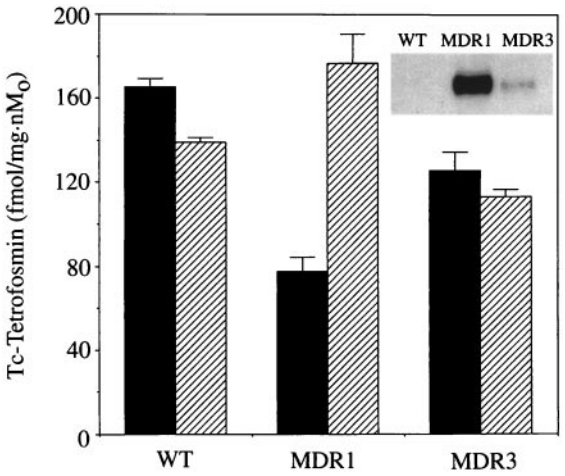

FIG. 6. Effects of various Pgp isoforms on accumulation of Tc-Tetrofosmin in Sf9 cells. Sf9 cells infected with wild-type baculovirus (WT), recombinant virus with *MDR1*, or recombinant virus with *MDR3* were used for transport assays 44–48 hr after infection. Infected Sf9 cells were incubated for 30 min in buffer containing Tc-Tetrofosmin without (solid bars) or with 300 nM GF120918 (hatched bars). Columns are the means of 4 determinations; error bars represent SEM. Inset: Expression of Pgp (M_r 140,000; core glycosylated) in Sf9 cells infected with wild-type baculovirus, recombinant MDR1 baculovirus, and recombinant *MDR3* baculovirus as determined by western blots of plasma membrane preparations with mAb C219; data reproduced from Ref. 42.

TABLE 3. Effect of GSH depletion on Tc-Tetrofosmin uptake in H69 and H69AR cells

	Intracellular GSH (nmol/10^6 cells)			Tc-Tetrofosmin uptake [fmol (mg protein)$^{-1}$ (nM_o)$^{-1}$]		
	Basal	BSO treatment	BSO/Basal ratio	Basal	BSO treatment	BSO/Basal ratio
H69	10.2 ± 1.9	2.8 ± 0.1	0.27	91.9 ± 2.9	145 ± 7.1	1.58
H69AR	4.4 ± 0.8	1.4 ± 0.3	0.31	8.1 ± 1.0	25.6 ± 0.6	3.16

Accumulation of Tc-Tetrofosmin in H69 and H69AR cells without or with depletion of GSH. Control cells or cells pretreated for 24 hr with BSO (25 μM) were incubated for 30 min in buffer containing Tc-Tetrofosmin. GSH was determined with Ellman's reagent in cells grown in the same culture under each condition in two independent experiments. Values represent the means ± range (N = 2) or ±SEM of 3–4 determinations from a representative experiment.

MDR3-containing baculovirus or wild-type baculovirus. Thus, although a slight cross-reactivity of Tc-Tetrofosmin with *MDR3* Pgp was possible, the data confirmed the specificity of GF120918 for *MDR1* Pgp.

Cross-reactivity of Tc-Tetrofosmin with Other ABC Transporters

To evaluate the impact of different levels of *MDR1* Pgp expression and to survey cross-reactivity of Tc-Tetrofosmin with other ABC superfamily members, cell transport assays were performed with the tracer using a variety of other cell lines (Table 1). Comparison transport data for Tc-Sestamibi are shown in Table 2. Levels of Pgp or MRP1 expression levels for each cell line are shown in Fig. 2. Human Dox 6 cells and the highly drug-selected CEM-Vbl300 cells expressed modest and very high levels of *MDR1* Pgp compared with undetectable levels in parental 8226 and CEM cells, respectively. The 8226/Dox 6 and CEM/CEM-Vbl300 uptake ratios for Tc-Tetrofosmin accumulation were 13.0 and 11.5, respectively, consistent with *MDR1* Pgp-mediated efflux transport of Tc-Tetrofosmin. The addition of GF120918 (300 nM) significantly enhanced tracer accumulation in Dox 6 cells. However, at this test concentration of GF120918, no enhancement of the tracer was observed in the CEM-Vbl300 cells. While complex interactions and collateral toxicity arising from unknown cross-reacting targets cannot be excluded, the lack of reversal in CEM-Vbl300 cells likely reflected a rightward shift of the concentration–effect curve in these highly drug-selected cells. Thus, the test concentration in this cell survey was not sufficient to inhibit Pgp-mediated transport of the tracer. A similar rightward shift for PSC 833 and cyclosporin A has been demonstrated unequivocally in these cells using either [^{3}H]daunorubicin or Tc-Sestamibi as the Pgp transport probe [52].

Control murine NIH3T3 fibroblasts expressed a small, but readily immunodetectable level of murine Pgp (Fig. 2), whereas significantly increased levels of Pgp were detected in cells stably transfected with human *MDR1*. The NIH3T3/NIH3T3-MDR1 Tc-Tetrofosmin uptake ratio was 10.9, and the addition of GF120918 enhanced Tc-Tetrofosmin uptake beyond that observed in the NIH3T3 cells alone, consistent with GF120918-mediated inhibition of both the native murine and the transfected human Pgp. NIH3T3 fibroblasts stably transfected with human *MRP1* modestly excluded Tc-Tetrofosmin. Note that GF120918 had only minimal effect on Tc-Tetrofosmin accumulation in NIH3T3-MRP1 cells, confirming the specificity of GF120918 for modulating Pgp. Human H69AR cells were confirmed to express high levels of MRP1 (in the absence of *MDR1* Pgp) and showed an H69/H69AR ratio of 7.0, again suggesting that Tc-Tetrofosmin had modest cross-reactivity with MRP1. However, GF120918 had no effect on Tc-Tetrofosmin accumulation in these MRP1-expressing cells. Human colon carcinoma S1M1–80 and breast carcinoma MCF-7-AdVp3000 cells are non-Pgp, non-MRP expressing cells that express high levels of BCRP/MXR/ABCP compared with undetectable levels of this half-transporter in parental S1M1 and MCF-7 cells, respectively [11]. The S1M1/S1M1–80 and MCF-7/MCF-7-AdVp3000 uptake ratios for Tc-Tetrofosmin accumulation were all $<$ 1, consistent with lack of recognition of Tc-Tetrofosmin by the BCRP/MXR/ABCP transporter. Addition of the inhibitor GF120918 (300 nM) had no significant enhancing effect in these cells, while cyclosporin A slightly enhanced accumulation, perhaps attributable to effects of the drug on mitochondrial energetics [67]. For all cells in the survey, similar trends were observed with Tc-Sestamibi (Table 2).

MRP1 may function as a GSH-drug co-transporter. For example, in inside-out membrane vesicle preparations, GSH can stimulate MRP1-mediated uptake of unconjugated substrates such as vincristine and aflatoxin B1 [9, 68], and whole tissue levels of GSH are elevated in *mrp1(−/−)* mice [69]. Because Tc-Tetrofosmin contains a fixed cationic charge and is neither conjugated nor significantly metabolized,* the tracer provides an interesting model substrate to further test the MRP1 co-transport model. To examine the contribution of intracellular GSH to Tc-Tetrofosmin transport, the effect of BSO-mediated depletion of GSH on net accumulation of Tc-Tetrofosmin in H69 and MRP1-expressing H69AR cells was determined (Table 3). Basal intracellular GSH concentrations were 10.2 ± 1.9 and 4.4 ± 0.8 nmol/10^6 cells in H69 and H69AR cells, respectively, consistent with expectations of reduced GSH content in *MRP1*-expressing cells [9, 70]. Basal Tc-Tetrofosmin net cell uptake also was lower in the H69AR cells. When cells were pretreated with BSO (25 μM) for 24 hr, GSH was reduced to approximately 30% of

* M. Marmion, Mallinckrodt, Inc., personal communication. Cited with permission.

the respective control values for each cell line. However, BSO-induced enhancement of Tc-Tetrofosmin uptake was 316% in H69AR cells, compared with only 158% in H69 cells, consistent with a preferential reduction in outward co-transport of Tc-Tetrofosmin in the GSH-depleted MRP1-expressing cell line.

Overall, expression of *MDR1* Pgp conferred much greater differences in Tc-Tetrofosmin cell uptake than expression of MRP1, whereas *MDR3* Pgp and BCRP/MXR/ABCP had little or no effect. Data were consistent with a GSH-drug outward co-transport function for MRP1. Furthermore, GF120918 showed greater and more consistent effects on tracer enhancement than cyclosporin A, and a strong preference of GF120918 for *MDR1* Pgp was confirmed.

Functional Interrogation of Pgp at the Blood–Brain Barrier In Vivo *with Tc-Tetrofosmin*

Pgp (*MDR1* in humans and *mdr1a* in mice) has an important physiologic function at the blood–brain barrier, limiting entry of many different amphipathic compounds into the central nervous system [57, 71]. To characterize the potential use of Tc-Tetrofosmin as a marker of the transport function and modulation of Pgp *in vivo*, we determined the biodistribution of Tc-Tetrofosmin in blood and brain tissue of FVB mice following intravenous injection of the tracer and a modulator (Fig. 7). To inhibit Pgp at the capillary blood–brain barrier, GF120918 (250 mg/kg) was administered by oral gavage to FVB mice 4 hr prior to injection of Tc-Tetrofosmin; this dosing regimen produces serum levels of drug that maximally inhibit Pgp [19]. Blood pharmacokinetics of Tc-Tetrofosmin were not changed significantly following the administration of GF120918 [AUC_{5-120} = 27.4 ± 5.5 vs 30.3 ± 5.9 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min in the absence and presence of GF120918, respectively, $P > 0.5$]. Penetration of Tc-Tetrofosmin into brain tissue of wild-type FVB mice was very limited, with an AUC_{5-120} of 7.4 ± 1.8 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min (N = 10), demonstrating exclusion of the tracer relative to blood. By comparison, in mice treated with GF120918, the AUC_{5-120} for Tc-Tetrofosmin in brain tissue increased to 15.1 ± 1.9 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min (N = 11), a value 204% above control ($P < 0.02$). Thus, increased accumulation and retention of Tc-Tetrofosmin in brain tissue following treatment with GF120918 were consistent with expectations for inhibition of Pgp at the capillary endothelium and could not be attributed to differences in blood content.

Mice have two isoforms of Pgp that confer multidrug resistance, but only *mdr1a* is expressed in endothelial cells of capillaries at the blood–brain barrier [71]. Thus, *mdr1a(−/−)* mice are thought to have no drug-transporting Pgp at the blood–brain barrier. To further demonstrate that drug-transporting Pgp at the blood–brain barrier excludes Tc-Tetrofosmin from the brain, we analyzed initial uptake and retention of the tracer in *mdr1a(−/−)* mice (Fig. 7). Comparison was made to wild-type FVB as well as *mdr1a/1b(−/−)* mice. Relative to wild-type mice, *mdr1a(−/−)* mice showed approximately 230% more Tc-Tetrofosmin in brain parenchyma 5 min after injection of the Tc-complex. Additionally, the AUC_{5-120} of Tc-Tetrofosmin in brain significantly increased to 24.1 ± 3.6 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min (N = 9), a value 327% greater than wild-type mice ($P < 0.005$). In *mdr1a/1b(−/−)* mice, the AUC_{5-120} of Tc-Tetrofosmin in brain was 27.7 ± 3.6 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min (N = 9), a value 374% greater than wild-type, but not significantly different from *mdr1a(−/−)* mice ($P > 0.5$). By contrast, blood retention of Tc-Tetrofosmin in *mdr1a(−/−)* and *mdr1a/1b(−/−)* mice was 25.2 ± 4.7 and 29.9 ± 8.4 μCi (injected μCi)$^{-1}$ (g tissue)$^{-1}$ × 100 × min, only 92 and 109% of the FVB control, respectively ($P > 0.5$ each). Net penetrations of Tc-Tetrofosmin into brain tissues of

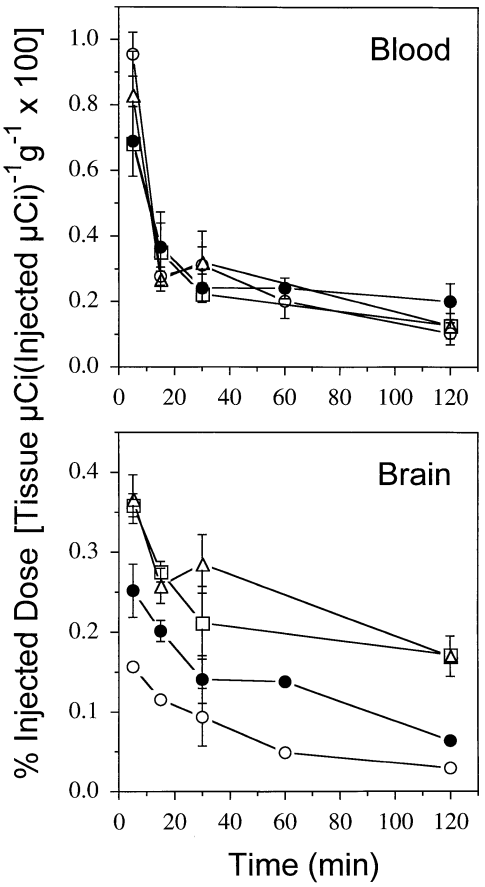

FIG. 7. Pharmacokinetics of ^{99m}Tc-Tetrofosmin in blood and brain of FVB mice. Parental FVB mice were administered GF120918 (250 mg/kg, ●) or vehicle alone (○), and *mdr1a(−/−)* mice (□) as well as *mdr1a/1b(−/−)* mice (△) also received vehicle alone by oral gavage 4 hr prior to intravenous bolus injection of ^{99m}Tc-Tetrofosmin. Data are expressed as percent of injected dose of radioactivity per gram tissue at each respective time point. For parental mice treated with vehicle alone or GF120918, data points represent the means of 3–4 determinations; for *mdr1a(−/−)* and *mdr1a/1b(−/−)* mice, data points represent the means of 2–3 determinations each; bars represent ± range (N = 2) or ± SEM (N = 3)when larger than the symbol.

mdr1a(−/−) and *mdr1a/1b(−/−)* mice also were 160 and 183%, respectively, of that found in FVB mice treated with GF120918 ($P < 0.05$). Because blood flow to the brain does not differ between wild-type and *mdr1a(−/−)* mice [72, 73], the observed enhancement in penetration and retention of Tc-Tetrofosmin in brains of *mdr1a(−/−)* and *mdr1a/1b(−/−)* mice cannot be attributed to differences in cerebral perfusion. These data support the hypothesis that Tc-Tetrofosmin is a substrate for drug-transporting Pgps and can be used as a marker of function and inhibition of these proteins *in vivo*. Additionally, these pharmacokinetic data provide functional evidence consistent with *mdr1a* as the dominant Pgp isoform at the blood–brain barrier.

DISCUSSION

Cellular Mechanisms of Localization of Tc-Tetrofosmin

The mechanisms of uptake and retention of selected hydrophobic cationic Tc-based complexes analogous to Tc-Tetrofosmin have been studied extensively in a variety of cellular and subcellular preparations *in vitro*. Net cell content of these Tc-based agents generally is a function of both passive potential-dependent influx and transporter-mediated efflux. For example, biophysical analysis has shown that Tc-Sestamibi is a high fidelity probe of transmembrane potential [50, 62], with passive inward movement of this lipophilic cation being driven (in the absence of cell surface transporters) by the transmembrane potentials generated in living cells [50, 74]. The complex is sequestered reversibly within mitochondria by the serial thermodynamic driving forces of the negative plasma membrane and mitochondrial inner membrane potentials [63]. While Tc-Tetrofosmin also has been shown to possess significant membrane potential-dependent uptake properties [44, 59, 75], the data would indicate that it does not respond in as robust a manner as Tc-Sestamibi. The reduced uptake of Tc-Tetrofosmin in steady-state may be due to decreased permeability arising from membrane interactions with the hydrophobic and positive character of Tc-Tetrofosmin such that full inward translocation across bilayers in response to imposed transmembrane potentials is prohibited. Outward transport of Tc-Tetrofosmin, but not Tc-Sestamibi, by another undetermined transporter expressed in drug-sensitive cells cannot be excluded.

Opposing passive influx is the action of efflux transporters such as *MDR1* Pgp [27, 54]. For example, baculoviral expression of recombinant human *MDR1* in insect cells confers decreased accumulation of Tc-Sestamibi [54], and, furthermore, MDR cells expressing Class I Pgp accumulate decreased Tc-Sestamibi in inverse proportion to the amount of immunodetectable transporter [27–31, 76]. Tc-Furifosmin, another Tc-based cationic agent, also behaves as a transport substrate for *MDR1* Pgp in a variety of MDR human and rodent cells [43, 44], but with a significantly lesser net difference between drug-sensitive and MDR cells. Other, more complex physicochemical interactions with the radiotracer due to the effects of membrane potential and pH [77] also cannot be excluded. Overall, net cellular accumulation of these tracers is decreased in general proportion to the level of Pgp expression, and enhancement of radiotracer net content is observed upon administration of modulators of Pgp such as verapamil, cyclosporin A, or the new high-potency agents such as GF120918 or PSC 833 [38, 42–44]. Addition of an MDR modulator that blocks selectively the function of *MDR1* Pgp and thus enables the tracer to accumulate within the cell forms the basis for a functional approach to assessment of *MDR1* Pgp in patients. Indeed, recent clinical studies with cancer patients have confirmed the utility of ^{99m}Tc-Sestamibi to functionally detect Pgp-mediated resistance in tumors *in vivo* using planar scintigraphy or single-photon emission computed tomography (SPECT) [32–37, 39, 40].

Recently, Tc-Tetrofosmin has been identified as another Tc-complex with potential *MDR1* Pgp-mediated transport properties [44, 49]. In the current study, we extended preliminary observations by characterizing the biochemical pharmacology of Tc-Tetrofosmin in mammalian cell lines expressing differing levels of ABC transporters and in a recombinant baculovirus system overexpressing isoforms of Pgp. Data from these systems supported the hypothesis that Tc-Tetrofosmin is outwardly transported to the extracellular space by the *MDR1* Pgp. In summary, (i) net cellular accumulation of Tc-Tetrofosmin was inverse to expression of Pgp in KB tumor cells and NIH3T3 cells transfected with *MDR1* Pgp; (ii) known modulators of Pgp increased cell contents of Tc-Tetrofosmin in multidrug-resistant cells to levels observed in drug-sensitive cells; (iii) EC_{50} values for potent and selective MDR modulators were consistent with known values for half-maximal inhibition of *MDR1* Pgp and were comparable for Tc-Tetrofosmin as those previously reported for Tc-Sestamibi, Tc-Q58, and Tc-Q63, other previously validated radioprobes of Pgp transport [27, 28, 42]; (iv) cell content of Tc-Tetrofosmin was decreased relative to control in Sf9 cells expressing recombinant human *MDR1* Pgp in a GF120918-dependent manner; and (v) cytotoxic agents not included in the MDR phenotype did not alter accumulation of Tc-Tetrofosmin in KB cells.

Cross-Reactivity

MDR in tumors also can be due to overexpression of *MRP1*, another member of the ABC transporter family [6]. *MRP1* and its protein product, MRP1, have subsequently been shown to be overexpressed in many cell lines that display the MDR phenotype ([78, 79] and references therein), and is widely expressed in various normal tissues, including heart, skeletal muscle, lung, kidney, testes, and epithelia [7]. *MDR1* Pgp and MRP1 transport a variety of structurally unrelated toxic xenobiotics, natural product chemotherapeutic drugs, phospholipids, and hydrophobic charged compounds [9]. While substrates transported by *MDR1* Pgp generally, but not always, are hydrophobic and cationic [4], MRP1 typically transports compounds that are amphipathic and anionic, such as leukotriene C_4 or compounds that

have been conjugated to GSH [80]. However, studies have shown that natural product cytotoxic agents that are neutral or cationic in character also can be transported by MRP1 [69, 70]. Furthermore, such compounds appear to be co-transported with but not conjugated to GSH [68, 70]. Indeed, *mrp1(−/−)* gene-disrupted mice show elevated levels of glutathione in lung, heart, kidney, muscle, and colon, but no change in organs known to express little if any *mrp1*, such as liver and small intestine [69].

Tc-Tetrofosmin also appears to be recognized as a transport substrate by MRP1, but to a lesser extent than *MDR1* Pgp. Specifically, (i) net cellular accumulation of Tc-Tetrofosmin was inverse to expression of MRP1 in H69 and H69AR tumor cells; (ii) net cellular accumulation of Tc-Tetrofosmin was inverse to expression of MRP1 in NIH3T3 cells transfected with recombinant human *MRP1*; and (iii) depletion of GSH in *MRP1*-expressing H69AR cells resulted in enhanced cellular accumulation of Tc-Tetrofosmin. Overall, 4- to 7-fold differences in cellular accumulation were observed with Tc-Tetrofosmin between control and MRP1-expressing cells, significantly less than the 10- to 40-fold differences observed with *MDR1* Pgp-expressing cells. Thus, whereas data suggested that Tc-Tetrofosmin may be transported by human MRP1, the differences in Tc-Tetrofosmin net uptake produced by *MRP1* expression were significantly *less* than differences produced by *MDR1* Pgp expression under similar conditions. Furthermore, while net accumulation levels (and putatively efflux) of Tc-Tetrofosmin from tumors have the potential to be functionally affected by expression of MRP1, this may depend further on intrinsic levels of GSH within a given tumor. As with Tc-Sestamibi, also putatively co-transported with GSH on MRP1 [76, 81, 82], MRP1-mediated transport is a double-edged sword. On the one hand, this reduces the apparent specificity of the tracers for functional imaging of the *MDR1* Pgp in tissues and tumors; but on the other hand, this property may favorably enable these Tc-complexes to be general probes of transporter-mediated MDR in cancer. Herein, modulator-induced reversal of tissue transport kinetics as determined by performing a second imaging exam, for example, after administration of LY335979, would provide specificity for *MDR1* Pgp *in vivo*.

Two other ABC transporters did not affect Tc-Tetrofosmin significantly. Uptake of Tc-Tetrofosmin in Sf9 cells expressing recombinant human *MDR3* Pgp was changed minimally as compared with control infection by wild-type baculovirus. Also, cell uptakes of Tc-Tetrofosmin (as well as Tc-Sestamibi) were not affected by expression of *BCRP/MXR/ABCP*, a recently cloned ABC half-transporter [10, 11], in two different cell lines that overexpress the transporter.

Functional Assessment of Pgp at the Blood–Brain Barrier

MDR1 Pgp is expressed in capillary endothelial cells in the brain, where the protein is a major component of the blood–brain barrier [83]. Gene-deleted *mdr1a(−/−)* mice are an excellent model system for investigating the role of Pgp in maintaining the blood–brain barrier. Recently, mice deficient in both *mdr1a* and *mdr1b* [*mdr1a/1b(−/−)*] also have been generated, and these animals showed no significant differences in accumulation of digoxin within brain parenchyma compared with *mdr1a(−/−)* mice, demonstrating that *mdr1a* dominates Pgp-mediated function at the blood–brain barrier in mice [84]. The present study showed that function and modulation of Pgp at the blood–brain barrier can be detected readily with tracer concentrations of Tc-Tetrofosmin within 5 min of injection of the agent and confirmed *mdr1a* as the dominant mechanism. As was reported with Tc-Q58 [42], increased brain accumulation of Tc-Tetrofosmin in *mdr1a(−/−)* mice relative to FVB mice treated with GF120918 was again observed. This may reflect differences between the complete absence of drug-transporting Pgp in the former animals versus the pharmacological inhibition of *mdr1a* Pgp in the latter. Although it is unlikely, lack of complete drug penetration into an active site cannot be excluded. Note that the majority of the differences between mice can be attributed to the initial penetration levels of the tracer leading to greater overall brain retention in knockout mice. Net brain washout kinetics, being quite similar for all mice, must be mediated by a Pgp-independent mechanism.

In conclusion, Tc-Tetrofosmin may be useful as a surrogate marker for detection of transporter-mediated MDR in tumors and tissues *in vivo*. Effective inhibition following administration of highly potent and specific modulators can be detected *in vitro* and *in vivo* and may provide a means for specific detection of *MDR1* Pgp [34, 35]. Thus, pharmacokinetic mapping of Pgp with Tc-complexes potentially can be used to improve therapy for patients treated with cytotoxic drugs of the MDR phenotype. Non-invasive detection of successful inhibition of Pgp- or MRP-mediated transport in tumors may also assist in evaluating the success of therapy with targeted modulators. Recent clinical data also have demonstrated the feasibility of imaging various levels of Pgp expression in bone marrow with Tc-Sestamibi [85]. Given that bone marrow is the target organ for *MDR1* expression in gene therapy directed at hematopoietic cell protection during cancer chemotherapy, these observations demonstrate the feasibility of using radiopharmaceuticals such as Tc-Tetrofosmin and Tc-Sestamibi for the noninvasive, repetitive monitoring of ectopic expression of *MDR1* Pgp in chemoprotective gene therapy. All of these uses potentially could guide successful chemotherapy in patients with cancer.

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