Expression of Recombinant Human Multidrug Resistance P-Glycoprotein in Insect Cells Confers Decreased Accumulation of Technetium-99m-Sestamibi

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The multidrug-resistant P-glycoprotein is a M, 170,000 plasma membrane protein encoded by the mammalian multidrug resistance gene (MDR) which appears to function as an efflux transporter of a variety of potent chemotherapeutic agents. Methods: To directly demonstrate that 99mTc-sestamibi is recognized by the human P-glycoprotein, we overexpressed recombinant human MDR1 P-glycoprotein in host Sf9 insect cells using a baculoviral vector and correlated expression of the gene product with 99mTc-sestamibi accumulation. Results: In parental Sf9 cells and in wild-type baculovirus infected (control) cells, 99mTc-sestamibi accumulation asymptotically approached a plateau of 650 femtols (mg protein)^{-1} (nM)^{-1} and 337 femtols (mg protein)^{-1} (nM)^{-1}, respectively. In MDR1 baculovirus infected cells, P-glycoprotein expression was maximal at 72 hr postinfection, while 99mTc-sestamibi accumulation was reduced to 12 femtols (mg protein)^{-1} (nM)^{-1}. Verapamil (500 µM), the classical MDR modulator, produced an ~300% enhancement of 99mTc-sestamibi accumulation in Sf9 cells expressing MDR1 P-glycoprotein, but only a 50% enhancement in parental Sf9 cells, consistent with verapamil-induced inhibition of P-glycoprotein-mediated 99mTc-sestamibi efflux. Conclusions: These data demonstrate that the recombinant protein is transiently expressed in a functional state capable of drug transport in Sf9 cell membranes and that 99mTc-sestamibi is a transport substrate recognized by the human MDR1 P-glycoprotein. Technetium-99m-sestamibi may prove useful for functionally characterizing P-glycoprotein expression in human tumors in vivo.

Key Words: multidrug resistance; P-glycoprotein; baculovirus; isonitrile complex; verapamil, technetium-99m-sestamibi


Resistance of malignant tumors to multiple chemotherapeutic agents is a major cause of treatment failure (1–6). Cells or tissues obtained from tumors and grown in the presence of a selecting cytotoxic drug can result in cross-resistance to other drugs in that class, as well as other classes of drugs, including anthracyclines, Vinca alkaloids, taxol and epipodophyllotoxins (1,7). One major mechanism of multidrug resistance in mammalian cells involves the increased expression of the M, 170,000 plasma membrane P-glycoprotein (2,8). Transfection of cloned P-glycoprotein is sufficient to cause multidrug resistance in experimental systems (9), and it is believed that by transporting chemotherapeutic agents out of the cells, P-glycoprotein renders tumors resistant to chemotherapy.

Increased levels of P-glycoprotein or P-glycoprotein messenger RNA have been detected in all forms of human cancers, including leukemias, lymphomas, sarcomas and carcinomas (10). In relapsing patients, an increased level of P-glycoprotein has been observed in postchemotherapy tumor biopsies; this is especially true with regard to neuroblastoma (11,12). Increased levels of P-glycoprotein have also been seen in late stage ovarian and breast carcinomas (13,14).

One surprising aspect of multidrug resistance is the apparent capacity of P-glycoprotein to recognize and transport a large group of cytotoxic compounds sharing little or no structural or functional similarities (12,15), other than being relatively small, hydrophobic and cationic (16). In this regard, hexakis(2-methoxyisobutyl isonitrile) technetium(I) (99mTc-sestamibi), a lipophilic cationic radiopharmaceutical useful in myocardial perfusion imaging, has been shown to have lower accumulation in P-glycoprotein-enriched hamster cell lines compared to their respective parental drug-sensitive cell lines (17).

Ambiguity exists, however, because such studies do not unequivocally prove that 99mTc-sestamibi is transported by the P-glycoprotein. These cells have been enriched in P-glycoprotein by growth selection in the presence of the cytotoxic agent doxorubicin, and thus, undetected co-expressed gene products or regulators could confound interpretation of the results. More definitive evidence would result from insertion of the target gene into an expression system to directly investigate the effect of the protein prod-
uct on transport and binding of the pharmaceutical. We chose to take advantage of the helper-independent baculovi-
ral expression system to directly demonstrate P-glycopro-
tein-mediated transport of $^{99m}$Tc-sestamibi.

Host *Spodoptera frugiperda* (S9) insect cells were infected with a recombinant *Autographa californica* nuclear polyhedrosis baculovirus, containing the human MDR1 gene under the control of the strong polyhedron promoter (18), to achieve high levels of expression of the multidrug transporter. Several properties of the host S9 cells could be exploited, including: (1) cells could be grown in monolayer culture, thereby facilitating transport assays; (2) parenteral S9 cells have little or no natural expression of P-glycoprotein, providing a convenient baseline for $^{99m}$Tc-sestamibi accumulation; and (3) baculovirus-infected S9 cells are able to perform many higher eukaryotic post-translational modifications, such as glycosylation and phosphorylation, and therefore, have been extensively characterized for the successful overexpression of a variety of cytoplasmic and integral membrane proteins in active functional states (19). We report that $^{99m}$Tc-sestamibi accumulation is significantly decreased in S9 cells expressing the human MDR1 gene.

**METHODS**

**Materials**

*Autographa californica* nuclear polyhedrosis baculovirus containing the human MDR1 (V185) gene under control of the poly-
henron promoter (BV-MDR1) (18) was kindly provided by Michael Gottesman (National Cancer Institute, Bethesda, MD). Wild-type *Autographa californica* baculovirus and host *Spodoptera frugiperda* (S9) insect cells were kindly provided by Helen Piwnica-Worms (Harvard Medical School, Boston, MA). Drug-sensitive V79 and multidrug-resistant 77A and LZ cells (20) were kindly provided by James Cross and grown as previously described (17). Anti-P-glycoprotein monoclonal antibody (Mab) C219 was purchased from Signet (Dedham, MA). Secondary an-
tibodies were purchased from Promega (Madison, WI).

Synthesis of the radiolabeled compound $^{99m}$Tc-sestamibi was performed with a one-step kit formulation (Cardiolite, kindly pro-
vided by E.I. Du Pont, Medical Products Division, Billerica, MA) containing solid stannous chloride (0.075 mg) as a reducing agent for the technetium and MIBI as the Cu(MIBI)$_2$BF$_4$ salt, as described (21).

**Viral Infection and S9 Cell Membrane Preparation**

S9 cells were grown and infected with wild-type baculovirus or recombinant BV-MDR1 using the general protocols and methods of Summers and Smith (19,22). Parental or virus-infected S9 cells were cultured in P150 flasks and 100-mm culture dishes containing seven 25-mm glass coverslips on the bottom (to serve as substrates for cell growth). Flasks and dishes were seeded with $\sim$0.9–1.0 $\times$ 10$^7$ cells per 10 ml of IPL-41 medium supplemented with 5% (V/V) fetal bovine serum. The virus was typically added at a multiplicity of infection of 5 PFU per cell.

Cell membranes of S9 cells were prepared 24, 48, 72 and 96 hr postinfection according to the procedure described by Sarkadi et al. (23). Briefly, cells were harvested by scraping them into Tris-
mannitol buffer (300 mM mannitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM Tris, pH 7.0 titrated with HCl). Cells were ho-
mogenized using a glass-Teflon tissue homogenizer in TMEP (50 mM Tris, pH 7.0 with HCl, 50 mM mannitol, 2 mM EGTA, 10 $\mu$g/ml leupeptin, 8 $\mu$g/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride and 2 mM beta-mercaptoethanol), and undisrupted cells and nuclear debris were removed by centrifugation at 500 $\times$ g for 10 min. The supernatant was then centrifuged at 100,000 $\times$ g for 1 hr; the resulting pellet containing membranes was resuspended in TMEP and stored at $-70^\circ$C.

**Western Blotting**

SDS-PAGE and Western blotting was performed according to standard protocols (24,25). Briefly, 100 $\mu$g of membrane fractions were mixed with Laemml sample buffer for 20 min at room temperature before loading onto 7% SDS-polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (0.76 M glycine, 20% methanol, 2.5 mM Tris, pH 8) and proteins were transferred to nitrocellulose sheets using a blotting apparatus (0.5 A at 100 V, 4°C) for 1–3 hr. Blots were blocked for 1 hr at room temperature in TBST (0.05% Tween 20, 0.15 M NaCl, 10 mM Tris, pH 8.0) and 10% bovine milk powder, followed by incubation with the primary antibody overnight at 4°C. Blots were washed for 10 min in TBST $\times$ 3, incubated in goat anti-mouse antibody conjugated to alkaline phosphatase for 1 hr, then washed for 10 min in TBST $\times$ 3. Specific antigen-antibody complexes were revealed by incubation with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine and nitroblue tetrazolium (Sigma). The dil-
utions of the antibodies were as follows: Mab C219 (1:150, i.e., 0.66 $\mu$g/ml), goat anti-mouse (1:7,500).

**Cell Transport Studies**

Control buffer 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$_2$PO$_4^-$, 0.8 SO$_4^{2-}$, 5.6 dextrose, 4.0 HEPES and 1% bovine calf serum (vol/vol); pH 7.4 $\pm$ 0.05.

Coverslips with S9 cells were removed from culture media, and preequilibrated for 40–60 sec in control buffer. Uptake and retention experiments were initiated by immersion of coverslips in 60-mm glass Pyrex dishes containing 4 ml of loading solution consisting of MEBSS with 0.02–0.6 nM [Tc-sestamibi] (5–9 pmols/mCi; 3–100 $\mu$Ci/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 sec each to clear extracellular spaces, and extracted in 2 ml of a 1% sodium dodecylsulfate, 10 mM Na-
borate solution. Aliquots of the loading buffer and stock solutions were then obtained for standardizing cellular data with extracellu-
lar concentrations (nM$_o$) of $^{99m}$Tc-sestamibi. Cell extracts, stock solutions and extracellular buffer samples were assayed for gamma activity in a well-type gamma counter (Packard, Minaxi Autogamma 5000). Cell samples were then quantified for protein content by the method of Lowry (26), using bovine serum albumin as the protein standard. Knowledge of the elution history of the generator and activity of stock solutions allowed use of generator equilibrium equations to calculate the absolute concentration of total Tc-sestamibi in the solutions (27). Transport data are pre-
sented as fmole $\cdot$ (mg protein)$^{-1}$ $\cdot$ (nM$_o$)$^{-1}$. For S9 cells, there are 2.9 $\times$ 10$^6$ cells (mg protein)$^{-1}$.

**Curve Fit and Statistical Analysis**

All data points in any panel were determined in quadruplicate with preparations obtained from the same culture. Values are presented as mean $\pm$ s.e.m. Curves were computer fit to the following equation: $A = A_n(1 - e^{-kt})$, where $A_n$ is final steady-
state cell accumulation, k is the influx rate constant, and t is time. Multiple comparisons were made by one-way analysis of variance (28). Pairs were compared by the Student’s t-test. Values of p < 0.05 were considered significant.

RESULTS

Sf9 insect ovarian cells were infected with a recombinant baculovirus carrying the human MDR1 cDNA which had been constructed and characterized previously (18). Uninfected Sf9 cells and cells infected with wild-type baculovirus served as control preparations. At various times postinfection, cells were disrupted by homogenization and crude plasma membrane preparations were collected by centrifugation. The time course of expression of human MDR1 P-glycoprotein in Sf9 cells is shown in Figure 1 as detected by immunoblotting with C219, an anti-P-glycoprotein monoclonal antibody. C219 shows detectable immunoreactivity with a band of M, 130,000 by 24 hr postinfection and reaches maximum intensity by 72 hr postinfection. Parental Sf9 cells and mock-infected cells have been shown to have no immunodetectable P-glycoprotein with this Mab (23). The M, 130,000 immunoreactive protein confirms the time course of expression of the coreglycosylated form of MDR1 P-glycoprotein as previously reported in Sf9 cells with this recombinant baculovirus (18,23).

To directly determine the functional state of the coreglycosylated P-glycoprotein expressed in the Sf9 cells, radiotracer accumulation curves were determined with 99mTc-sestamibi. Figure 2 shows the time course of accumulation of 99mTc-sestamibi in parental Sf9 cells, wild-type baculovirus 70 hr postinfection cells, and MDR1 baculovirus 70 hr postinfection cells. In parental Sf9 cells, computer fit of the data revealed that 99mTc-sestamibi approached a plateau with a half-time ~17 min and attained a steady-state of 650 fmole (mg protein)⁻¹ (nMₜ)⁻¹. In wild-type baculovirus-infected cells, the time course of 99mTc-sestamibi accumulation was similar, however, the plateau was reduced to 337 fmole (mg protein)⁻¹ (nMₜ)⁻¹. This likely reflected the lytic effect of viral infection, thereby partially depolarizing mitochondrial and plasma membrane potentials. In MDR1 baculovirus-infected cells, net accumulation of 99mTc-sestamibi was further reduced to only 12 fmole (mg protein)⁻¹ (nMₜ)⁻¹. Thus, expression of the recombinant human MDR1 gene in Sf9 cells reduced 99mTc-sestamibi net uptake by 97%, a result directly predicted by P-glycoprotein-mediated efflux of the lipophilic cationic radiotracer.

To correlate the relative expression of P-glycoprotein with the functional state of the transporter as probed with this radio-substrate, 60-min 99mTc-sestamibi accumulation values in Sf9 cells were determined at daily intervals during the course of MDR1 baculoviral infection (Fig. 3). In general, 60-min tracer accumulation declined (Fig. 3A) as membrane expression of P-glycoprotein increased (Fig. 1). However, the largest interval decrease in tracer accumulation occurred between 22 and 46 hr postinfection despite the observation that the most dramatic increase in immunodetectable P-glycoprotein expression occurred between 46 and 70 hr postinfection. Similar trends in transport data were obtained for 2-min uptakes, the shortest time interval examined (data not shown). Overall, these data indicated a nonlinear relationship between membrane expression of P-glycoprotein and substrate transport, and thus, implied that not all of the expressed gene product was in a functional state.

This was further explored by evaluation of the effects of inhibition of P-glycoprotein on 99mTc-sestamibi transport. To determine if Sf9 cell content of 99mTc-sestamibi could be increased by inhibition of P-glycoprotein, verapamil, the classical MDR reversal agent (12), was added to the
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The attempt at maximal concentrations of verapamil (500 μM) demonstrated potent enhancing effects on 60-min 99mTc-sestamibi accumulation. The effect peaked with 46 hr postinfection MDR1 Sf9 cells, and was significantly less with 22-hr postinfection cells, 70-hr postinfection cells, and parental Sf9 cells. Thus, the potency of the MDR reversal effect of verapamil did not directly correlate with the expressed protein content, but instead, the maximum functional blockade of P-glycoprotein-mediated efflux of the radiotracer occurred at ~46 hr postinfection, a time earlier than maximal gene expression.

The data developed in this transient expression system indicated a transport model where net cell uptake of 99mTc-sestamibi was driven by the summed effects of membrane potential-mediated influx countered by P-glycoprotein-mediated efflux. To test if any changes occurred in the final compartment size for 99mTc-sestamibi accumulation, we attempted to block P-glycoprotein-mediated efflux with a maximal concentration of verapamil and net uptake of the radiotracer determined. As P-glycoprotein expression increased, there was a decline in net 99mTc-sestamibi accumulation (Fig. 4A), even with these maximal reversing verapamil concentrations. However, and as shown in 99mTc-sestamibi in MDR1 baculovirus infected Sf9 cells. (B) 99mTc-sestamibi 60-min accumulation in the presence of 500 μMverapamil in MDR1 baculovirus infected Sf9 cells. (B) 99mTc-sestamibi 60-min accumulation in the presence of 100 μMverapamil in V79, 77A and LZ cells. Each bar represents the mean ± s.e.m. of 3–4 determinations each. Asterisks indicate p < 0.05 compared to 22-hr postinfection cells (A) or V79 cells (B).

radiotracer assay buffer. As shown in Fig. 3B, maximal concentrations of verapamil (500 μM) demonstrated potent enhancing effects on 60-min 99mTc-sestamibi accumulation. The effect peaked with 46 hr postinfection MDR1 Sf9 cells, and was significantly less with 22-hr postinfection cells, 70-hr postinfection cells, and parental Sf9 cells. Thus, the potency of the MDR reversal effect of verapamil did not directly correlate with the expressed protein content, but instead, the maximum functional blockade of P-glycoprotein-mediated efflux of the radiotracer occurred at ~46 hr postinfection, a time earlier than maximal gene expression.

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Net accumulation and unidirectional influx of 99mTc-sestamibi are thermodynamically responsive to negative mitochondrial inner membrane potentials (ΔΨ) and plasma membrane potentials (E_m) (29). The agent is concentrated within nonMDR cells such as heart and within liposomes in a manner similar to other selected lipophilic cationic probes of membrane potential (30,31). Recent studies have also shown 99mTc-sestamibi accumulation in various tumors in vivo (32,33) and human carcinoma cell lines in vitro (34). It is possible that the variable accumulation identified in these tissues may be, in part, attributable to P-glycoprotein-mediated efflux.

**Technetium-99m-Sestamibi as an MDR1 P-Glycoprotein Substrate**

Baculoviruses have emerged as a powerful system for overproducing biologically active recombinant proteins in eukaryotic cells for the study of protein function and for receptor-targeted drug development (19,22,35,36). Overproduction and biochemical characterization of MDR1 P-glycoprotein in the baculoviral expression system was recently reported (18). The recombinant protein was specifically recognized by a battery of antibodies directed against different regions of the human transporter, and was localized by immunohistochemical analysis to the plasma membrane.
membrane of the host cells. Putative precursor forms were identified in the Golgi apparatus. The membrane-bound protein was able to bind a P-glycoprotein-directed photoaffinity probe, \(^{3}H\)azidopine, and known modulators of P-glycoprotein were able to displace the probe. In addition, other investigators reported that the overexpressed protein results in a drug-stimulated ATPase activity (23). However, because baculoviral infection is lytic to the host SF9 cells, previous studies had not been able to directly demonstrate the ability of the recombinant transporter to confer multidrug resistance nor to demonstrate drug transport. We now report that baculoviral expression of the MDR1 gene generates a functionally active transporter able to mediate the efflux transport of the lipophilic cationic P-glycoprotein substrate \(^{99m}Tc\)-sestamibi.

The data indicated that the transporter was expressed in its most active functional state between 24 and 48 hr postinfection. However, total P-glycoprotein content as determined by immunoreactivity with C219 was highest at 72 hr postinfection. This is consistent with the known viral life cycle and its impact on host cell protein synthesis. The baculovirus tends to shut down host function by 24 hr postinfection and divert cell metabolism to viral protein production (22). As late phase infection proceeds, the expression of the desired protein is maximal, but many forms of host cell post-translational processing such as endoplasmic reticulum-dependent protein maturation, glycosylation or phosphorylation may be compromised. Thus, the \(^{99m}Tc\)-sestamibi data demonstrating maximal verapamil-enhanced accumulation 46 hr postinfection would be consistent with optimal expression of functional MDR1 P-glycoprotein during a time interval when a variety of other expressed proteins have shown maximal biological activity (19). The functional \(^{99m}Tc\)-sestamibi transport data thus suggest that a significant portion of late infection P-glycoprotein is partially or perhaps completely inactive.

**Clinical Significance**

Increased levels of P-glycoprotein or mRNA have been detected in nearly all forms of human cancer and correlated with poor clinical outcome in many instances (10). The ability to assay in vivo the P-glycoprotein transporter non-invasively with a pharmacologically inert tracer substrate such as \(^{99m}Tc\)-sestamibi could provide a significant new tool for advancing the clinical understanding of the MDR phenotype in cancer patients. In addition, the low cost and ease of preparation of this class of SPECT agents could allow facile application of the imaging test. This approach may ultimately be used to guide chemotherapeutic protocols, assist clinical trials or screening of new MDR reversing agents, and direct tumor biopsies. The goal of this functional test is to directly assist patient management, or to tailor chemotherapy for the individual patient, and thus, need not be confined to pre-therapeutic diagnosis. For example, we envision a noninvasive imaging test to directly determine the best chemotherapeutic regimen for a given patient by imaging the effect of MDR reversing agents on net accumulation of \(^{99m}Tc\)-sestamibi in an individual tumor. In this context, \(^{99m}Tc\)-sestamibi could serve as a nontoxic surrogate chemotherapeutic agent. The true value of identifying the expression of P-glycoprotein may be to monitor the effect of chemotherapeutic regimens, not to locate or identify primary tumors. Many common extraabdominal tumors such as breast, lung, mediastinal lymphoma and limb sarcomas could easily benefit from this approach. Previous biodistribution studies with \(^{3}H\)colchicine (37) and \(^{99m}Tc\)-sestamibi (17) have demonstrated the feasibility of using radioactive MDR substrates to distinguish multidrug resistant and sensitive tumors in vivo. Furthermore, the clinical feasibility of planar and SPECT \(^{99m}Tc\)-sestamibi breast and lung imaging has already been validated in patients (38–40).

An important controversy in the field of multidrug resistance is whether identification of the expression of P-glycoprotein per se with antibodies or mRNA probes is sufficient, or is it necessary to assay directly the function of this transport protein. Given that regulatory pathways, such as protein kinase C-mediated phosphorylation (12), have been shown to modulate P-glycoprotein transport function, \(^{99m}Tc\)-sestamibi may provide an unique opportunity to directly assay protein function in vivo and in vitro. The relatively straight-forward radiotracer assay system described in this report may also provide a simple tool for functionally evaluating new chemotherapeutic agents or MDR modulators. In addition, this system should be a powerful tool to characterize and directly screen novel technetium-isonitrile complexes for high affinity interactions with the human MDR1 P-glycoprotein.

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