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Characterization of Rhodamine 123 Binding to P-Glycoprotein in Human Multidrug-Resistant Cells

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SUMMARY

The overexpression of P-glycoprotein is currently believed to be responsible for the enhanced efflux or decreased influx of cytotoxic drugs across the cell membrane in drug-resistant cells. P-glycoprotein has been proposed to mediate the efflux of a large number of structurally and functionally unrelated drugs. Although it has been suggested that P-glycoprotein binds directly to many lipophilic cations, it remains unclear whether one or more sites in P-glycoprotein mediate its broad substrate specificity. In this report, a photoactive derivative of rhodamine 123 (Rh123) [^{125}I-azidosalicylic acid (ASA)-Rh123] was synthesized and used in a photoaffinity labeling assay to demonstrate, for the first time, direct and specific binding to P-glycoprotein. The photoaffinity labeling of P-glycoprotein by ASA-Rh123 was specifically inhibited in the presence of vinblastine and verapamil but not in the presence of colchicine. Surprisingly, ASA-Rh123 photoaffinity labeled a 6-kDa V8 peptide in P-glycoprotein that was previously shown to be photoaffinity labeled by another multidrug resistance-associated drug, [^{125}I]iodoarylazidoprazosin. Photoaffinity labeling of mitochondria from drug-sensitive or -resistant cells with ^{125}I-ASA-Rh123 did not reveal significant differences in the mitochondrial proteins from sensitive or resistant cells. Interestingly, however, ^{125}I-ASA-Rh123 did photolabel a 66-kDa protein in mitochondria that was not detected in plasma membrane preparations with this assay. Taken together, our results demonstrate for the first time that Rh123 binds specifically to P-glycoprotein and that its binding site may be shared by other multidrug resistance-associated drugs.

The treatment of cancer patients with antineoplastic agents has been hampered significantly by the development of MDR to chemotherapeutic drugs. Such pleitropic drug resistance *in vitro* is believed to be mediated by the overexpression of a 170-kDa protein termed P-gp (1, 2). P-gp is thought to function as a drug efflux pump that derives energy from ATP hydrolysis, and its efflux action results in drug concentrations that are below cytotoxic levels in MDR cells (3–5). P-gp shares structural homology with other ATP-binding transport proteins such as the yeast STE6 transporter (6), the cystic fibrosis transmembrane conductance regulator (7), and the bacterial periplasmic membrane transporters (8). However, unlike other membrane transporters (6–8), P-gp appears to have a broad substrate specificity towards lipophilic compounds that share no apparent structural or functional similarities. The molecular mechanism of P-gp drug efflux is currently not understood; however, photoaffinity labeling of membranes from drug-resistant cells with photoactive drugs has demonstrated direct and specific binding of cytotoxic drugs to P-gp (9). Furthermore, certain nontoxic drugs, including the calcium channel blockers, quinidines, and cyclosporins, have been shown to bind P-gp and reverse its MDR-associated phenotype (10, 11).

The P-gp gene family in humans and rodents is made up of two or three structurally homologous but functionally distinct proteins (12). Only classes I and II in rodents and class I in humans have been shown to mediate the drug-resistant phenotype in transfectant cells (13, 14). The function of the class III isoform remains undefined (15, 16). The differential expression of P-gp isoforms has been demonstrated in normal tissues from humans and rodents (17–20). The class I isoform was predominantly overexpressed in the liver and small intestine, whereas the class III isoform was overexpressed in normal muscle tissue in mice and hamsters (19, 20). Based on this tissue distribution of P-gp, a transport function similar to that now accepted for P-gp in tumor drug-resistant cells has been proposed (21, 22). The recent demonstration of ATP-dependent drug transport in membrane vesicles from normal rat hepatocytes and intestinal mucosa provided additional evidence for the role of P-gp in mediating drug efflux in normal tissue. Thus,

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ABBREVIATIONS: MDR, multidrug resistant (resistance); Rh123, rhodamine 123; P-gp, P-glycoprotein; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; IAAP, iodoarylazidoprazosin; DMF, dimethylformamide; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ASA, azidosalicylic acid.

given the capacity of P-gp in normal tissue and MDR tumors to transport hydrophobic drugs, drug transport studies using the fluorescent probe Rh123 are currently used to predict the presence of an intrinsically active or chemotherapeutically induced P-gp drug efflux pump in tumors (23). More recently (24), Rh123 has been used in large screening assays to identify P-gp-specific MDR-reversing drugs.

Rh123, a cationic fluorescent dye, has been shown to accumulate selectively in the mitochondria of eukaryotic cells (25). Interestingly, P-gp-associated MDR cells that were selected for resistance to anticancer drugs were shown to extrude Rh123 more rapidly than their drug-sensitive parental cells (26, 27). Furthermore, the Rh123 efflux from drug-sensitive or -resistant cells was inhibited in the presence of the calcium channel blocker verapamil (26, 27). Based on these observations, it was suggested that Rh123 is a substrate for the P-gp efflux pump; however, no direct evidence for P-gp-Rh123 interactions was demonstrated. In addition, the specific localization of Rh123 to the mitochondria is thought to be dependent on the high membrane potential across the mitochondrial membrane (28). Consequently, changes in the mitochondrial membrane potential would likely affect the cellular accumulation of Rh123 (28). Thus, the possibility that the reduced accumulation of Rh123 in drug-resistant cells is mediated by a verapamil-sensitive mechanism other than P-gp cannot be ruled out. In this study, a photoactive derivative of Rh123 was synthesized and its interactions with P-gp and mitochondrial proteins in drug-sensitive or -resistant cells were evaluated in a photoaffinity labeling assay.

Experimental Procedures

Materials. Rh123 and NHS-ASA were obtained from Pierce Chemical Co. Vinblastine was from Aldrich, whereas verapamil and colchicine were from Sigma Chemical Co. [^{125}I]IAAP (2200 Ci/mmol) was purchased from DuPont-New England Nuclear. P-gp-specific mAb (C219) was a kind gift from Dr. Victor Ling of the Ontario Cancer Institute (Toronto, Canada). Drug-sensitive human lymphoma cells (CEM) (29) were a gift from Dr. W. Beck at St. Jude Children's Research Hospital (Memphis, TN). The CEM/VLB^{1000} line was established from the CEM/VLB^{100} line obtained from Dr. W. Beck.[1] All other chemicals used were of the highest grade available.

Synthesis and characterization of ASA-Rh123. ASA-Rh123 was synthesized by reacting Rh123 and NHS-ASA in DMF. Briefly, 5 mg (16.5 μmol) of Rh123 were dissolved in 250 μl of DMF, and 5 μl of triethylamine were added. The mixture was vortex mixed and 250 μl (7.5 mg, 27 μmol) of NHS-ASA in DMF were added. The reaction was allowed to proceed for 48 hr at room temperature, with mixing. The solvent was removed by vacuum drying and the oily residue was dissolved in 250 μl of methanol. The photoactive derivative of Rh123, ASA-Rh123, was purified by high performance liquid chromatography using a Vydac 201HS54 C_{18} reverse phase column (4.6 mm × 25 cm), with a 30-min gradient of 20–100% acetonitrile in 0.025 M ammonium acetate buffer, pH 5.5. The products were monitored at 505 nm or 290 nm. Under these conditions Rh123, NHS-ASA, and ASA-Rh123 had retention times of 15.50, 18.80, and 20.05 min, respectively. The final purification of ASA-Rh123 was done in the absence of UV detection, to avoid photodestruction. The ASA-Rh123 fractions were collected, vacuum dried, and stored at −20° until needed. Fig. 1A shows the chemical structures of unmodified and NHS-ASA-modified Rh123. The absorption spectrum of ASA-Rh123 before and after UV irradiation is shown in Fig. 1B. UV irradiation abolished the absorbance at 269 nm

[1] V. Ling, unpublished observations.

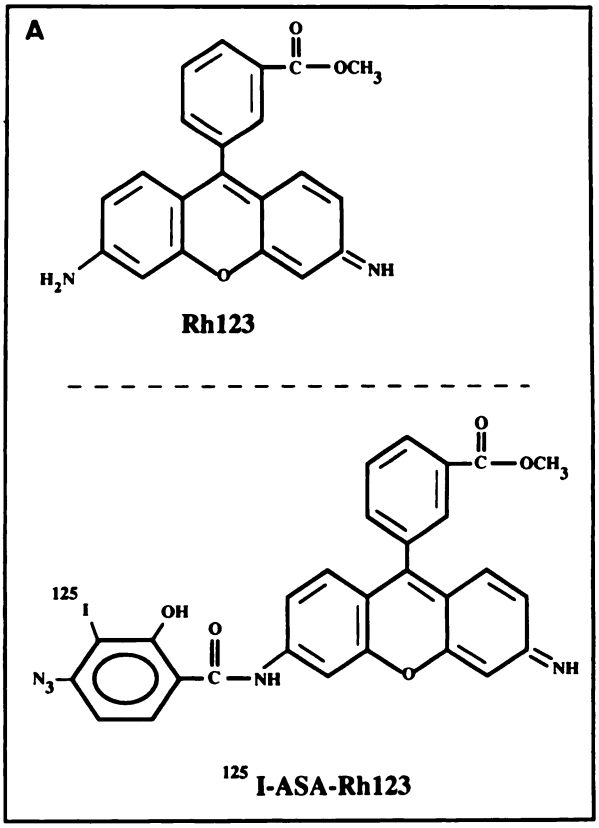

Fig. 1. A, Chemical structures of unmodified Rh123 and its photoactive derivative ^{125}I-ASA-Rh123. B, Absorbance profile of ASA-Rh123 in methanol before and after UV irradiation at 254 nm for 2 min. The absorbance peaks due to the ASA moiety and Rh123 are marked.

(ASA moiety), whereas the 505-nm peak of Rh123 remained relatively stable.

Iodination of ASA-Rh123. The method of Hunter and Greenwood (30) was used to prepare the ^{125}I-ASA-Rh123 derivative. ASA-Rh123 (1–2 μg), in 5 μl of dimethylsulfoxide, was added to 50 μl of freshly prepared chloramine-T (3 μg/μl) solution. Carrier-free Na^{125}I (2 mCi) in 20 μl of 0.01 M sodium phosphate, pH 8.5, was added to the mixture. The reaction was allowed to proceed for 2 min at room temperature and was quenched with 50 μl of 5% sodium metabisulfate. The mixture was diluted with sodium phosphate buffer and loaded onto a SepPak cartridge (Millipore). The cartridge was washed with 0.01 M sodium phosphate, pH 8.5 containing 10% methanol until no detectable radioactivity eluted. ^{125}I-ASA-Rh123 was eluted with 5 ml of methanol and the solvent was evaporated to dryness under nitrogen. The mixture

was redissolved in methanol and purified by high performance liquid chromatography as described for the unlabeled drug.

Tissue culture and plasma membrane preparation. Drug-sensitive (CEM) and -resistant (CEM/VLB^{1000}) human leukemic cell lines were grown in α-minimal Eagle's medium as described previously (29). Plasma membrane vesicles were prepared using the calcium precipitation procedure, essentially as described by Lin *et al.* (31). Briefly, CEM or CEM/VLB^{1000} cells were washed in phosphate buffer, pH 7.4, containing 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 and were suspended in a hypotonic buffer containing protease inhibitors (50 mM mannitol, 5 mM HEPES, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, 30 μM leupeptin), on ice. Cells were homogenized in a glass Dounce homogenizer and the cell suspension was made up to 10 mM calcium chloride. The calcium-induced membrane aggregates were removed by centrifugation at 3000 × *g* in a Sorval centrifuge, using a SS34 rotor. The slightly turbid supernatant containing the plasmalemma vesicles was collected by a 100,000 × *g* centrifugation for 1 hr, using a Beckmen SW50 rotor. The resultant pellet was washed with 10 mM Tris·HCl, pH 7.4, and stored at −80° until needed. Protein concentration was measured using bovine serum albumin as a standard (32).

Preparation of mitochondrial fraction. CEM or CEM/VLB^{1000} cells were resuspended in a hypotonic buffer and homogenized as described above for the preparation of membrane vesicles, but without the addition of calcium chloride. The homogenate was centrifuged at 660 × *g* for 10 min at 4° to remove unbroken cells and nuclei. The supernatant was removed and centrifuged at 8820 × *g* for 15 min. The resultant pellet was suspended in 1 ml of 10 mM Tris·HCl, pH 7.4, containing 250 mM sucrose and was centrifuged at 8820 × *g* for 15 min, using the SS34 rotor. The final pellet was suspended in Tris buffer and stored at −80° until required.

Photoaffinity labeling. Aliquots (20 μg) of plasma membrane fractions from drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells were incubated with 50 nM ^{125}I-ASA-Rh123 (2 × 10^6 cpm) for 1 hr in the dark at room temperature. For drug inhibition, membrane fractions were preincubated with a 500- or 1000-fold molar excess of Rh123, verapamil, vinblastine, or colchicine for 30 min before the addition of the labeled drug. At the end of the incubation period the mixture was maintained on ice for 10 min, followed by UV irradiation for 10 min at 254 nm (Stratagene UV cross-linker; Stratagene, La Jolla, CA).

Immunoprecipitation of photoaffinity-labeled proteins. Immunoprecipitation of ^{125}I-ASA-Rh123-photolabeled P-gp was carried out as described previously (33). Briefly, 100 μg of ^{125}I-ASA-Rh123-photolabeled plasma membranes from CEM or CEM/VLB^{1000} cells were incubated with 10 μg of mAb C219 (34) or an irrelevant second antibody. Proteins bound to mAb C219 or IgG2a were isolated using Protein A-Sepharose beads (Pharmacia). The ^{125}I-ASA-Rh123-photoaffinity-labeled proteins were eluted from Protein A-Sepharose beads with 5% SDS/10 mM dithiothreitol and resolved by SDS-PAGE.

Rh123 uptake. Aliquots of CEM or CEM/VLB^{1000} cells (1 × 10^6) were incubated with 0.1 μM Rh123 or ASA-Rh123 in the absence or presence of 100 μM vinblastine, verapamil, or colchicine. Cells were incubated for 1 hr at 37° and then washed three times in ice-cold phosphate-buffered saline. The cell pellets were lysed with 100 μl of 0.5 N NaOH. The cell lysate was neutralized with an equal volume of 0.5 N HCl, and the fluorescence intensity was determined using a fluorescence spectrophotometer with settings at 515-nm excitation and 535-nm emission. Fluorescence measurements were corrected for autofluorescence from cells not exposed to Rh123.

Protease cleavage. Plasma membrane fractions from CEM/VLB^{1000} cells were suspended in 10 mM Tris·HCl, pH 7.4, 250 mM sucrose, and photolabeled with 50 nM ^{125}I-ASA-Rh123 or 20 nM [^{125}I] IAAP, as described earlier. After SDS-PAGE, ^{125}I-ASA-Rh123- or [^{125}I] IAAP-photolabeled P-gp was digested with *Staphylococcus aureus* V8 protease (5–10 μg) in gel slices loaded in wells of a 15% acrylamide gel, essentially as described by Cleveland *et al.* (35).

PAGE. Membrane protein fractions were resolved by SDS-PAGE using the Fairbanks gel system (36). For Cleveland maps samples were run on a 15% Laemmli gel system (37). After electrophoresis gel slabs were fixed in 40% methanol/10% acetic acid and dried. Dried slabs were exposed to XAR film at −80° overnight.

Results

Rh123 is thought to transverse the cell membrane by passive diffusion and selectively accumulate in the mitochondria of cells *in vitro* (25). Earlier reports (26, 27) using Rh123 had demonstrated a reduced accumulation of Rh123 in MDR cells, which was potentiated in the presence of verapamil. Based on these observations, it was suggested that Rh123 may be a substrate for P-gp. However, it was not clear from those studies whether Rh123 interacts directly with P-gp or its efflux from MDR cells is mediated via a verapamil-sensitive transport mechanism other than P-gp. To address these questions a photoactive derivative of Rh123 was synthesized (see Experimental Procedures) (Fig. 1) and used in a photoaffinity labeling assay. Plasma membranes from drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells were incubated in the presence of 50 nM ^{125}I-ASA-Rh123 and UV irradiated (see Experimental Procedures). The results in Fig. 2 show that a 170-kDa protein was specifically photolabeled with ^{125}I-ASA-Rh123 in membranes from drug-resistant cells (Fig. 2, *lane 2*), whereas no 170-kDa protein was photolabeled with ^{125}I-ASA-Rh123 in plasma membranes from drug-sensitive cells (Fig. 2, *lane 1*). The identity of the 170-kDa ^{125}I-ASA-Rh123-photoaffinity-labeled protein in drug-resistant cells was confirmed by immunoprecipitation of ^{125}I-ASA-Rh123-photolabeled membrane proteins with P-gp-specific mAb C219. Fig. 2, *lane 4*, shows a 170-kDa ^{125}I-ASA-Rh123-photoaffinity-labeled protein specif-

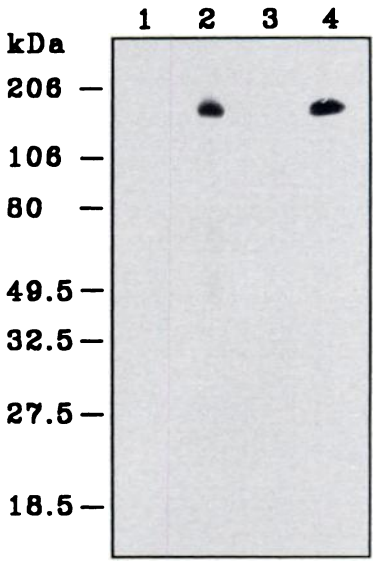

Fig. 2. Photoaffinity labeling of P-gp with ^{125}I-ASA-Rh123. Plasma membranes from drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells were photoaffinity labeled with 50 nM ^{125}I-ASA-Rh123. *Lanes 1* and *2*, photoaffinity labeling of plasma membranes from drug-sensitive and -resistant cells, respectively. A 170-kDa protein was photoaffinity labeled with ASA-Rh123 in plasma membranes from drug-resistant cells. *Lanes 3* and *4*, immunoprecipitation of ^{125}I-ASA-Rh123-photoaffinity-labeled proteins from CEM/VLB^{1000} cell membranes with an irrelevant IgG2a antibody or mAb C219, respectively. A 170-kDa ^{125}I-ASA-Rh123-photoaffinity-labeled protein, P-gp, was immunoprecipitated with mAb C219 (*lane 4*) but not with an irrelevant antibody (*lane 3*). The positions of the molecular weight markers are indicated to the *left*.

ically immunoprecipitated with mAb C219 from drug-resistant plasma membranes. No ^{125}I-ASA-Rh123-photoaffinity-labeled 170-kDa protein was immunoprecipitated with an irrelevant IgG2a (Fig. 2, *lane 3*). These results demonstrate that the 170-kDa protein photoaffinity-labeled with ^{125}I-ASA-Rh123 is P-gp, as confirmed by its specific binding to mAb C219 (19, 34).

To determine the binding specificity of ^{125}I-ASA-Rh123 for P-gp, photoaffinity labeling of CEM/VLB^{1000} cells was carried out in the presence of increasing concentrations of ^{125}I-ASA-Rh123 (Fig. 3). Fig. 3, *inset*, shows ^{125}I-ASA-Rh123-photoaffinity-labeled P-gp after SDS-PAGE. Saturation of ^{125}I-ASA-Rh123 binding was obtained in the range of 0.05–8 μM drug. To further confirm the specificity of ASA-Rh123 binding to P-gp, photoaffinity labeling of plasma membranes was carried out in the presence of excess Rh123 (Fig. 4). Fig. 4, *lanes 1* and *2*, shows photoaffinity-labeled plasma membranes from CEM and CEM/VLB^{1000} cells, respectively. Similar photoaffinity labeling in the presence of a 500- or 1000-fold molar excess of Rh123 resulted in a marked decrease in the photoaffinity labeling of P-gp (Fig. 4, *lanes 3* and *4*). To further characterize the specificity of Rh123 binding to P-gp and the effect of other MDR-associated drugs on this binding, membrane fractions from drug-resistant cells were incubated with ^{125}I-ASA-Rh123 in the absence or presence of a 1000-fold molar excess of verapamil, vinblastine, or colchicine before UV irradiation. The results in Fig. 5 show a decease in the photoaffinity labeling of P-gp with ^{125}I-ASA-Rh123 in the presence of vinblastine and verapamil (Fig. 5, *lanes 3* and *4*). Colchicine at a similar concentration did not reduce the photoaffinity labeling of P-gp with ASA-Rh123 (Fig. 5, *lane 5*).

Results on the specificity of ^{125}I-ASA-Rh123 binding were further supported by drug uptake studies, which compared the intracellular concentrations of unmodified Rh123 or ^{125}I-ASA-Rh123 in drug-sensitive and -resistant cells in the absence or presence of a 100 μM concentration of colchicine, verapamil, or

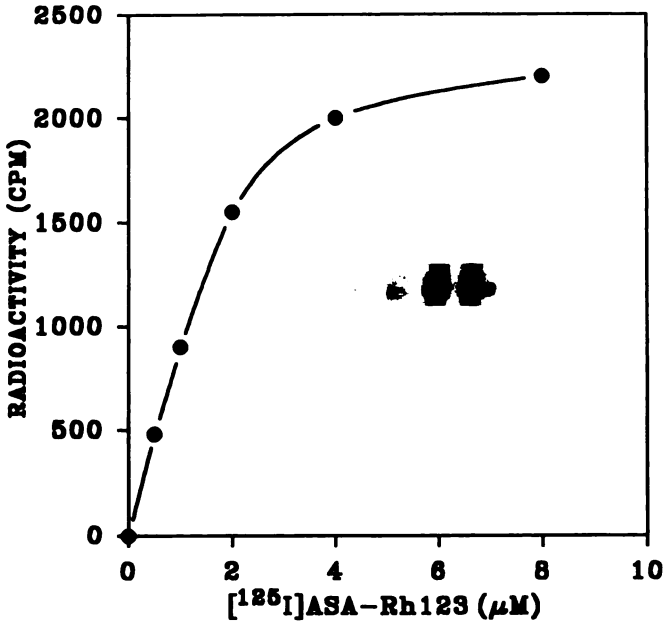

Fig. 3. Saturability of ^{125}I-ASA-Rh123 binding to CEM/VLB^{1000} cell plasma membranes. Plasma membranes (20 μg) were labeled with increasing concentrations (0–10 μM) of ^{125}I-ASA-Rh123, appropriately diluted with unlabeled Rh123. After SDS-PAGE the bands corresponding to P-gp (see *inset*) were excised and quantified by γ counting.

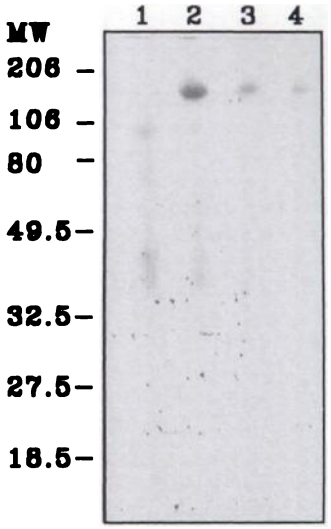

Fig. 4. Effects of Rh123 on the photoaffinity labeling of P-gp with ^{125}I-ASA-Rh123. Plasma membranes from drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells were photolabeled with 50 nM ^{125}I-ASA-Rh123. *Lanes 1* and *2*, photoaffinity labeling of membrane fractions from drug-sensitive and -resistant cells, respectively. *Lanes 3* and *4*, decreased photoaffinity labeling of P-gp from CEM/VLB^{1000} cells in the presence of a 500- and 1000-fold molar excess Rh123, respectively.

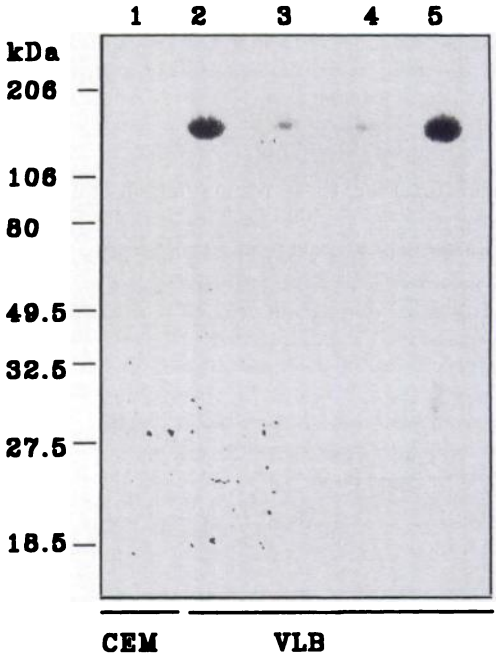

Fig. 5. Effects of vinblastine, verapamil, and colchicine on the photoaffinity labeling of P-gp with ^{125}I-ASA-Rh123. Plasma membranes from CEM/VLB^{1000} (VLB) cells were photoaffinity labeled with 50 nM ^{125}I-ASA-Rh123 in the absence (*lane 2*) and in the presence of a 1000-fold molar excess of verapamil (*lane 3*), vinblastine (*lane 4*), or colchicine (*lane 5*). Vinblastine and verapamil inhibited the photoaffinity labeling of P-gp by ^{125}I-ASA-Rh123, whereas colchicine did not. Photoaffinity labeling of plasma membranes from drug sensitive (CEM) cells with ^{125}I-ASA-Rh123 is shown in *lane 1*.

vinblastine. The results in Fig. 6A show high levels of Rh123 in drug-sensitive but not drug-resistant cells. Vinblastine and verapamil at 100 μM final concentrations increased the accumulation of Rh123 in drug-resistant cells, whereas colchicine caused only a slight increase in Rh123 uptake in drug-resistant cells. Similar results were also observed when ASA-Rh123 was

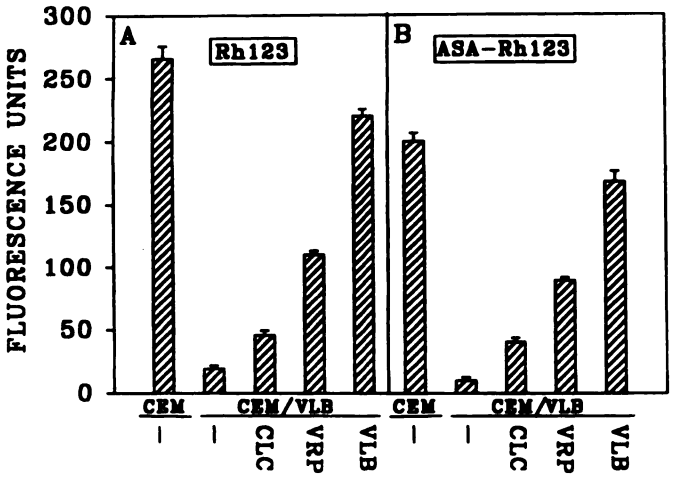

Fig. 6. Rh123 or ASA-Rh123 uptake. Drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells were incubated for 1 hr in α-minimal Eagle's medium in the presence of 0.1 μM Rh123 (A) or noniodinated ASA-Rh123 (B), in the absence or presence of 100 μM colchicine (*CLC*), verapamil (*VRP*), or vinblastine (*VLB*). The total accumulation of Rh123 or ASA-Rh123 was determined using fluorescence spectroscopy. The fluorescence values are expressed as a percentage of control CEM uptake (mean ± standard error of a representative of three experiments carried out triplicate).

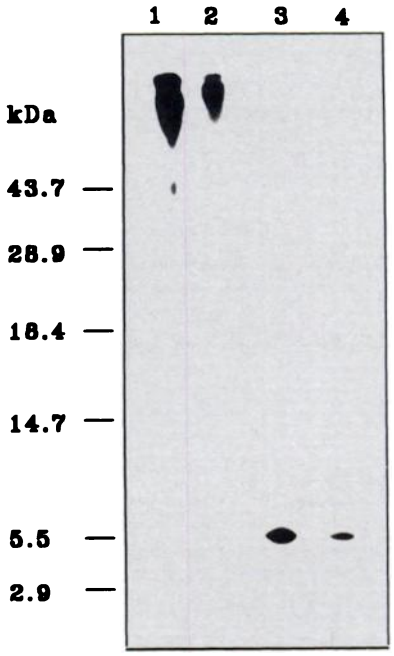

Fig. 7. Cleveland maps of ^{125}I-ASA-Rh123- or [^{125}I]IAAP-photoaffinity-labeled P-gp. Plasma membranes from CEM/VLB^{1000} cells were photoaffinity labeled with [^{125}I]IAAP or ^{125}I-ASA-Rh123 and exhaustively digested in gel slices with V8 protease. *Lanes 1* or *2* and *3* or *4* show undigested ^{125}I-IAAP- or ^{125}I-ASA-Rh123-photoaffinity-labeled P-gp, and their V8-digested products, respectively. A 6-kDa peptide in P-gp was photoaffinity labeled with [^{125}I]IAAP or ^{125}I-ASA-Rh123.

used in the drug uptake studies (Fig. 6B). The presence of colchicine, verapamil, or vinblastine did not significantly increase the uptake of Rh123 in drug-sensitive cells (data not shown). Thus, the modification of Rh123 with NHS-ASA did not affect its uptake properties in drug-sensitive or -resistant cells. Taken together, these results demonstrate for the first time a direct and specific binding between Rh123 and P-gp in plasma membranes from drug-resistant cells.

IAAP, an α_1-adrenergic probe, was previously shown to specifically photoaffinity label P-gp in plasma membranes from drug-resistant cells (38). Protease cleavage of [^{125}I]IAAP-photoaffinity-labeled P-gp yielded a major peptide (≈6 kDa) that was also shown to be photoaffinity labeled with [^{3}H]azidopine, a calcium channel blocker. More recently (39), two tryptic peptides, with apparent molecular masses of 4 and 5 kDa, were shown to contain the [^{125}I]IAAP photoaffinity label. Given the specificity of ^{125}I-ASA-Rh123 binding to P-gp and the molecular structure of ^{125}I-ASA-Rh123, which is different from the structures of [^{125}I]IAAP and [^{3}H]azidopine, it was of interest to compare the peptides in P-gp that are photoaffinity labeled with ^{125}I-ASA-Rh123 and those previously identified using [^{125}I]IAAP or [^{3}H]azidopine (38). Photoaffinity-labeled (with ^{125}I-ASA-Rh123 or [^{125}I]IAAP) P-gp was digested with *Staphylococcus aureus* V8 protease and the digestion products were resolved on SDS-PAGE. The results in Fig. 7 show a single peptide, with an apparent molecular mass of ≈6 kDa, photoaffinity labeled with ^{125}I-ASA-Rh123 or [^{125}I]IAAP (Fig. 7, *lanes 4* and *3*). These results indicate that ASA-Rh123 and IAAP, which are structurally different, photoaffinity label similar peptides in P-gp.

The specific localization of Rh123 to the mitochondria is thought to be due to the high negative membrane potential across the mitochondrial membrane (28). Thus, the addition of an arylazido group onto one of the two amino groups in Rh123 (see Fig. 1A) is expected to reduce the net cationic moment of the modified Rh123 and could alter its localization to the mitochondria. To determine whether the modification of Rh123 alters its cellular target, CEM cells were incubated with 0.1 μM Rh123 or ASA-Rh123 at 37° for 30 min and cells were viewed under a fluorescence microscope. Rh123 and ASA-Rh123 revealed similar staining patterns, with the majority of the fluorescence being concentrated in the mitochondria (data not shown). These results indicate that the chemical modification of Rh123 with an arylazido group does not affect its targeting to the mitochondria, and they may suggest the presence of other structural moieties in Rh123, in addition to the cationic charges, that are recognized by mitochondrial receptors. Although the aforementioned data in this study indicate a specific interaction between P-gp and Rh123, it was of interest to ascertain whether changes in mitochondrial receptors could be responsible for the differential accumulation of Rh123 in drug-sensitive and -resistant cells. Photoaffinity labeling of mitochondrial preparations from drug-sensitive (CEM) or -resistant (CEM/VLB^{1000}) cells with ^{125}I-ASA-Rh123 is shown in Fig. 8. Fig. 8, *lanes 1* and *2*, show ^{125}I-ASA-Rh123-photoaffinity-labeled mitochondrial proteins from drug-sensitive and -resistant cells, respectively. Two proteins, with apparent molecular masses of 54 kDa and 66 kDa, were photoaffinity labeled with ASA-Rh123 (Fig. 8, *lanes 1* and *2*). Although some differences were observed in the intensity of ASA-Rh123-photolabeled proteins between drug-sensitive and -resistant cells, these differences were not consistently observed and were generally variable. More interesting was the presence of a 66-kDa protein that was photoaffinity labeled with ^{125}I-ASA-Rh123 in the mitochondrial preparations but was not detected in the plasma membranes (Fig. 8, *lanes 1* and *2* versus *lanes 3* and *4*). The other ASA-Rh123-photoaffinity-labeled protein in the mitochondrial preparations (54 kDa) was also detected in the plasma

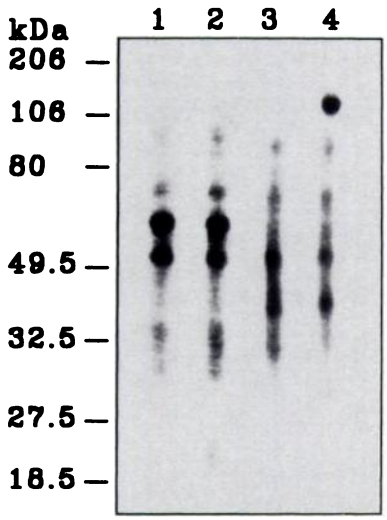

Fig. 8. Photoaffinity labeling of mitochondria with ^{125}I-ASA-Rh123. Mitochondrial proteins (*lanes 1* and *2*) or plasma membrane proteins (*lanes 3* and *4*) from drug-sensitive or -resistant cells, respectively, were photoaffinity labeled with 50 nM ^{125}I-ASA-Rh123. Two mitochondrial proteins, with apparent molecular masses of 66 kDa and 54 kDa, were photoaffinity labeled with ^{125}I-ASA-Rh123. The 66-kDa ^{125}I-ASA-Rh123-photoaffinity-labeled mitochondrial protein was not detected in plasma membranes from drug-sensitive or -resistant cells using this assay.

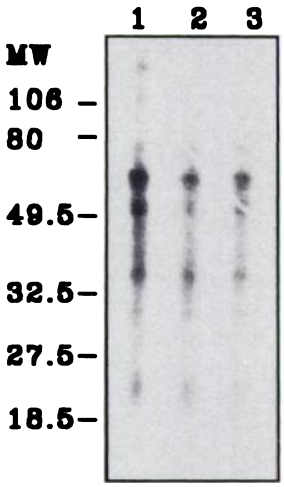

Fig. 9. Effects of Rh123 on the photoaffinity labeling of mitochondria with ^{125}I-ASA-Rh123. Mitochondrial preparations from CEM/VLB^{1000} cells were photoaffinity labeled with 50 nM ^{125}I-ASA-Rh123 in the absence (*lane 1*) or in the presence of a 500- or 1000-fold molar excess of Rh123 (*lanes 2* and *3*, respectively). Photoaffinity labeling of mitochondrial proteins was inhibited in the presence of Rh123.

membrane preparations, albeit with lower intensities (Fig. 8, *lane 1* versus *lane 3*). It should be pointed out that, given the purity of our mitochondrial preparation, which is likely to contain other cellular organelles such as the lysosomes, it could not be established whether some or all of the ASA-Rh123-photoaffinity-labeled proteins are of mitochondrial origin. Based on these preliminary photoaffinity-labeling experiments, the photoaffinity labeling of mitochondrial preparations from drug-sensitive and -resistant cells shows no significant differences and thus is consistent with the role of P-gp in mediating the enhanced efflux of Rh123 from drug-resistant cells. Photoaffinity labeling of the 66-kDa mitochondrial protein was specifically inhibited in the presence of unlabeled Rh123 (Fig. 9). Fig. 9, *lane 1*, shows photoaffinity labeling of mitochondrial preparations in the absence of Rh123, whereas Fig. 9, *lanes 2* and *3*, show photolabeling in the presence of a 500- and 1000-fold molar excess of Rh123, respectively. These results demonstrate that the intereactions between Rh123 and the 66-kDa mitochondrial protein are specific, as indicated by the reduction in the photoaffinity labeling of the protein with ^{125}I-ASA-Rh123 in the presence of excess amounts of unmodified Rh123 or nonradiolabeled photoactive analogue (ASA-Rh123).

Discussion

In this study we have demonstrated for the first time that a photoactive derivative of Rh123, ^{125}I-ASA-Rh123, binds specifically to P-gp in drug-resistant cells. Although other proteins were photoaffinity labeled with ASA-Rh123, P-gp was the major receptor for Rh123 and was consistently photoaffinity labeled in membranes from drug-resistant cells. Moreover, the inhibition of P-gp photoaffinity labeling with ^{125}I-ASA-Rh123 in the presence of excess Rh123, vinblastine, or verapamil further demonstrated the specificity of ^{125}I-ASA-Rh123 photolabeling of P-gp. These results are in agreement with earlier reports (26, 27) relating to the role of P-gp in mediating the enhanced efflux of Rh123 in drug-resistant cells, and they provide direct evidence for P-gp-Rh123 interactions. The capacity of verapamil and vinblastine but not colchicine to inhibit the ASA-Rh123 photoaffinity labeling of P-gp is consistent with earlier observations using photoactive derivatives of verapamil (40), vinblastine (41), and cyclosporin A (42). It is not clear whether the differences between the effects of vinblastine or verapamil and those of colchicine on the photoaffinity labeling of P-gp via ASA-Rh123 are due to differences in the drug binding sites or other physicochemical differences.

The drug binding sites of P-gp are currently not known. Using mutational analysis, amino acid sequences in two putative transmembrane domains (transmembrane domains 6 and 11) were recently shown to affect the substrate specificity of P-gp toward MDR-associated drugs (43, 44). It is not known whether these amino acid residues are part of the drug binding sites or affect P-gp drug binding through conformational changes. The latter possibility may be likely, in view of an earlier report (45) in which a point mutation at position 185 (glycine to valine) in the intracellular domain was also shown to modulate the drug-binding specificity of P-gp. By another approach, using photoactive drug analogues, it was shown that a 6-kDa proteolytic fragment of P-gp was photoaffinity labeled with [^{125}I]IAAP (α-adrenergic probe) and [^{3}H]azidopine (calcium channel blocker) (38). Our results demonstrate that ^{125}I-ASA-Rh123 and [^{125}I]IAAP photoaffinity labeled similar V8 peptides, as determined from their co-migration on SDS-PAGE. These results further demonstrate the specificity of Rh123 binding to P-gp and suggest the possibility that a single drug-binding domain in P-gp is likely to accommodate many structurally and functionally diverse compounds. Although it remains to be determined whether the V8 peptides photoaffinity labeled with [^{125}I]IAAP and ^{125}I-ASA-Rh123 are the same, using antipeptide antisera Greenberger (39) demonstrated that sequences in P-gp immediately to the carboxyl termini of transmembrane domains 6 and 12 are likely to encode at least one of the aforementioned photoaffinity-labeled peptides. When these findings are taken together, it remains to be seen whether other photoactive drug analogues photo-

affinity label the same V8 peptide in P-gp and how these sequences code for the permissive drug binding site.

Using a photoactive derivative of Rh123, we have been able to test directly whether differences in the mitochondrial proteins in drug-sensitive and -resistant cells, in addition to P-gp, may be responsible for Rh123 efflux. Our results show no significant differences in the intensities of Rh123-photoaffinity-labeled proteins in mitochondria from drug-sensitive or -resistant cells. Although these results confirm the role of P-gp in mediating the efflux of Rh123 in drug-resistant cells, the possibility of other changes not detected by our photoaffinity labeling assay cannot be ruled out. However, differential sensitivity of susceptible and drug-resistant Friend leukemia cells to Rh123 does correlate with inhibition of oxidative phosphorylation (46). Our current results further support this observation, in that the patterns of ^{125}I-ASA-Rh123 photoaffinity labeling are similar for mitochondria from sensitive and MDR cell lines. The presence of a 66-kDa protein that specifically interacts with Rh123 suggests that accumulation of this compound in mitochondria may be mediated by specific receptor(s). However, further characterization of this mitochondrial protein is required to determine its role as a Rh123 receptor. The early findings by Summerhayes *et al.* (47) relating to the differences in the retention time of Rh123 between muscle cells (3–5 days) and untransformed bladder epithelia cells (1 hr) remain unclear. One possibility is that a non-P-gp mechanism may mediate the clearance of Rh123 in normal cells and drug-resistant cells. Alternatively, the class I isoform of P-gp, which has been detected in certain normal tissues, may be sufficient to cause Rh123 efflux (15, 16). In this respect it is interesting that muscle tissue, which expresses the class III isoform of P-gp, retains Rh123 for several days (19, 20). We are currently assessing whether the latter possibility is responsible for the differential accumulation of Rh123 in different normal tissues.

In summary, the results in this study demonstrate that the differential accumulation of Rh123 in drug-sensitive and -resistant cells is mediated via a direct and specific interaction between P-gp and Rh123. Interestingly, Rh123 appears to share a binding site in P-gp similar to that shown for other MDR-associated drugs. In addition, although it is likely that the mitochondria encode some Rh123 receptors, as determined from our photoaffinity labeling assay using ASA-Rh123, no significant differences in the intensities of these proteins were detected between drug-sensitive and -resistant cells.

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