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Most Drugs that Reverse Multidrug Resistance Also Inhibit Photoaffinity Labeling of P-Glycoprotein by a Vinblastine Analog

SHIN-ICHI AKIYAMA, MARILYN M. CORNWELL, MICHIHIKO KUWANO, IRA PASTAN, and MICHAEL M. GOTTESMAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 (M.M.C., I.P., M.M.G.), Department of Cancer Chemotherapy, Institute of Cancer Research, Faculty of Medicine, Kagoshima University, 1208-1 Usuki-cho, Kagoshima 890, Japan (S.-I.A.), and Department of Biochemistry, Oita Medical School, Hazama-cho, Oita 879-56, Japan (M.K.)

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SUMMARY

Multidrug-resistant human KB carcinoma cells express a 170,000-dalton membrane glycoprotein (P-glycoprotein) that can be photoaffinity labeled with the vinblastine analog *N*-(*p*-azido-[3-^{125}I]salicyl]-*N'*-(*β*-aminoethyl)vindesine. Several agents that suppress the multidrug-resistant phenotype, including *N*-solanesyl-*N*,*N'*-bis(3,4-dimethylbenzyl)ethylenediamine, cepharanthine, quinidine, and reserpine, were found to inhibit photolabeling of P-glycoprotein at doses comparable to those that reverse multidrug resistance. However, the phenothiazines chlorpromazine and trifluoperazine, which also effectively reverse multidrug resistance, were poor inhibitors of the photoaffinity labeling of P-glycoprotein. Chloroquine, propranolol, or atropine, which only partially reversed the drug resistance, also did not inhibit photolabeling. Naphthalene sulfonamide calmodulin inhibitors, W7 and W5, as well as many other drugs that did not circumvent multidrug resistance, did not inhibit photolabeling. These studies suggest that most, but not all, agents that phenotypically suppress multidrug resistance also inhibit drug binding to a site on P-glycoprotein with which a photoaffinity analog of vinblastine interacts.

Multidrug-resistant human tumor cell populations pose a major obstacle to effective cancer chemotherapy (1). Drug-resistant sublines of human KB carcinoma cells (2, 3) and other cells (reviewed in Ref. 4) have been established and used to determine the molecular basis of the multidrug-resistant phenotype. Resistance is due to drug efflux (5) resulting from a membrane glycoprotein of 170,000 daltons (P-glycoprotein) (4) encoded by the *MDR*1 gene in human cancer cells (6). The human (7), mouse (8), and hamster (9) *mdr* genes have been sequenced and shown to encode a membrane glycoprotein with 12 transmembrane domains and two nucleotide-binding sites. Transfer of cloned mouse (10) and human (11) *mdr* genes confers multidrug resistance on sensitive cells.

We have previously demonstrated the specific binding of ^{3}H-vinblastine to membrane vesicles from multidrug-resistant cells and the specific labeling of P-glycoprotein with a photoaffinity analog of vinblastine, ^{125}I-NASV (12–14). Some drugs that reverse multidrug resistance, such as the calcium channel blockers, and quinidine, which is not a calcium channel blocker, block labeling of P-glycoprotein by ^{125}I-NASV (13, 14). One possible hypothesis is that agents that reverse multidrug resistance do so by competing with cytotoxic drugs for a binding site on P-glycoprotein, the drug efflux pump.

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Recent studies have indicated that phenothiazine calmodulin inhibitors, synthetic isoprenoids, lysosomotropic agents, and bisbenzylisoquinoline alkaloids also overcome multidrug resistance in multidrug-resistant KB cells (15–18). All of these agents inhibit the active efflux of anticancer drugs (15–18). Although the mechanism by which these agents overcome multidrug resistance is not known, they are all amphipathic, lipophilic drugs that could react with a site or sites on P-glycoprotein similar to that with which cytotoxic drugs interact. In this study, we report that most, but not all of these agents, inhibit the photoaffinity labeling of P-glycoprotein in membrane vesicles prepared from multidrug-resistant human KB cells.

Materials and Methods

Cell culture and cell lines. Human epidermal KB carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). The multidrug-resistant mutant KB-V1 was selected with increasing concentrations of vinblastine and maintained as described previously (2, 3).

Membrane vesicle preparation. Membrane vesicles from KB-V1 cells were prepared as described (12, 19) from cells grown in 24 × 24 cm dishes (GIBCO) under standard growth conditions (2, 19). Protein concentrations were determined by the method of Bradford (20).

Photoaffinity labeling. Membrane vesicles were incubated with 3.8 μM ^{125}I-NASV (5×10^5 dpm) (13, 21) for 15 min at room temperature in the presence or absence of various drugs. After continuous irradiation

ABBREVIATIONS: P-glycoprotein, membrane glycoprotein of 170,000 daltons; ^{125}I-NASV, *N*-(*p*-azido-[3-^{125}I]salicyl-*N'*-(*β*-aminoethyl)vindesine; SDB-ethylenediamine, *N*-solanesyl-*N*,*N'*-bis(3,4-dimethylbensyl)ethylenediamine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

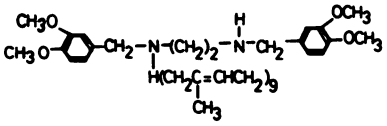
of samples at 366 nM for 20 min at 25°, samples were solubilized in sodium dodecyl sulfate sample buffer as described by Debenham *et al.* (22).

Sodium dodecyl sulfate gel electrophoresis. Samples labeled with ^{125}I-NASV were fractionated by electrophoresis on an sodium dodecyl sulfate/polyacrylamide/urea gel using a modification of the system described by Debenham *et al.* (22) on a 5% polyacrylamide/4.5 M urea gel, pH 7.6, without a stacking gel. Proteins were stained with Coomassie blue [0.25% in 50% (w/v) trichloroacetic acid].

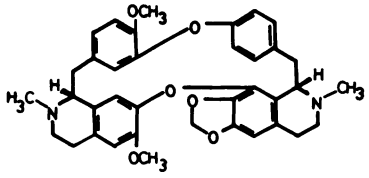
Drugs and chemicals. Cepharanthine was obtained from Kaken Pharmaceutical Co., Ltd. (Osaka, Japan). SDB-ethylenediamine was synthesized, purified, and used as described previously (13). W7 and W5 were gifts of Drs. H. Hidaka and T. Tanaka (Mie University). Other agents were purchased from Sigma Chemical Co. (St. Louis, MO). Structures of some of these agents are shown in Fig. 1.

Results

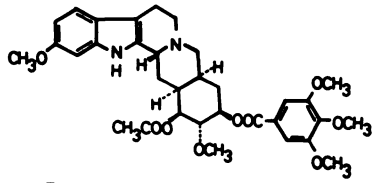
The 150- to 170-kDa P-glycoprotein in membrane vesicles from a multidrug-resistant KB cell line, KB-V1, is specifically labeled with ^{125}I-NASV (Fig. 2, *first lane* in each *panel*). This protein is overexpressed in multidrug-resistant cell lines, and it is not detectable in drug-sensitive parental or revertant cells by photolabeling (13, 14). In Fig. 2, we show that cationic and amphipathic or lipophilic agents, such as SDB-ethylenediamine, cepharanthine, and phenothiazine calmodulin inhibitors, which can completely overcome multidrug resistance in human KB cells (15–18), inhibit the photolabeling of P-glycoprotein in KB-V1 vesicles. Cepharanthine and SDB-ethylenediamine at 10–100 μM almost totally inhibited the photolabeling activity. The phenothiazine, thioridazine, at 200 μM only partially inhibited this activity (Fig. 2). The calmodulin inhibitor W7, which cannot reverse drug resistance, did not inhibit photolabeling even at 200 μM.



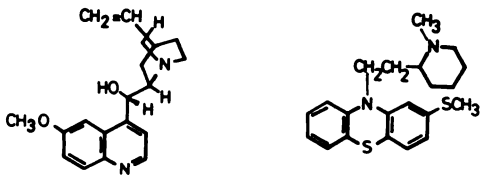
SDB–ethylenediamine



Cepharanthine



Reserpine



Quinidine Thioridazine

Fig. 1. Structural formulas of some drugs used in this study.

Reserpine and quinidine are known to reverse drug resistance in P388 mouse leukemia cells (23, 24). They also inhibited the photolabeling of P-glycoprotein in KB-V1 vesicles. Quinine, a stereoisomer of quinidine, inhibited photolabeling about 10-fold less efficiently than quinidine.

The data were quantitated by cutting out gel slices corresponding to the bands on the autoradiograms to determine their content of radioactivity (Fig. 3). SDB-ethylenediamine, reserpine, and quinidine inhibited photolabeling at concentrations similar to that of vinblastine, with half-maximal inhibition of the labeling between 1 and 10 μM. This concentration range is similar to the range at which these agents reverse multidrug resistance (Table 1). In contrast, a phenothiazine calmodulin inhibitor, thioridazine, inhibited photolabeling to about 50% at 100 μM, whereas it overcame multidrug resistance at 5 μM. Chloroquine, a cationic and amphipathic lysosomotropic agent, partially overcomes multidrug resistance at 6 μM (14), but it did not inhibit photolabeling even at 100 μM (Table 1). Other lysosomotropic agents that partially reverse drug resistance, atropine, atenolol, and propranolol, also did not inhibit photoaffinity labeling. Nifedipine, a potent calcium channel blocker which is a poor reversor of drug resistance, was also a poor inhibitor of photoaffinity labeling. Colchicine, a drug to which multidrug-resistant cells are cross-resistant, did not inhibit photoaffinity labeling at a concentration of 100 μM (13).

As summarized in Table 2, we also investigated many other agents that do not reverse multidrug resistance, including dexamethasone, amiloride, choline chloride, cyanin 863, probenecid, *p*-aminohippuric acid, epinephrine, and norepinephrine. None of these drugs showed any inhibition of the photoaffinity labeling even at 100 μM concentration.

Discussion

In this work we screened a series of agents known to reverse the multidrug-resistant phenotype in human KB cells for their ability to inhibit ^{125}I-NASV labeling of the 150- to 170-kDa P-glycoprotein in membrane preparations from multidrug-resistant cells. We had previously shown that verapamil (13, 14), diltiazem, desmethoxyverapamil, and quinidine (14), which reversed drug resistance, efficiently inhibit P-glycoprotein labeling by ^{125}I-NASV, and we wanted to determine whether this was a general phenomenon. The results demonstrate that most agents that can reverse multidrug resistance also inhibit ^{125}I-NASV labeling. The specificity of the inhibition of ^{125}I-NASV labeling was demonstrated by showing that many other compounds that do not reverse drug resistance, including amines and other hydrophobic agents such as dexamethasone, did not inhibit labeling (Table 2).

Many agents that reverse drug resistance, such as reserpine, SDB-ethylenediamine, and cepharanthine, are not calcium channel blockers. Nifedipine, a potent calcium channel blocker, is a poor reversor of drug resistance. Thus, the results reported in the current study support our earlier conclusions that the

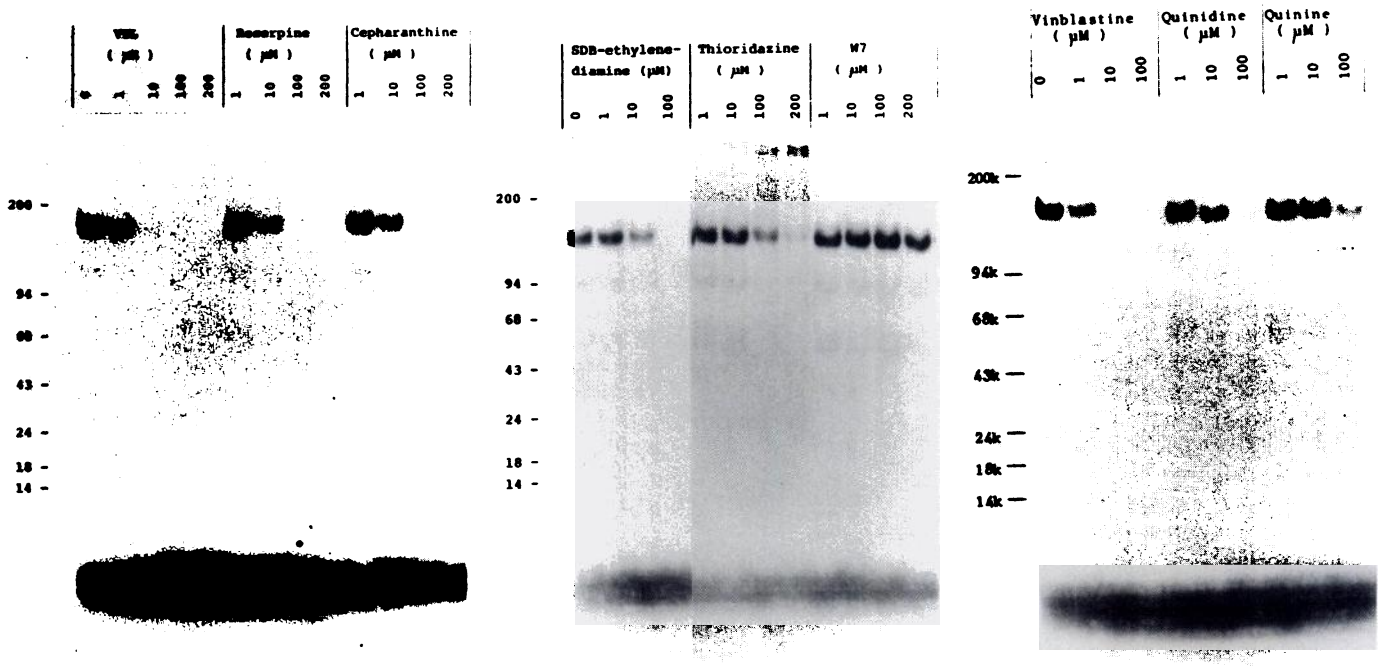


Fig. 2. The inhibition of ^{125}I-NASV labeling of the 150- to 170-kDa P-glycoprotein in KB-V1 vesicles by various agents. KB-V1 vesicles (190 μg of protein per lane) were incubated with ^{125}I-NASV in the absence or presence of the indicated concentrations of the drugs. Autoradiograms were developed after a 16-hr exposure. Molecular size markers at the *left* are in kilodaltons.

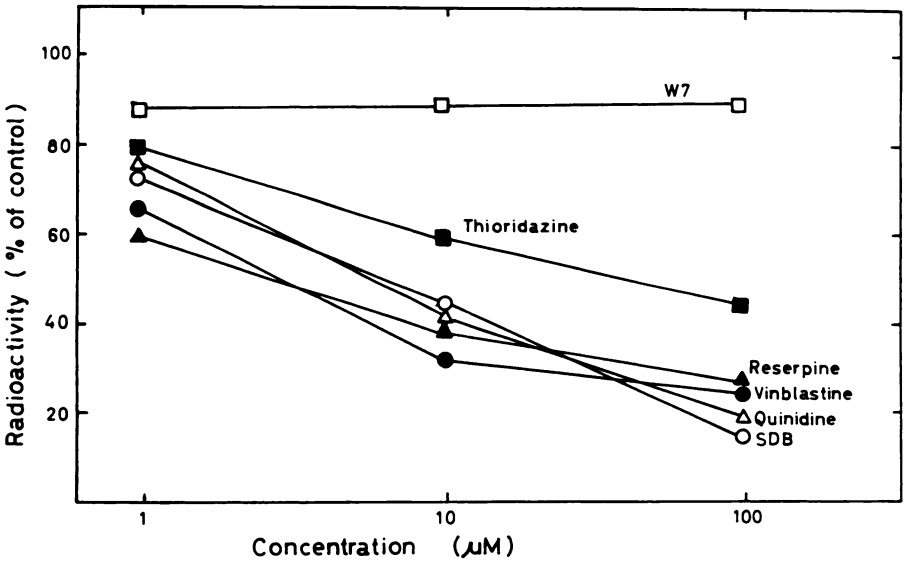


Fig. 3. Quantitative measurement of the inhibition of ^{125}I-NASV photolabeling. The gel slices corresponding to the bands on the autoradiogram were cut out, their radioactivity was determined, and background radioactivity from a random segment of the gel was deducted. Data are expressed as percentage of the radioactivity of the photolabeling of P-glycoprotein in the absence of drugs.

abilities to reverse drug resistance and to inhibit ^{125}I-NASV labeling are not directly related to calcium channel blockade (14). All of the reversing agents have been shown to increase accumulation of toxic drugs in multidrug-resistant cells. Some of the drugs that reverse resistance (i.e., verapamil, diltiazem, and desmethoxyverapamil) have been shown to bind specifically to membranes from multidrug-resistant cells, presumably to P-glycoprotein (14). Therefore, it seems likely that the mechanism of action of some or all of these agents is to bind to P-glycoprotein and to inhibit binding of the drugs to which the multidrug-resistant cells are resistant. We presume that the hydrophobic character of the reversing agents allows them to bind to P-glycoprotein and inhibit ^{125}I-NASV labeling, perhaps by competing for the same sites as the drugs that are substrates for this efflux pump. Recently, Safa *et al.* (25) have demonstrated directly that ^{3}H-azidopine, a dihydroperidine calcium channel blocker which can reverse multidrug resistance, binds directly to P-glycoprotein, and this binding could be inhibited by vinblastine and other calcium channel blockers.

Three phenothiazines (thioridazine, trifluoperazine, and chlorpromazine) and the lysosomotropic agent chloroquine are moderately effective at overcoming drug resistance but do not inhibit ^{125}I-NASV labeling at concentrations at which they reverse resistance. One possibility is that these agents act in different ways to reverse multidrug resistance, such as by interacting with a polar lipid (18) and changing the environment of the membrane in which the P-glycoprotein transport system is functioning. A second possibility is that these agents

TABLE 1

Concentration of agents that overcome multidrug resistance and inhibit photolabeling by ^{125}I-NASV

Agents	Concentration for 50% inhibition	
	To overcome multidrug resistance	To inhibit photoaffinity labeling
	μM	
SDB-ethylenediamine	18–53	10
Cepharantine	3–5	1–10
Reserpine	10	1–10
Quinidine	3–10	1–10
Thioridazine	3–5	100
Trifluoperazine	3–5	>20
Chlorpromazine	6–9	>20
Chloroquine	3–6	>100

TABLE 2

Inhibition of ^{125}I-NASV labeling of P-glycoprotein

Reversing agents that are effective inhibitors	Agents that do not reverse drug resistance with no effect on ^{125}I-NASV labeling	
SDB-ethylenediamine	W7	Cyanin 863
Cepharanthine	W5	Probenecid
Quinidine	Dexamethasone	*p*-Aminohippuric acid
Quinine	Amiloride	Epinephrine
Reserpine	Choline chloride	Norepinephrine

interact with P-glycoprotein at a site different from the one which binds ^{125}I-NASV or that they interact at a P-glycoprotein site which results in no inhibition of labeling under the conditions of the *in vitro* labeling assay. The existence of a second drug-binding site or a site that is not recognized under *in vitro* assay conditions is suggested by the observation that colchicine, to which multidrug-resistant cells are highly resistant, also does not inhibit ^{125}I-NASV labeling or vinblastine binding (12, 13). Colchicine is presumed to be transported by P-glycoprotein, since cells into which the cloned *mdr*1 gene has been transfected become resistant to colchicine as well as vinblastine (11).

These results suggest that it is possible to screen for agents that might overcome multidrug resistance by measuring inhibition of ^{125}I-NASV labeling of P-glycoprotein. This assay is much quicker than tissue culture assays for agents that reverse drug resistance. Some agents, however, will be missed by this screen. Other assays, based on inhibition of labeling by photoaffinity analogs of drugs such as colchicine, may reduce the false negative rate of this assay.

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Send reprint requests to: Dr. Michael Gottesman, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.