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Synthetic Isoprenoid Photoaffinity Labeling of P-Glycoprotein Specific to Multidrug-Resistant Cells

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SUMMARY

The synthetic isoprenoid *N*-solanesyl-*N,N'*-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB) is known to reverse drug resistance in human multidrug-resistant KB cells. SDB inhibits the photolabeling of P-glycoprotein with the vinblastine analog *N*-(*p*-azido-(3-^{125}I)salicyl)-*N'*-β-aminoethylvindesine. We synthesized photoactive radioactive SDB and used it to photoaffinity label membrane vesicles from human KB cells and their multidrug-resistant subline KB-C2 cells. A 150 to 170 kDa protein in membrane vesicles from KB-C2 cells was specifically labeled by the photoanalog of SDB. The labeled band was not detectable in parenteral drug-sensitive cells. The photolabeled 150 to 170 kDa protein was immunoprecipitated with a monoclonal antibody (C219) specific to P-glycoprotein. P-glycoprotein labeling was inhibited by anticancer agents, vinblastine, vincristine, actinomycin D, and daunomycin, with half-maximal inhibition at 2.0, 2.3, 18, and 23 μM, respectively. Only 33 and 18% of the labeling was inhibited by 100 μM Adriamycin and colchicine, respectively. The labeling was also inhibited by agents that reverse multidrug resistance, such as verapamil, reserpine, cepharanthine, and SDB. The existence of other molecules that specifically bind to ^{125}I-SDB-photoanalog was suggested in both KB and KB-C2 membrane vesicles. The fact that we could identify the synthetic isoprenoid acceptor in membrane vesicles from multidrug-resistant cells confirms that P-glycoprotein plays a role in the multidrug resistance phenotype and provides an explanation for the fact that SDB circumvents multidrug resistance.

The development of drug resistance and especially of multiple resistance to agents such as *Vinca* alkaroids and anthracyclines has been recognized as one of the major obstacles to successful cancer chemotherapy (1). Multidrug resistance is due to enhanced drug efflux (2, 3) resulting from a membrane glycoprotein of 170,000 Da (P-glycoprotein) (4) encoded by the *mdr*1 gene in human cancer cells (5). *mdr* genes from human cells have been sequenced and shown to encode a membrane protein with 12 transmembrane domains and two nucleotide binding sites (6).

It has been demonstrated that a photoaffinity analog of vinblastine, ^{125}I-NASV, specifically labels P-glycoprotein (7, 8). Most drugs that reverse multidrug resistance, such as the calcium channel blocker verapamil and the synthetic isoprenoid SDB, block drug efflux from cells and also inhibit the photoaffinity labeling of P-glycoprotein by the vinblastine analog (9).

Agents that reverse multidrug resistance appear to compete with hydrophobic anticancer agents for a binding site on P-glycoprotein. In this study, we report that a photoanalog of a reversing agent, SDB, binds to P-glycoprotein and that some anticancer agents and reversing agents inhibit this binding. These results suggest that SDB interacts directly with the binding site of anticancer agents on P-glycoprotein to reverse multidrug resistance.

Materials and Methods

Synthesis

A photoactive analog of SDB, *N*-[2-{(3-azido-2-hydroxy)benzoylamino}ethyl] - *N,N'* - bis{(3,4-dimethoxyphenyl)methyl} - *N* - (3,7,11,15, 19,23,27,31,35 - nonaenyl - 2,6,10,14,18,22,26,30,34 - hexatriacontanonaenyl)-1,2-ethanediamine (**VI**) was synthesized through five steps, as shown in Fig. 1.

***N,N'* - bis{(3,4 - Dimethoxyphenyl)methyl} - 1,2 - ethanediamine (II)** Compound **II** was prepared by the reaction of veratraldehyde (343.2 g) and ethylenediamine (62 g) in the presence of sodium borohydride (82.4 g). The crude product was purified by recrystallization with acetone to give 273 g of compound **II** as white crystals (melting point, 103.0–104.5°).

Solanesyl bromide (VII). Solanesol was isolated from the waste of blue-cured tobacco (*Nicotiana tobacco*) following the method devel-

ABBREVIATIONS: ^{125}I-NASV, *N*-(*p*-azido-(3-^{125}I)-salicyl)-*N'*-β-aminoethylvindesine; SDB, *N*-solanesyl-*N,N'*-bis(3,4-dimethoxybenzyl)ethylenediamine; SDS, sodium dodecyl sulfate; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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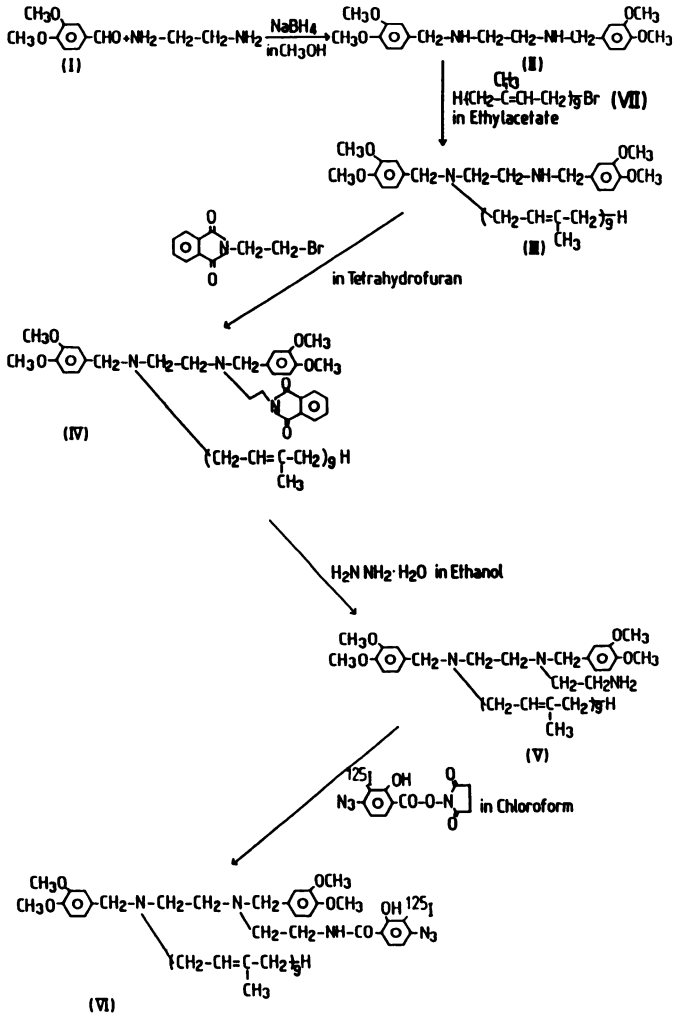

Fig. 1. Synthesis of ^{125}I-SDB-photoanalog.

oped by Rowland *et al.* (10). The lipids extracted with *n*-hexane were saponified and the unsaponified materials were extracted with isopropyl ether. Solanesol was separated from unsaponified material by centrifugal molecular distillation, crystallized from acetone, and recrystallized from *n*-hexane. Further purification was performed by column chromatography on silica gel with increasing concentrations of ethyl ether in *n*-hexane. Crude solanesyl bromide (545 g) was obtained from 825 g of solanesol, described above, and 130 g of phosphorus tribromide, according to the procedure of Noller and Dinsmore (11).

***N,N'*-bis{(3,4-Dimethoxyphenyl)methyl}-*N*-solanesyl-1,2-ethanediamine (III).** Compound **II** (273 g) and 200 g of solanesyl bromide (**VII**) were used to synthesize compound **III**, as described by Craig and Young (12). The crude product was purified by column chromatography on silica gel with ethyl acetate, to give 124.8 g of compound **III**.

***N,N'*-bis{(3,4-Dimethoxyphenyl)methyl}-*N*-(2-phthalimide) ethyl-*N'*-solanesyl-1,2-ethanediamine (IV).** Compound **IV** was synthesized as described by Craig and Young (12); the crude product of 7 g of compound **III** and 7 g of *N*-(2-bromoethyl)phthalimide was purified by column chromatography on silica gel, with increasing concentrations of methanol in chloroform, to give 3.2 g of compound **IV** as a pale yellow oil.

***N*-(2-Aminoethyl)-*N,N'*-bis{(3,4-dimethoxyphenyl)methyl}-N'-solanesyl-1,2-ethanediamine (V).** Compound **V** was synthesized as described by Ing and Manske (13); the crude product of 3.2 g of compound **IV** and 20 ml of 80% hydrated hydrazine was used. Crude product was purified by column chromatography on silica gel with ethanol to give 1.3 g of compound **V** as a pale yellow oil.

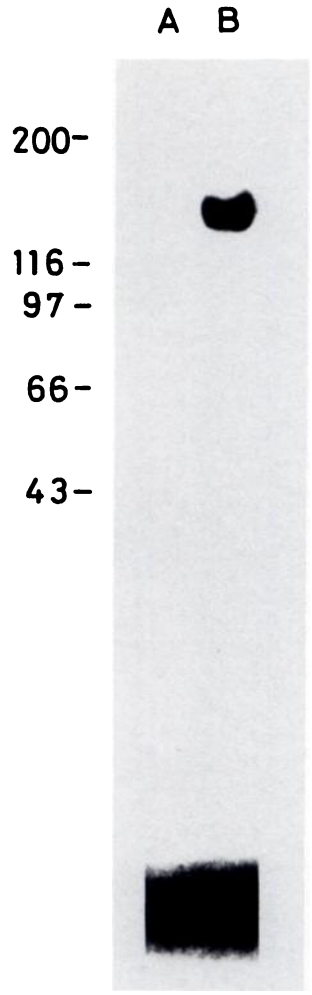

Fig. 2. Photolabeling with ^{125}I-SDB-photoanalog of a 150 to 170 kDa protein in membrane vesicles from KB-C2 cells. Membrane vesicles from KB and KB-C2 cells were incubated with 0.8 μM ^{125}I-labeled photoanalog of SDB. After photoactivation at 366 nm for 20 min at 25°, vesicles were solubilized with solubilizing buffer and electrophoresed on a SDS/urea gel. The autoradiogram was developed after 24-hr exposure. *Lane A*, KB vesicles; *lane B*, KB-C2 vesicles.

***N* - [2 - {(3 - Azido - 2 - hydroxy)benzoylamino}ethyl] - *N,N'*-bis{(3,4-dimethoxyphenynyl)methyl}-*N'*-solanesyl-1,2-ethanediamine (VI).** Three hundred milligrams of compound **V** and 50 mg of *N*-(3-azido-2-hydroxyphenylcarboxyl)succinimide in chloroform were stirred at room temperature for 3 hr. The reaction mixture was purified by thin layer chromatography (Merck $60F_{254}$ silica gel plate 5717, with chloroform/methanol, 20:1) to give 110 mg of compound **VI** as a pale yellow oil. NMR (in $CDCl_3$) (rpm); 6.77–6.0 (m, 12 H), 5.25 (t, 1 H), 5.12 (t, 8 H), 3.83, 3.77, 3.73 (s, 12 H), 3.5–3.4 (br, 2 H), 3.10 (d, 2 H), 2.7–2.5 (m, 6 H), 2.2–1.85 (br, 32 H), 1.68 (s, 3 H), 1.60 (s, 27 H). IR (neat) (con^{-1}): 3400 (OH), 2120 (N_3), 1650 (double bond), 1600, 1520.

^{1}H NMR spectra of the products of each step were measured with a JEOL FX 200 spectrometer in a $CDCl_3$ solution. The IR spectra were obtained on a Hitachi IR-810 spectrometer. The mass spectra were measured with a Hitachi M-80 double-focussing spectrometer. For the iodination, 16.7 nmol of NHS-ASA was mixed with 5 mCi of $Na^{125}I$ (0.29 mg of iodine) and iodinated with chloroamine T. Compound **V** was incubated with ^{125}I-labeled NHS-ASA in $CHCl_3$ and purified with reverse phase high pressure liquid chromatography as follows: column, 4.6-mm i.d. × 15-cm TSK gel ODS-80TM; particle size, 5 μm (Toyo Soda); mobile phase, acetonitrile/0.17% ammonium acetate in ethanol/methanol, 5:3:2. Three radioactive peaks were detected after fraction-

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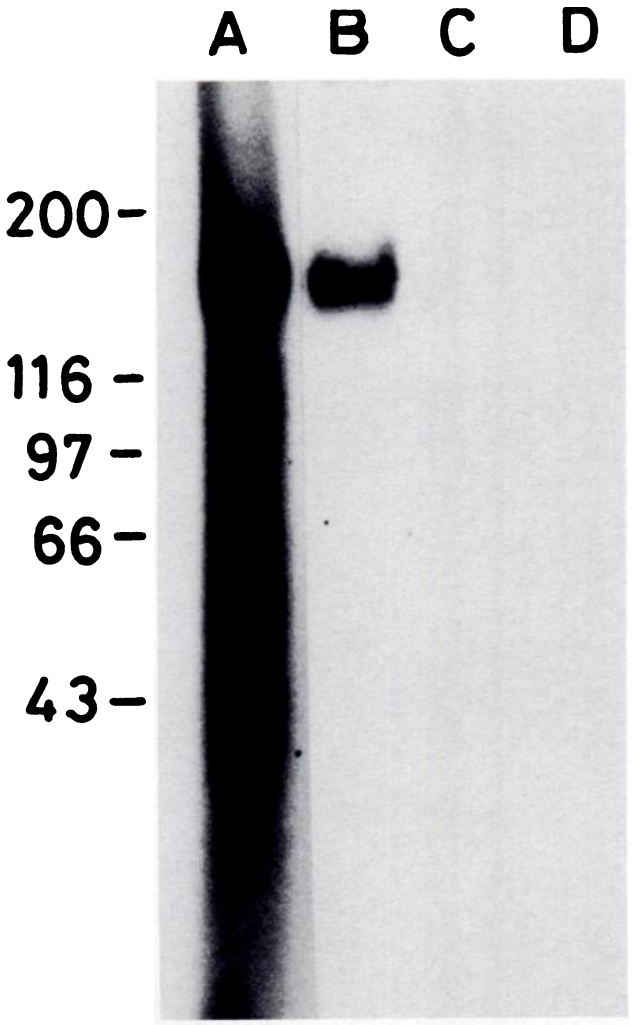

Fig. 3. SDS/urea/polyacrylamide gel electrophoresis of immunoprecipitates of ^{125}I-SDB-photoanalog-photolabeled, detergent-solubilized, KB-C2 membrane vesicles. Photoaffinity labeling was carried out in the presence (*lane C*) and absence (*lanes A, B,* and *D*) of 200 μM SDB. After solubilization, photolabeled proteins were analyzed by SDS/urea/polyacrylamide gel electrophoresis (*lane A*). Photolabeled membrane vesicles were solubilized with a buffer containing a detergent, CHAPS, and immunoprecipitated with C219 (*lanes B* and *C*) or nonimmunized mouse IgG (D) and then P-glycoprotein was analyzed by SDS/urea/polyacrylamide gel electrophoresis. Molecular size markers at the *left* are in kDa. Autoradiograms were developed after 2-day exposure.

ation and the R_F value of the main component in the second peak was the same as that of authentic noniodinated photoanalog of SDB, developed with a solvent system consisting of chloroform and methanol (20:1).

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-C2 was selected with increasing concentrations of colchicine and was maintained as described previously (14, 15).

Membrane Vesicle Preparation

Membrane vesicles from KB-C2 cells were prepared as described (16, 17) from cells grown in 24 × 24 cm dishes (GIBCO) under standard growth conditions (14). Protein concentrations were determined by the method of Bradford (18).

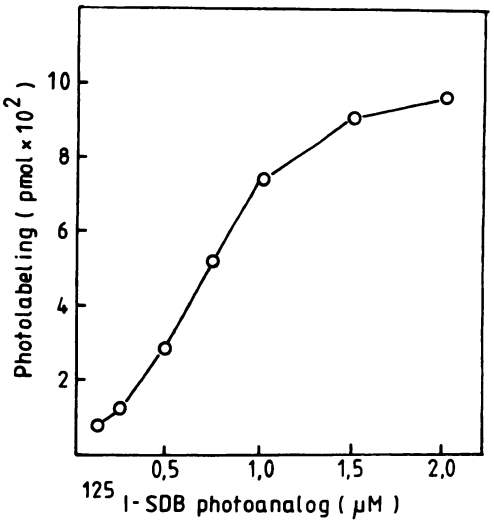

Fig. 4. Relationship of dose of the ^{125}I-SDB-photoanalog to photolabeling. The KB-C2 membrane vesicles were photolabeled with increasing concentrations of ^{125}I-SDB-photoanalog. The data were quantified by cutting out gel slices corresponding to the P-glycoprotein bands on the autoradiogram and determining their radioactivity. The background radioactivity in equal areas of the gel in each lane was measured and subtracted from the radioactivity of the band. Each *point* represents the mean of duplicate determinations.

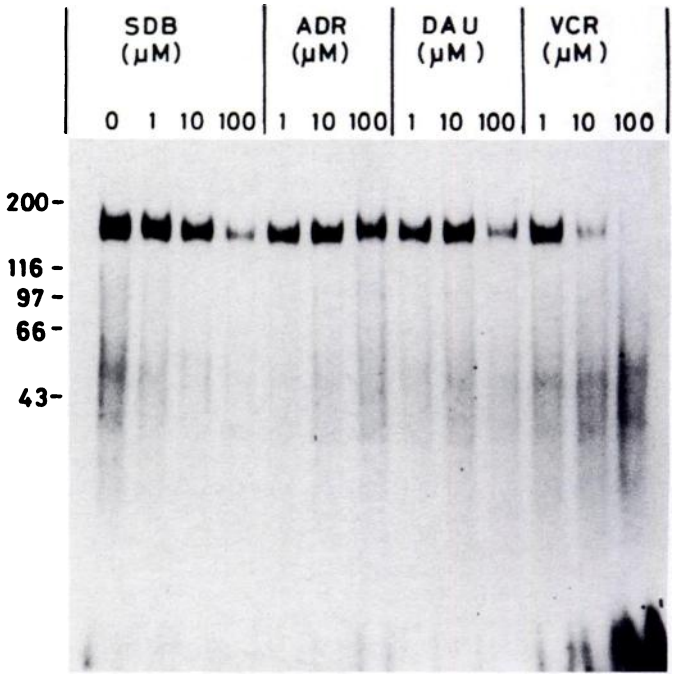

Fig. 5. Inhibition of the photolabeling of P-glycoprotein in membrane vesicles from KB-C2 cells by SDB and anticancer agents. KB-C2 vesicles were incubated with ^{125}I-SDB-photoanalog in the presence and absence of the indicated concentrations of SDB, Adriamycin (*ADR*), daunomycin (*DAU*) and vincristine (*VCR*). Autoradiograms were developed after 24-hr exposure.

Photoaffinity Labeling

Membrane vesicles (160 μg of protein) were incubated with 0.8 μM concentrations of the photoanalog of SDB labeled with ^{125}I (2 × 10^6 cpm), for 15 min at room temperature, in the presence or absence of various drugs. After continuous irradiation of samples at 366 nm for 20 min at 25°, samples were solubilized in a SDS sample buffer, as described by Debenham *et al.* (19).

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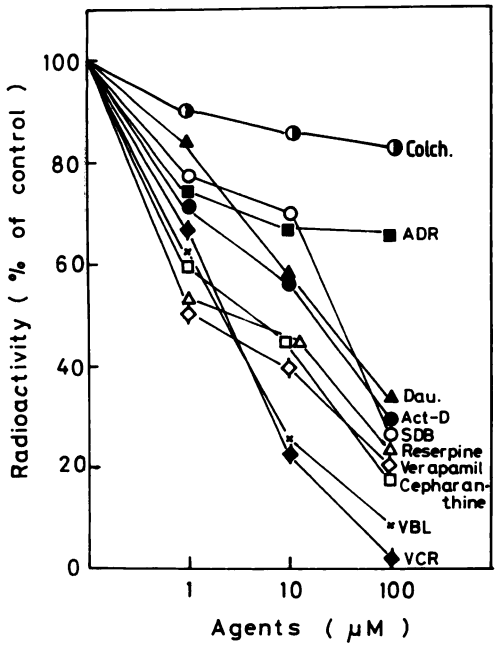

Fig. 6. Quantitative measurements of the inhibition of ^{125}I-SDB-photoanalog labeling by anticancer agents and other agents that reverse multidrug resistance. The data were quantified as described in the legend to Fig. 4. Data are expressed as the percentage of radioactivity measured in the absence of any agents. ■, Adriamycin; ▲, daunomycin; ●, actinomycin D; ○, SDB; △, reserpine, ◇, verapamil; □, cepharanthine; ×, vinblastine; ♦, vincristine; ◑, colchicine. Each *point* represents the mean of duplicate determinations.

SDS Gel Electrophoresis

Samples labeled with the ^{125}I-labeled photoanalog of SDB were fractionated by electrophoresis on a SDS/polyacrylamide/urea gel, using a modification of the system described by Debenham *et al.* (19), on a 5% polyacrylamide/4.5 M urea gel, pH 7.6, without a stacking gel.

Immunoprecipitation

Membrane vesicles containing 160 μg of protein were photolabeled as described above. Labeled membrane vesicles were suspended in 2 ml of buffer A (50 mM Tris·HCl, pH 8.0, 150 mM NH_4Cl, 2 mM $MgCl_2$) containing 1% CHAPS and were incubated for 30 min at 4° (20). The membrane vesicles were incubated with 4 μg of monoclonal antibody C-219, at 4° for 2 hr. and then a suspension of 200 μl of 20% Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) in buffer A was added. The mixture was incubated for 30 min at 4° with continuous mixing, and the precipitates were washed four times with buffer A containing 1% CHAPS. The precipitates were then used for SDS/urea/polyacrylamide gel electrophoresis analysis.

Reversible ^{125}I-SDB-Photoanalog Binding Studies

Binding of the photoanalog with vesicles was measured by filtration, essentially as described by Cornwell *et al.* (16). Vesicles containing 80 μg of protein were incubated with the photoanalog in the dark at 25° in 0.01 M Tris·HCl, pH 7.5, 0.125 M sucrose, 5 mM $MgCl_2$ (transport buffer), in a total volume of 100 μl. The reaction was stopped by placing the samples on ice and immediately adding 4 ml of ice-cold transport buffer. Samples were collected by filtration on glass fiber filters (Whatman GF/C) that were pretreated with 10% fetal calf serum, and then the radioactivity of each filter was measured. Nonspecific absorption was determined by dilution of the membranes with transport buffer before the addition of ^{125}I-SDB-photoanalog and a 1000-fold molar excess of SDB-ethylenediamine.

Immunoblotting

Monoclonal antibody against hamster P-glycoprotein (C219), which was originally isolated by Kartner *et al.* (21), was obtained from Centocor, Inc. (Malvern, PA). Membrane vesicles were suspended in 50 μl of electrophoresis buffer containing 0.5% (w/v) SDS and 10% (v/v) glycerol. Electrophoresis on 7.5% polyacrylamide gel was carried out according to the method of Laemmli (22), without heating the sample, as previously described (23, 24). Transfer to nitrocellulose paper was performed as previously described (25). For immunoblotting, the nitrocellulose paper was blocked with 2.5% skim milk in buffer B (0.4 M NaCl, 50 mM Tris·HCl, pH 8.0, 0.05% Tween-20) for 1 hr at room temperature and then incubated for 5 hr with 5 μg/ml C219 in buffer B containing 2% skim milk. The paper was washed four times with buffer B and then incubated for 1 hr with 0.5 μCi/ml ^{125}I-labeled anti-mouse IgG (ICN Radiochemicals) in buffer B containing 2% skim milk. Then the paper was washed four times with buffer B, dried, and autoradiographed for 5 days at −80° on Kodak XAR-5 film with an intensifying screen.

Drugs and Chemicals

SDB was synthesized, purified, and used as described previously (26). NHS-ASA was purchased from Pierce Chemical Co. (Rockford, IL). Iodine-125 (15.5 mCi/μg) was obtained from Amersham. Other agents were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

We have previously demonstrated that the synthetic isoprenoid SDB reverses multidrug resistance in human KB carcinoma cells (27). SDB has a structure partially similar to verapamil but no calcium channel-blocking activity (27). SDB inhibits photolabeling with ^{125}I-NASV of P-glycoprotein in KB-V1 vesicles (9). SDB also inhibits labeling with ^{125}I-NASV of P-glycoprotein in vesicles from KB-C2 multidrug resistant cells (data not shown).

We examined specific labeling with the ^{125}I-SDB-photoanalog (Fig. 1, **VI**) of membrane vesicles made from KB-C2 cells and drug-sensitive KB cells. As shown in Fig. 2, the 150–170 kDa component was radiolabeled in drug-resistant KB-C2 cells (Fig. 2, *lane B*). No labeled band was detected in vesicles from the drug-sensitive KB cells (Fig. 2, *lane A*).

To identify the 150–170 kDa component, photolabeled membrane vesicles from KB-C2 were solubilized with CHAPS and immunoprecipitated with monoclonal antibody to P-glycoprotein, C-219 (Fig. 3). The labeled protein was immunoprecipitated by the monoclonal antibody (Fig. 3, *lane B*) but not by nonimmunized mouse IgG (Fig. 3, *lane D*). Photolabeling in the presence of 200 μM SDB remarkably reduced radioactive 150–170 kDa protein in the immunoprecipitate (Fig. 3, *lane C*).

Specific photoaffinity labeling was characterized in further detail by photolabeling KB-C2 membrane vesicles in the presence of increasing concentrations of ^{125}I-SDB-photoanalog. The photolabeling increased linearly between 0.25 and 1 μM ^{125}I-SDB-photoanalog and reached a plateau at higher concentrations of the photoanalog (Fig. 4). The half-maximal saturable concentration of the SDB photoanalog was 0.8 μM.

To characterize further the labeling of P-glycoprotein with ^{125}I-labeled SDB photoanalog, KB-C2 vesicles were incubated with the photoanalog in the presence of increasing concentrations of various anticancer agents and reversing agents (Fig. 5). SDB inhibited photolabeling in a dose-dependent manner. Adriamycin and daunomycin also partially inhibited labeling. Vincristine completely inhibited labeling at 100 μM.

The data were quantified by cutting out gel slices corresponding to the bands on the autoradiograms and measuring their radioactivity (Fig. 6). Among anticancer agents, alkaloids isolated from *Vinca rosea Linn.*, vincristine and vinblastine, in-

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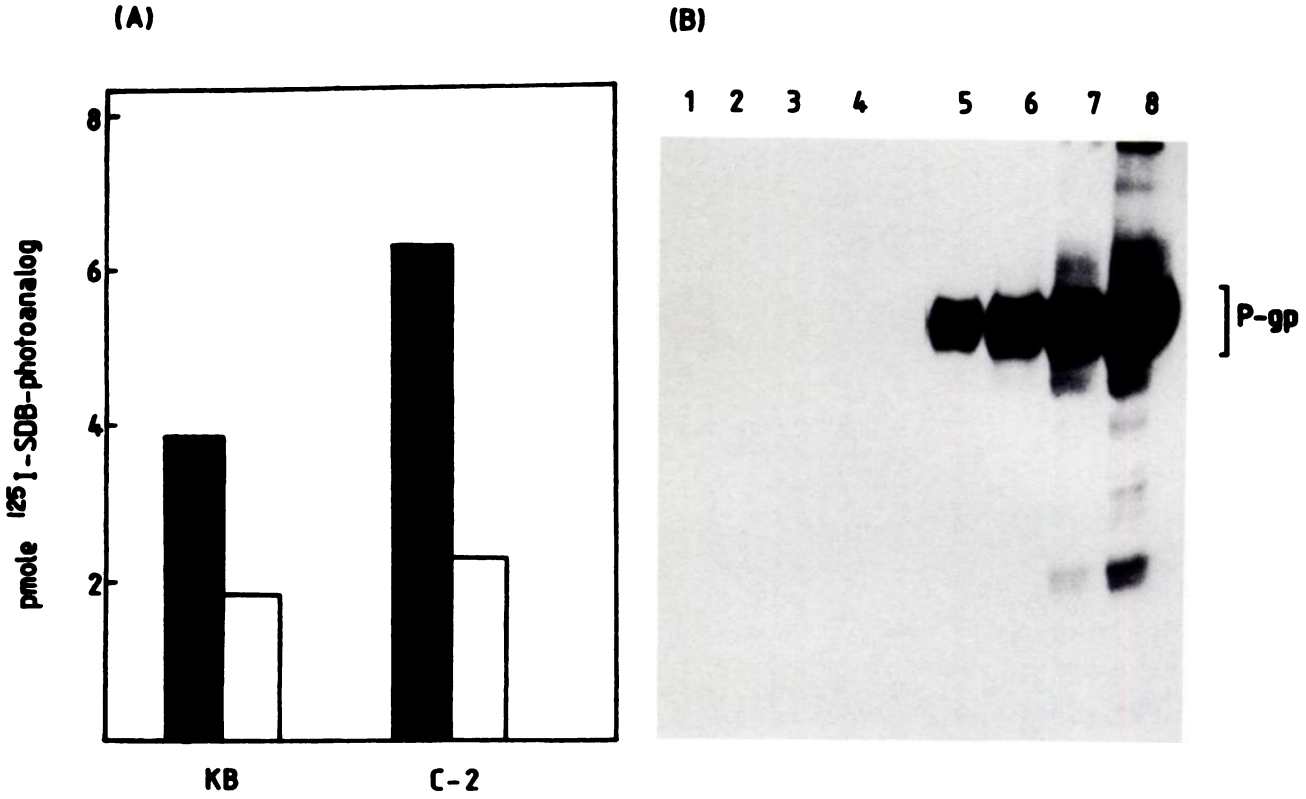

Fig. 7. A comparison of ^{125}I-SDB-photoanalog binding to membrane vesicles from sensitive and multidrug-resistant cells, and the expression of P-glycoprotein in both cell lines. A, Membrane vesicles (80 μg of protein) from KB and KB-C2 cells were incubated at 25° in the transport buffer containing 0.13 μM ^{125}I-SDB-photoanalog. Vesicles were collected and washed by filtration and the radioactivity of ^{125}I-SDB-photoanalog was measured on the filters. ■, Total; □, nonspecific. Data are expressed as the means of duplicate determinations. B, P-glycoprotein content of membrane vesicles from KB and KB-C2. Twenty five micrograms (*lanes 1* and *5*), 50 μg (*lanes 2* and *6*), 100 μg (*lanes 3* and *7*), and 200 μg (*lanes 4* and *8*) per lane of membrane vesicle protein from KB (*lanes 1* to *4*) or KB-C2 (*lanes 5* to *8*) cells were run on a 7.5% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose paper. The paper was treated with C219, washed, and then incubated with ^{125}I-labeled anti-mouse IgG to identify P-glycoprotein bands as described in Materials and Methods. *P-gp*, P-glycoprotein.

hibited photolabeling most strongly, with half-maximal inhibition of the labeling at 2.3 and 2.0 μM, respectively. Actinomycin D and daunomycin also inhibited labeling, with half-maximal inhibition at 18 and 23 μM, respectively. Adriamycin and colchicine, however, inhibited only 33 and 18% of the labeling at 100 μM. Verapamil, reserpine, and cepharanthine inhibited the labeling by 50% at between 1 and 5 μM.

Cornwell *et al.* (16) previously demonstrated the specific binding of [^{3}H]vinblastine to membrane vesicles from multidrug-resistant KB-V1 cells. We measured ^{125}I-SDB-photoanalog binding to membrane vesicles from KB and KB-C2 cell lines in the dark, according to the method of Cornwell *et al.* (16). Specific binding of the photoanalog with vesicles from KB-C2 cells was about twice as great as with KB vesicles, reflecting the higher drug resistance of KB-C2 cells (Fig. 7A). Fig. 7B shows Western blot analysis of KB and KB-C2 vesicles with C219, a monoclonal antibody that recognizes the C-terminal portion of P-glycoprotein. A large amount of P-glycoprotein was detected in KB-C2 vesicles, but we could not detect any P-glycoprotein in KB vesicles. The data suggest that little of the specific binding of ^{125}I-SDB-photoanalog to KB vesicles and not all of the specific binding of the photoanalog to KB-C2 vesicles are related to P-glycoprotein

Discussion

The synthetic isoprenoid SDB reverses the multidrug-resistant phenotype in KB-Ch^R-24 cells and reverses resistance to vincristine in mouse leukemia P388 cells *in vitro* and *in vivo* (27, 28). This isoprenoid enhances the accumulation of hydrophobic anticancer agents in drug-resistant cells by inhibiting the efflux of the agents from the cells (27).

The molecular basis for the reversal of drug-resistance by SDB was not known until recently. In 1986, however, Cornwell *et al.* (16) reported that the 150–170 kDa protein in multidrug-resistant cells is labeled by the radioactive photoactive analog of vinblastine ^{125}I-NASV and that the labeled protein is immunoprecipitated by the monoclonal antibody for P-glycoprotein (7). SDB completely inhibited the photolabeling of P-glycoprotein with ^{125}I-NASV (8). We hypothesize that reversal of multidrug resistance occurs when SDB binds to P-glycoprotein and inhibits the binding of drugs to which multidrug-resistant cells are resistant.

In this study, we detected the 150–170 kDa protein, which is specifically photolabeled with the photoanalog of SDB in membrane vesicles from KB-C2 cells but not from drug-sensitive KB cells. The protein was immunoprecipitated by the monoclonal antibody specific to P-glycoprotein. The 150–170 kDa protein labeled with the ^{125}I-labeled SDB photoanalog coelectrophoresed exactly with P-glycoprotein labeled with ^{125}I-NASV (data not shown). These observations establish that P-glycoprotein is an acceptor protein for SDB in the membrane of multidrug-resistant cells. P-glycoprotein was recently identified as an acceptor of the calcium channel blockers azidopine

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and verapamil, which reverse multidrug resistance (29, 30). SDB has a structure partially similar to verapamil and reverses multidrug resistance but has no calcium channel-blocking activity (28). Our data show that P-glycoprotein is also an acceptor of a synthetic isoprenoid that has no calcium channel-blocking activity.

Inhibition experiments provide evidence that P-glycoprotein recognizes hydrophobic anticancer agents to which the cells display multidrug resistance and also some agents that reverse multidrug resistance. These agents display varying abilities to inhibit photolabeling. It may be that P-glycoprotein has an acceptor site with broad drug specificity and that affinities of the agents for this acceptor site are different (30). Alternatively, there may be multiple drug binding sites that are very close or overlap and have different affinities for each agent. We need to determine the exact binding site of each agent to determine whether SDB and any of the other inhibitors bind to the same site or to separate sites.

Our results indicate that P-glycoprotein has multidrug binding capacity and participates in the process by which SDB inhibits the efflux of some anticancer agents and reverses multidrug resistance.

The reversible binding of ^{125}I-SDB-photoanalog with membrane vesicles from drug-sensitive and multidrug-resistant KB cells was also studied. The results show substantial differences in specific binding of the photoanalog by vesicles from KB and KB-C2 cells. Although the specific binding of the analog with KB vesicles was about 50% of that with KB-C2 vesicles, considerable binding occurred in KB vesicles. Cornwell *et al.* (16) also observed a considerable amount of specific binding of [^{3}H] vinblastine to KB membrane vesicles. We detected a large amount of P-glycoprotein in KB-C2 cells but could not detect any expression of P-glycoprotein in KB cells. These data indicate that the SDB photoanalog binds specifically with other molecules than P-glycoprotein in KB vesicles. In autoradiograms developed after long exposure, we detected photolabeled bands (20–60 kDa) other than P-glycoprotein from both KB and KB-C2 vesicles. Increasing the concentration of nonradioactive SDB in the reaction mixture decreased the density of these bands in a dose-dependent manner (data not shown). These molecules and other molecules, such as anionic polar lipids in the membrane vesicles (31), may account for the binding of the SDB photoanalog in KB vesicles. Further study is needed of these molecules and of their roles in the reversal of multidrug resistance with SDB.

Acknowledgments

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