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Interaction of Forskolin with the P-Glycoprotein Multidrug Transporter†

Diane I. Morris,‡ Lisa A. Speicher,§ Arnold E. Ruoho,‖ Kenneth D. Tew,§ and Kenneth B. Seamon*,‡

Molecular Pharmacology Laboratory, Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, Maryland 20892, Department of Pharmacology, Fox Chase Cancer Research Center, Philadelphia, Pennsylvania 19104, and Department of Pharmacology, University of Wisconsin—Madison, Madison, Wisconsin 53706

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ABSTRACT: Forskolin and 1,9-dideoxyforskolin, an analogue that does not activate adenylyl cyclase, were tested for their ability to enhance the cytotoxic effects of adriamycin in human ovarian carcinoma cells, SKOV3, which are sensitive to adriamycin and express low levels of P-glycoprotein, and a variant cell line, SKVLB, which overexpresses the P-glycoprotein and has the multidrug resistance (MDR) phenotype. Forskolin and 1,9-dideoxyforskolin both increased the cytotoxic effects of adriamycin in SKVLB cells, yet had no effect on SKOV3 cells. Two photoactive derivatives of forskolin have been synthesized, 7-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]-7-deacetylforskolin, ^{125}I-7-AIPP-Fsk, and 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]forskolin, ^{125}I-6-AIPP-Fsk, which exhibit specificity for labeling the glucose transporter and adenylyl cyclase, respectively (Morris et al., 1991). Both photolabels identified a 140-kDa protein in membranes from SKVLB cells whose labeling was inhibited by forskolin and 1,9-dideoxyforskolin. There was no specific labeling of proteins in membranes from the SKOV3 cells. The overexpressed 140-kDa protein in SKVLB membranes was identified as the P-glycoprotein by immunoblot analysis and immunoprecipitation using anti-P-glycoprotein antiserum. Total inhibition of photolabeling of the P-glycoprotein was observed with verapamil, nifedipine, diltiazem, and vinbalastine, and partial inhibition was observed with colchicine and cytochalasin B. Forskolin was less effective at inhibiting the photolabeling of the P-glycoprotein than 1,9-dideoxyforskolin or a lipophilic derivative of forskolin. The data are consistent with forskolin binding to the P-glycoprotein analogous to that of other chemosensitizing drugs that have been shown to partially reverse MDR. The ability of forskolin photolabels to specifically label the glucose transporter, the adenylyl cyclase, and the P-glycoprotein suggests that these proteins may share a common binding domain for forskolin analogues.

Multidrug resistance (MDR)[1] is a phenomenon whereby tumor cells acquire resistance to a variety of structurally and functionally unrelated cytotoxic drugs (Endicott & Ling, 1989). This frequently is associated with the overexpression of the P-glycoprotein, an integral membrane glycoprotein whose molecular weight has been reported to be between 130 and 180 kDa (Juliano & Ling, 1976). The protein is believed to act as an energy-dependent efflux pump capable of transporting drugs out of a cell (Willingham et al., 1986). Thus, overexpression of the P-glycoprotein would maintain a low intracellular concentration of cytotoxic drugs, which would be associated with reduced cytotoxic consequences.

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* To whom correspondence should be addressed.
‡ Food and Drug Administration.
§ Fox Chase Cancer Research Center.
‖ University of Wisconsin—Madison.

[1] Abbreviations: MDR, multidrug resistant; ^{125}I-AIPPS, *N*-[3-(4-azido-3-[^{125}I]iodophenyl)propionyl]succinimide; ^{125}I-6-AIPP-Fsk, 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]forskolin; ^{125}I-7-AIPP-Fsk, 7-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)-propionamido]ethyl]carbamyl]-7-deacetyl forskolin; 6-HPP-Fsk, 6-*O*-[[2-[3-(4-hydroxyphenyl)propionamido]ethyl]carbamyl]forskolin.

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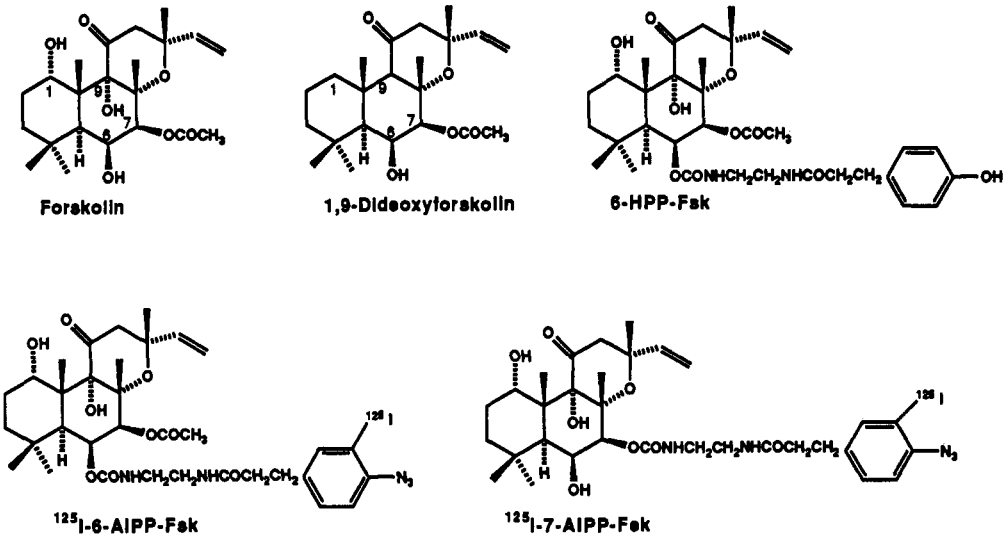

FIGURE 1: Structures of forskolin and forskolin derivatives.

Many compounds can reverse MDR via binding and inhibition of the P-glycoprotein. These diverse agents whose only similarity appears to be their hydrophobic nature include verapamil, progesterone, reserpine, and phenothiazines (Endicott & Ling, 1989; Naito et al., 1989; Qian & Beck, 1990; Pearce et al., 1989). The exact mechanism of action of these reversing agents is not known; however, some of these compounds inhibit drug efflux from resistant cell lines, and it has been suggested that the reversing agents bind to the P-glycoprotein. Support for this proposal derives from the ability of photoactive derivatives of drugs that reverse MDR to bind to the P-glycoprotein and the ability of cytotoxic drugs to inhibit this labeling. These include photoactive derivatives of calcium channel blockers such as azidopine and verapamil, and derivatives of other diverse compounds, such as vinblastine and prazosin (Safa, 1988; Safa et al., 1986, 1990; Greenberger et al., 1990). The ability of cytotoxic drugs to inhibit labeling correlates well with their cytotoxic potencies.

The P-glycoprotein is structurally homologous with bacterial transport proteins (Juranka et al., 1989). The amino acid sequence predicts that the protein consists of two domains each of which contains six transmembrane helices and an ATP-binding site (Chen et al., 1986). A similar structural organization is predicted for adenylyl cyclase (Krupinski et al., 1989), the cystic fibrosis transmembrane conductor (Riordan et al., 1989), and the yeast STE-6 gene product (McGrath & Varshavsky, 1989). Other transmembrane transport proteins such as the facilitated glucose transporter (Mueckler et al., 1985), bacterial sugar transporters (Henderson & Maiden, 1990), and voltage-gated ion channels are also composed of domains of six transmembrane helices (Catterall, 1988). The P-glycoprotein and adenylyl cyclase contain consensus sequences for binding nucleotides and the glucose transporter has been shown to bind nucleotides (Krupinski et al., 1989; Chen et al., 1986; Carruthers & Helgerson, 1989). Other than containing domains composed of six helical segments, there is no biochemical evidence to indicate functional homologies between the P-glycoprotein adenylyl cyclase, the voltage-gated ion channels, or the facilitated glucose transporters.

Forskolin, a natural product diterpene, is an activator of adenylyl cyclase (Seamon & Daly, 1986). Forskolin can also affect other membrane proteins in a cyclic-AMP-independent manner (Laurenza et al., 1989). For example, forskolin inhibits the glucose transporter (Kashiwagi et al., 1983; Joost et al., 1988), voltage-gated potassium channels (Hoshi et al., 1988), and ligand-gated ion channels (Heuschneider & Schwartz, 1989; Nishizawa et al., 1990). Photoactive derivatives of forskolin label the glucose transporter and adenylyl cyclase, consistent with a direct interaction of forskolin with these proteins (Wadzinski et al., 1987; Pfeuffer & Pfeuffer, 1989). We have recently developed two different iodinated photoactive derivatives of forskolin (Figure 1), 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]-forskolin (^{125}I-6-AIPP-Fsk) and 7-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]-7-deacetylforskolin (^{125}I-7-AIPP-Fsk) that show specificity for the glucose transporter and the adenylyl cyclase, respectively (Morris et al., 1991). Previous data from Wadler and Wiernik (1988) demonstrated that forskolin could affect drug transport in MDR cell lines. These data along with the structural homology displayed between adenylyl cyclase and the P-glycoprotein prompted us to investigate the direct interaction of forskolin with the P-glycoprotein. The ability of forskolin to affect the cytotoxicity of adriamycin in an MDR cell line was investigated and correlated with the ability of forskolin photoactive derivatives to label the P-glycoprotein.

Experimental Procedures

Materials and Cells. Verapamil, diltiazem, nifedipine, vinblastine, colchicine, D-glucose, β-mercaptoethanol, deoxycholate, and Tween 20 were from Sigma; cytochalasin B was from Aldrich; protein A agarose and SDS were from Pierce; aprotinin was from Boehringer Mannheim; ^{125}I-labeled protein A was from New England Nuclear; ^{14}C molecular weight standards were from Bethesda Research Laboratories. ^{125}I-6-AIPP-Fsk (2200 Ci/mmol) and ^{125}I-7-AIPP-Fsk (2200 Ci/mmol) were synthesized as described (Morris et al., 1991). Forskolin and 1,9-dideoxyforskolin were the generous gift of Dr. Richard Allen, Hoechst-Celanese, Somerville, NJ. 6-HPP-Fsk was synthesized as described (Robbins et al., 1991). The human ovarian carcinoma cell lines SKOV3 and SKVLB (1.0) were generously provided by Dr. Victor Ling (Bradley et al., 1989). The antiserum 4007 against the P-glycoprotein was the generous gift of Dr. Michael Gottesman, NCI, Bethesda, MD (Tanaka et al., 1990).

Cell Culture. SKOV3 and SKVLB (vinblastine resistant) were cultured in α-MEM supplemented with 15% fetal bovine serum, and incubated at 37 °C in a humidified 5% CO_2 atmosphere. SKVLB was passaged once a week at a 1:10 dilution and maintained in 1.0 ng/mL vinblastine.

Preparation of Membranes. Plasma membrane vesicles for P-glycoprotein analysis were isolated by following the procedures of Riordan and Ling (1979). Briefly, cells (5×10^7) were grown, washed with PBS, scraped from plates, and frozen as pellets until used. Cell pellets were quickly thawed in a 37 °C water bath and stored on ice. All subsequent steps were carried out at 4 °C. Cells were resuspended in approximately 5 mL of hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 2 mM phenylmethanesulfonyl fluoride, 10 mM Tris-HCl, pH 7.4, at 26 °C) and incubated for 30–45 min. Cells were homogenized, and the homogenate was centrifuged at 4000*g* for 10 min. The supernatant was collected and centrifuged at 100000*g* for 60 min and the membrane pellet was resuspended in 8.6% (w/v) sucrose in 5 mM Tris-HCl, pH 7.4, and stored at −80 °C.

Cytotoxicity Assays. The effect of forskolin on cytotoxicity of adriamycin in SKOV3 and SKVLB cell lines was determined by using colony forming assays. Cell monolayers were trypsinized from flasks, counted, and plated directly into media containing drug at a density of 500 cells/25 cm^2 tissue culture flask. Cells were maintained in constant exposure to drug at 37 °C in 5% CO_2 for 10–12 days. Surviving colonies (>32 cells) were fixed with methanol/acetic acid (90:10), stained with 1% crystal violet, and counted with a Biotran III automatic totalizer (New Brunswick Scientific, Edison, NJ). The percentage of surviving colonies was calculated by comparison to control flasks containing the drug solvent.

Photoaffinity Labeling. Membranes (5–10 μg) were preincubated in 1.5-mL microfuge tubes in the presence of either buffer alone (10 mM Tris-HCl, pH 7.4) or buffer containing drug in a total volume of 100 μL for 30 min on ice. ^{125}I-6-AIPP-Fsk or ^{125}I-7-AIPP-Fsk was added to the membranes such that a final concentration of approximately 3 nM was achieved. The labels were taken directly from the stock solutions of labels that were in ethanol, and the final concentration of ethanol in the assays did not exceed 2%. The membranes were incubated an additional 30 min on ice in the dark. Immediately before photolysis, 0.9 mL of the corresponding buffer solution containing drug was added, and the diluted samples were quickly photolyzed (energy = 200000 μJ) in a Stratagene UV Stratalinker. Following photolysis, 1% β-mercaptoethanol was added to each tube as a scavenger for any long-lived species. The membranes were microfuged at full speed for 15 min, and the supernatants were removed.

SDS–PAGE. Membrane pellets were resuspended in 1% SDS Laemmli electrophoresis sample buffer (Laemmli, 1970) and incubated for 10 min at 55 °C. The proteins were separated on 8% SDS–PAGE gels with the Bio-Rad Mini-Protean II apparatus run at a constant 200 V. Proteins were fixed in 12% trichloroacetic acid for 15 min, washed in water, and then stained by R-250 Commassie blue according to the Novex stain protocol. The gels were dried and exposed to XAR 5 film. The ^{14}C-labeled molecular weight standards were myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Calculated molecular weights were determined by semilogarithmic analysis with the above standards.

Immunological Analysis. For the immunodetection experiment, 10-μg samples of SKOV3 and SKVLB membranes were pelleted and run on an 8% SDS–PAGE gel as described above. After the gel was run, the proteins were electrophoretically transferred onto Immobilon at 50 V for 1 h with an LKB Midget MultiBlot apparatus in 25 mM Tris, 10 mM glycine, and 10% methanol. The membrane was blocked with 10% nonfat Carnation milk in PBS (10 mM phosphate, 0.15 M NaCl, pH 7.5) for 1 h at room temperature with shaking, incubated overnight with antiserum 4007 (1:400 dilution) in a milk solution at 4 °C, washed three times for 10 min with PBS containing 0.1% Tween 20 and once with PBS, and incubated with approximately 0.2 μCi/mL recombinant ^{125}I-labeled protein A in PBS milk solution for 1 h at room temperature. The blot was washed again three times for 10 min with PBS containing 0.1% Tween 20 and once with just PBS, dried, and exposed to XAR 5 film.

A modified protocol was used for the immunoprecipitation of the P-glycoprotein from SKOV3 and SKVLB membranes (Bruggemann et al., 1989). Membranes (5 μg) were photolabeled with ^{125}I-6-AIPP-Fsk as described under Photoaffinity Labeling. Ice-cold acetone (1 mL) was added to the photolabeled membranes and kept at −20 °C for 3 h and then microfuged for 15 min at 13000 rpm. To the photolabeled membranes, 20 μL of buffer (2% SDS, 100 mM Tris-HCl, pH 7.5, 10 mM EDTA) was added and incubated at 37 °C for 20 min. The membranes were then diluted with 180 μL of water and 200 μL of buffer containing 30 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 2% Triton X-100, 2% deoxycholate, 0.2% SDS, 2 mM EDTA, and 0.02% aprotinin. Rabbit antiserum 4007 (5 μL) was added to each and incubated overnight at 4 °C with rocking. A 20% solution of protein A agarose (120 μL) prewashed with PBS (without Ca and Mg), was added to each sample and placed on a rotator at room temperature for 2 h. The agarose was washed with 800 μL of cold RIPA buffer (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1% Triton-X100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 0.1% aprotinin), 800 μL of cold RIPA buffer containing 2.5 M KCl (without EDTA or aprotinin), and again with 800 μL of cold RIPA buffer. The agarose was centrifuged for 1 min in a microfuge between each wash, and the supernatants were discarded. Proteins were eluted from the agarose with 400 μL of buffer (100 mM Tris-HCl, pH 7.5, 2% SDS, 5% β-mercaptoethanol) by a 30-min incubation on a rotator at room temperature. After a 1 min spin in the microfuge, the supernatants, containing the eluted proteins, were removed from the agarose and saved. The eluted proteins were precipitated with 40 μL of 50% sucrose and 600 μL of ice-cold acetone at −70 °C overnight. After the samples were thawed in the freezer, electrophoresis sample buffer was added and the samples were run on an 8% SDS–PAGE gel as described above.

Results

Effect of Forskolin on Cytotoxicity of Adriamycin. The SKOV3 cell line is a human ovarian carcinoma cell line that through multiple selections with vinblastine produced a cell line, SKVLB, that is multidrug resistant (Bradley et al., 1989). A 170-kDa protein was detected in a plasma membrane preparation of SKVLB by using a monoclonal antibody that recognizes a highly conserved epitope in P-glycoprotein (Bradley et al., 1989). This protein was absent in the parental SKOV3 cell line. Forskolin and 1,9-dideoxyforskolin, a naturally occurring analogue that does not activate adenylyl cyclase, were tested for their cytotoxic effects on the SKOV3 and SKVLB cell lines. Cells were incubated with different concentrations of either forskolin or 1,9-dideoxyforskolin for 14 days, and cell survival was determined and compared to control cells (Table I). Forskolin and 1,9-dideoxyforskolin had little effect on cell survival at concentrations of 10 and 40 μM. However, higher concentrations of these drugs were associated with toxic effects on both cell lines.

The effects of forskolin and 1,9-dideoxyforskolin on adriamycin cytotoxicity in the sensitive SKOV3 and the resistant

Table I: Effect of Forskolin and 1,9-Dideoxyforskolin on Cell Survival

	% cell survival[a]			
	forskolin		1,9-dideoxyforskolin	
conc (μM)	SKOV3	SKVLB	SKOV3	SKVLB
10	102 ± 4	100 ± 5	108 ± 3	81 ± 5
40	100 ± 4	92 ± 6	110 ± 2	87 ± 6
50	67 ± 2	52 ± 1	77 ± 3	42 ± 4
75	nd[b]	nd	86 ± 2	10 ± 3

[a] The percent was calculated as the percentage of surviving cells after a 14-day incubation with the indicated concentration of forskolin or 1,9-dideoxyforskolin compared to control cells that were incubated with no additions. Results are means ±SD. [b] Not determined.

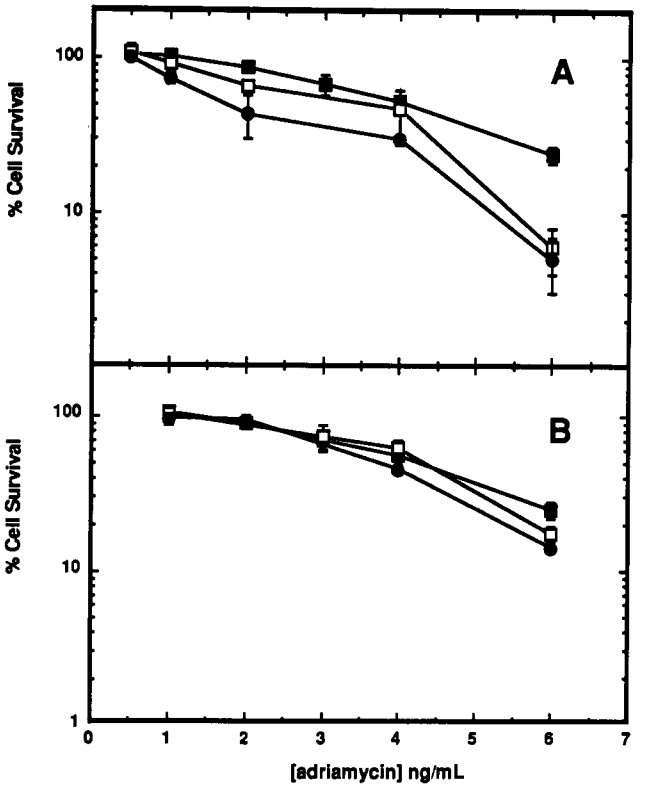

FIGURE 2: Effect of forskolin and 1,9-dideoxyforskolin on adriamycin cytotoxicity of SKOV3 cells. (A) SKOV3 cells were incubated with the indicated concentrations of adriamycin in the absence of forskolin (■) or in the presence of 10 (□) or 40 μM (●) forskolin. (B) SKOV3 cells were incubated with the indicated concentrations of adriamycin in the absence of 1,9-dideoxyforskolin (■) or in the presence of 10 (□) or 40 μM (●) 1,9-dideoxyforskolin. Cells were incubated for 10–12 days and the percent cell survival was determined as described under Experimental Procedures. Values are the means of duplicate determinations and the SD is indicated.

SKVLB cell lines were determined. Adriamycin was a potent cytotoxic agent in SKOV3 cells, causing over 70% cell death at a concentration of 6 ng/mL. Forskolin enhanced the cytotoxicity of adriamycin primarily at 6 ng/mL, the highest concentration of adriamycin tested (Figure 2A). Both concentrations of 1,9-dideoxyforskolin, 10 and 40 μM, produced a slight augmentation of cytotoxicity only at the highest dose of adriamycin (Figure 2B).

In contrast to the results observed with the SKOV3 cells, forskolin and 1,9-dideoxyforskolin produced a marked augmentation in the cytotoxic effects of adriamycin on SKVLB cells (Figure 3). Cell survival was reduced from 51%, at a concentration of 1 μg/mL adriamycin alone, to 3% and 1% in the presence of 10 and 40 μM forskolin, respectively (Figure 3A). Forskolin at a concentration of 40 μM reduced the cell

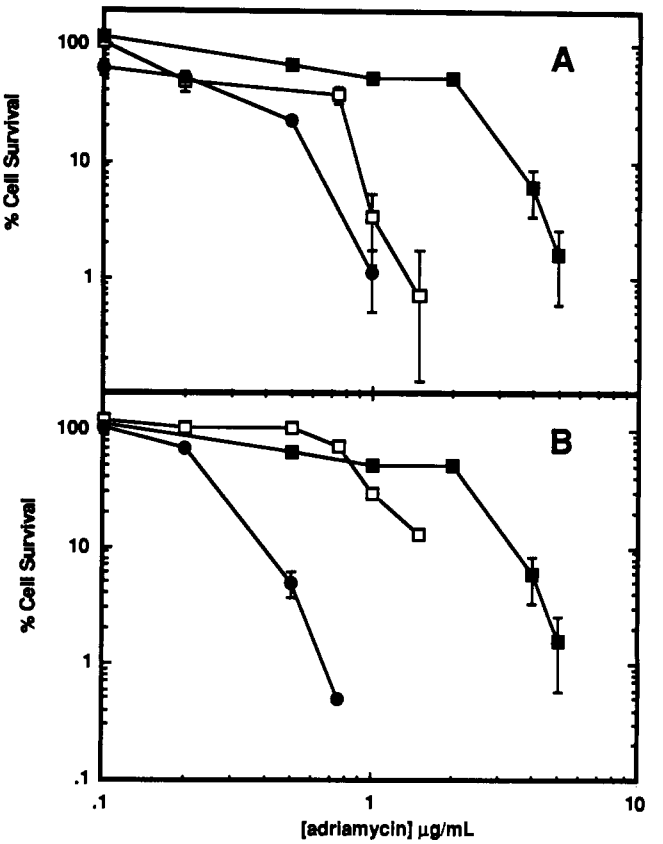

FIGURE 3: Effect of forskolin and 1,9-dideoxyforskolin on adriamycin cytotoxicity of SKVLB cells. (A) SKVLB cells were incubated with the indicated concentrations of adriamycin in the absence of forskolin (■) or in the presence of 10 (□) or 40 μM (●) forskolin. (B) SKVLB cells were incubated with the indicated concentrations of adriamycin in the absence of 1,9-dideoxyforskolin (■) or in the presence of 10 (□) or 40 μM (●) 1,9-dideoxyforskolin. Cells were incubated for 10–12 days and the percent cell survival was determined as described under Experimental Procedures. Values are the means of duplicate determinations and the SD is indicated.

survival from 100% to 63% in the presence of 0.1 μg/mL adriamycin. The potency of adriamycin to decrease cell survival was increased about 10-fold by 40 μM forskolin (Figure 3A). 1,9-Dideoxyforskolin exhibited qualitatively similar effects on adriamycin cytotoxicity of SKVLB cells as forskolin (Figure 3B). A concentration of 40 μM 1,9-dideoxyforskolin increased the potency of adriamycin about 10-fold and modulated the cytotoxicity of 0.5 μg/mL adriamycin from 68% to 5%.

The concentration dependence of the effect of forskolin and 1,9-dideoxyforskolin on adriamycin cytotoxicity was determined in SKOV3 and SKVLB cells. Cells were incubated with submaximal doses of adriamycin in the presence of increasing concentrations of forskolin or 1,9-dideoxyforskolin. SKOV3 cells incubated with 3 ng/mL adriamycin alone exhibited only about 10–20% cytotoxicity. There was relatively little effect of forskolin or 1,9-dideoxyforskolin up to concentrations of 50 μM on SKOV3 cell survival in the presence of 3 ng/mL adriamycin (Figure 4), consistent with the results shown in Figure 2. SKVLB cells incubated with 0.5 μg/mL of adriamycin alone exhibited 20–30% cytotoxicity. In contrast, forskolin and 1,9-dideoxyforskolin produced dose-dependent decreases in the survival of SKVLB cells with 50% of their effects being observed at concentrations of 10 and 20 μM, respectively (Figure 4).

Photolabeling of SKOV3 and SKVLB Membranes by ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk. Membranes from SKOV3 and SKVLB cells were preincubated with either

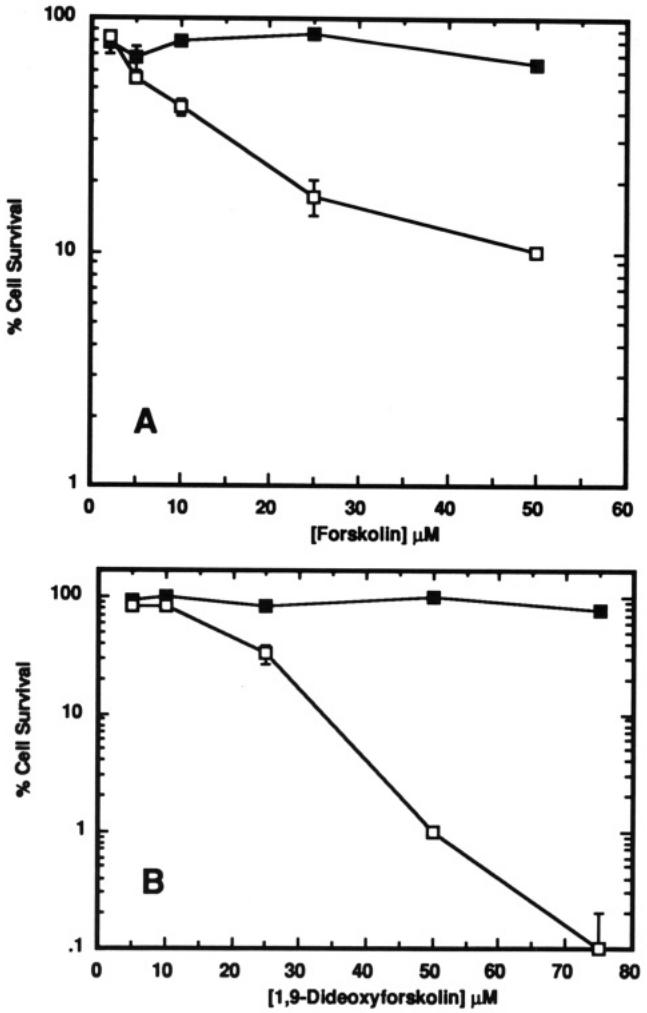

FIGURE 4: Concentration dependence of forskolin and 1,9-dideoxyforskolin augmentation of adriamycin cytotoxicity in SKVLB cells and SKOV3 cells. (A) SKOV3 cells (■) were incubated with 3 ng/mL adriamycin, and SKVLB cells (□) were incubated with 0.5 μg/mL adriamycin in the presence of the indicated concentrations of forskolin. (B) SKOV3 cells (■) were incubated with 3 ng/mL adriamycin and SKVLB cells (□) were incubated with 0.5 μg/mL adriamycin in the presence of the indicated concentrations of 1,9-dideoxyforskolin. Cells were incubated for 10–12 days and the percent cell survival was determined as described under Experimental Procedures. Values are the means of duplicate determinations and the SD is indicated.

buffer, 100 μM forskolin, or 100 μM 1,9-dideoxyforskolin and then incubated with ^{125}I-6-AIPP-Fsk or ^{125}I-7-AIPP-Fsk. The membranes were then photolyzed, and the proteins were separated on denaturing polyacrylamide gels. After the gels were dried, the labeled proteins were detected by autoradiography. There was low incorporation of ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk into proteins of 170 and 45 kDa in SKOV3 membranes. The labeling of these proteins was slightly inhibited by forskolin and 1,9-dideoxyforskolin (Figure 5A).

A 140-kDa protein was predominantly labeled by both ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk in the SKVLB membranes (Figure 5B). The labeling of the 140-kDa protein by both ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk was inhibited by forskolin and by 1,9-dideoxyforskolin. 1,9-Dideoxyforskolin inhibited more of the labeling than forskolin. There was some inhibition of photolabeling of other proteins; however, the extent of inhibition was not as large as that observed for the 140-kDa protein. Since ^{125}I-6-AIPP-Fsk was more efficient in labeling the 140-kDa protein than ^{125}I-7-AIPP-Fsk and appeared to be slightly more specific, ^{125}I-6-AIPP-Fsk was used in all further experiments.

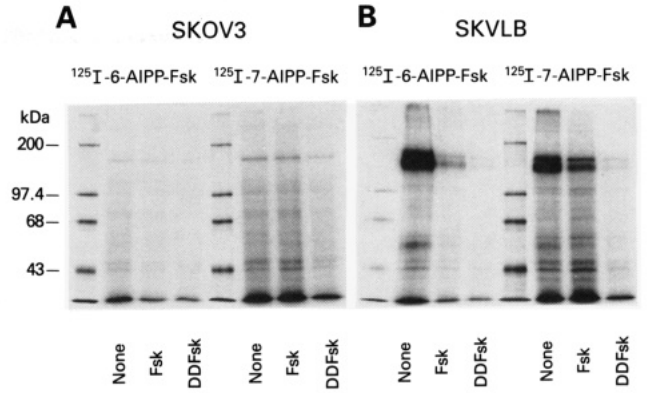

FIGURE 5: Photolabeling of SKOV3 and SKVLB membranes by ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk. SKOV3 and SKVLB membranes (10 μg) were pretreated with buffer (None); 100 μM forskolin (Fsk); or 100 μM 1,9-dideoxyforskolin (DDFsk). The membrane samples were incubated with either ^{125}I-6-AIPP-Fsk or ^{125}I-7-AIPP-Fsk and photolyzed as described under Experimental Procedures. The membrane proteins were incubated with electrophoresis sample buffer and run on two 8% SDS–PAGE gels. The gels were stained, dried, and exposed to XAR 5 film.

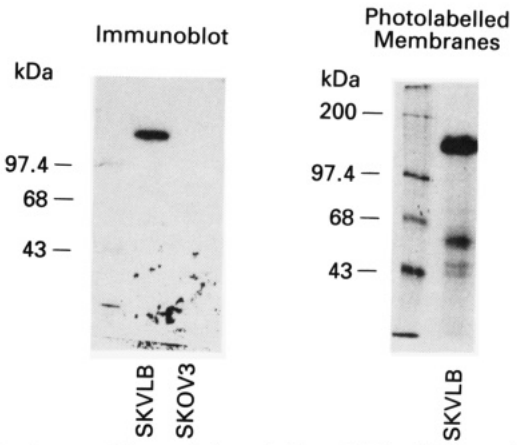

FIGURE 6: Immunoblot analysis and photoaffinity labeling of the P-glycoprotein. For the immunoblot analysis (Immunoblot), 10 μg of both SKOV3 and SKVLB membranes were pelleted, incubated with electrophoresis sample buffer, and run on an 8% SDS–PAGE gel. After the proteins were transferred onto Immobilon, the Immobilon was blocked and then incubated with rabbit antiserum 4007 overnight. The Immobilon was washed, incubated with ^{125}I-labeled protein A, washed, dried, and exposed to XAR 5 film all as described under Experimental Procedures. For photolabeling membranes (Photolabeling Membranes), 10 μg of SKVLB membranes were pretreated with buffer and then incubated with ^{125}I-6-AIPP-Fsk and photolyzed. The membrane proteins were incubated with electrophoresis sample buffer and run on the same gel as the samples for immunodetection. The gel half containing the photolabeled SKVLB membranes was cut out, stained, dried, and exposed to XAR 5 film.

Immunological Identification of the 140-kDa Protein. In order to identify the major 140-kDa labeled protein in the SKVLB membranes, immunoblot analysis was carried out. SKOV3 and SKVLB membranes were run on an 8% SDS–PAGE gel, proteins were transferred onto Immobilon, and the P-glycoprotein was immunodetected with rabbit antiserum 4007. The antiserum was raised against the carboxy-terminal half of the P-glycoprotein (Tanaka et al., 1990). SKVLB membranes were also incubated with ^{125}I-6-AIPP-Fsk and photolabeled. The photolabeled proteins were run concurrently with the membrane samples for immunodetection on the 8% SDS–PAGE gel. The protein detected with antiserum 4007, in SKVLB membranes, (Figure 6, Immunoblot) and the protein predominantly photolabelled (Figure 6, photolabeled membranes) migrated with the same apparent molecular mass

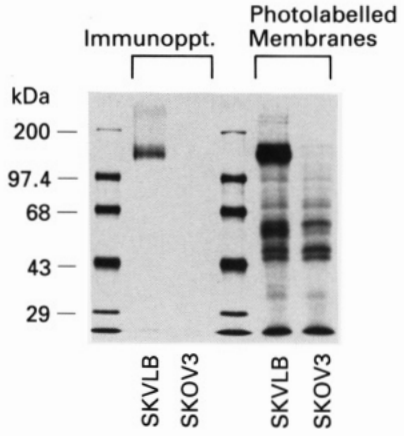

FIGURE 7: Immunoprecipitation and photoaffinity labeling of the P-glycoprotein. For the immunoprecipitation (Immunoppt.), SKOV3 and SKVLB membranes (5 μg) were incubated with ^{125}I-6-AIPP-Fsk and photolyzed. The membranes were then solubilized and immunoprecipitated with rabbit antiserum 4007 overnight as described under Experimental Procedures. The immunoprecipitated proteins were incubated with electrophoresis sample buffer and run on an 8% SDS–PAGE gel. The gel was stained, dried, and exposed to XAR 5 film. For the photolabeled membranes (Photolabeled Membranes), SKOV3 and SKVLB membranes (5 μg) were incubated with ^{125}I-6-AIPP-Fsk and photolyzed. The membrane proteins were incubated with electrophoresis sample buffer and run on the same gel as the immunoprecipitated proteins.

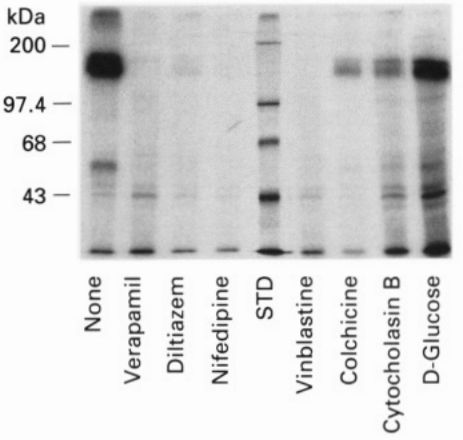

FIGURE 8: Specificity of photolabeling of P-glycoprotein with ^{125}I-6-AIPP-Fsk. SKVLB membranes (5 μg) were pretreated with either buffer (None) or various drugs, all at a concentration of 50 μM, except D-glucose, 500 mM, as indicated. The membranes were incubated with the ^{125}I-6-AIPP-Fsk label and photolyzed as described under Experimental Procedures. The membrane proteins were incubated with electrophoresis sample buffer and run on an 8% SDS–PAGE gel. The gel was stained, dried, and exposed to XAR 5 film.

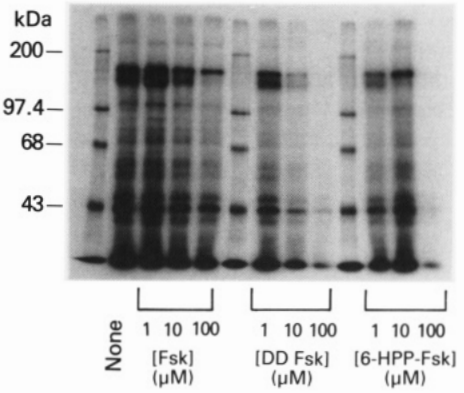

FIGURE 9: Concentration dependence of inhibition of photoaffinity labeling of P-glycoprotein. SKVLB membranes (5 μg) were pretreated with buffer (None) or increasing concentrations of forskolin (Fsk), 1,9-dideoxyforskolin (DDFsk), or 6-HPP-forskolin as indicated. The membranes were incubated with ^{125}I-6-AIPP-Fsk and photolyzed as described under Experimental Procedures. The membrane proteins were incubated with electrophoresis sample buffer and run on an 8% SDS–PAGE gel. The gel was stained, dried, and exposed to XAR 5 film.

of 140 kDa. A 140-kDa protein was not immunodetected in SKOV3 membranes by the antiserum. A 55-kDa protein was photolabeled in the SKVLB membranes (Figure 6, photolabeled membranes) and could be detected in the immunoblot after longer exposures (data not shown). The photolabeling of the 55-kDa protein is also evident in Figures 5B and 8. There was no detection of a 170-kDa protein by immunoblot analysis.

SKOV3 and SKVLB membranes were immunoprecipitated with 4007 antiserum to further verify that the 140-kDa protein was the P-glycoprotein. Both membranes were photolabeled in duplicate with ^{125}I-6-AIPP-Fsk. One set of the labeled membranes was kept as controls. The other set was solubilized and immunoprecipitated as described under Experimental Procedures with 4007 rabbit antiserum. The immunoprecipitated proteins were run on an 8% SDS–PAGE gel with the control photolabeled membranes. A 140-kDa protein was immunoprecipitated only in the SKVLB membranes (Figure 7, immunoprecipitate). A 170-kDa protein that was photolabeled in both SKOV3 and SKVLB membranes (Figure 7, photolabeled membranes) was not detected in the immunoprecipitates.

Inhibition of ^{125}I-6-AIPP-Fsk Labeling. A number of agents that bind to the P-glycoprotein were tested for their ability to inhibit the labeling of ^{125}I-6-AIPP-Fsk (Figure 8). The calcium channel blockers verapamil, diltiazem, and nifedipine, at concentrations of 50 μM, inhibited almost all of the labeling of the 140-kDa protein. These agents also inhibited labeling of the 55-kDa band. At a concentration of 50 μM, vinblastine inhibited all of the labeling of the 140-kDa protein, while colchicine was less effective. Cytochalasin B (50 μM) inhibited the labeling to about the same extent as colchicine; however, D-glucose (0.5 M) had no effect on the labeling of the 140-kDa protein. Cytochalasin B and D-glucose inhibit forskolin binding to the glucose transporter.

Initial experiments indicated that 1,9-dideoxyforskolin was slightly more effective than forskolin at inhibiting labeling of the P-glycoprotein by ^{125}I-6-AIPP-Fsk. The ability of different concentrations of forskolin, 1,9-dideoxyforskolin, and a lipophilic derivative of forskolin 6-*O*-[[2-[3-(4-hydroxyphenyl)-propionamido]ethyl]carbamyl]forskolin, 6-HPP-Fsk (Figure 1), to inhibit labeling of the P-glycoprotein was determined (Figure 9). A concentration of 100 μM forskolin was required to inhibit most of the labeling of the P-glycoprotein. In contrast, only 10 μM 1,9-dideoxyforskolin was needed to demonstrate similar inhibition. At concentrations of 100 and 10 μM, inhibition of labeling by 6-HPP-Fsk and 1,9-dideoxyforskolin were similar. However, 1 μM 6-HPP-Fsk was even more efficient at inhibiting labeling than 1 μM 1,9-dideoxyforskolin. These results suggest that a rank order of potency for inhibition of ^{125}I-6-AIPP-Fsk labeling of the P-glycoprotein is 6-HPP-Fsk > 1,9-dideoxyforskolin > forskolin. At concentrations of 100 μM, 1,9-dideoxyforskolin and 6-HPP-Fsk completely inhibited labeling of virtually all proteins, while forskolin appeared to be more specific at inhibiting the labeling of only the P-glycoprotein. At high concentrations of some protecting agents, a generalized reduction in nonspecific labeling was observed. This effect, which was variable between experiments, could be due to a scavenging effect of the protecting agents at these concentrations. However,

specific protection of photolabeling of the 140-kDa P-glycoprotein and not the 170-kDa protein was clearly observed at low concentrations of forskolin analogues.

Discussion

The P-glycoprotein binds a number of structurally diverse drugs that are similar only in that they are relatively hydrophobic. Photoactive derivatives of some of these drugs have been synthesized and used to label the P-glycoprotein. The ability of cytotoxic drugs to compete for the labeling has been suggestive that the photoactive agents and cytotoxic drugs may be binding at the same site. A previous study (Wadler & Wiernik, 1988) demonstrated that forskolin can effect drug efflux in a MDR cell line. Interest in this observation is strengthened by the suggestion that adenylyl cyclase and the P-glycoprotein may be structurally similar (Krupinski et al., 1989). It therefore seemed reasonable to determine if forskolin and derivatives of forskolin could interact directly with the P-glycoprotein and affect the MDR phenotype.

Reversal of Drug Resistance. The effects on cell survival and on the ability to augment the cytotoxic effects of adriamycin by forskolin and 1,9-dideoxyforskolin were determined on a wild-type ovarian carcinoma cell line, SKOV3, and a variant, SKVLB, that expresses the MDR phenotype. In the absence of other drugs, forskolin and 1,9-dideoxyforskolin are toxic to both cell lines at concentrations greater than 40 μM. In the presence of adriamycin, forskolin and 1,9-dideoxyforskolin have little effect on the ability of the drug to produce cytotoxic effects in the SKOV3 cell line, except at high concentrations of adriamycin. This cell line is sensitive to adriamycin, does not express MDR phenotype, and does not overexpress the P-glycoprotein. In contrast, forskolin and 1,9-dideoxyforskolin are able to augment adriamycin cytotoxicity in the SKVLB cell line. This line has been selected for resistance to vinblastine, is cross resistant to adriamycin, and overexpresses the P-glycoprotein. The ED_{50} for adriamycin cytotoxicity in SKVLB is about 2 μg/mL and is reduced 10-fold in the presence of either 40 μM 1,9-dideoxyforskolin or 40 μM forskolin. Forskolin and 1,9-dideoxyforskolin have also been shown to partially reverse adriamycin resistance in two MDR variants of the S180 murine sarcoma cell line, although the extent of reversal was not as great as that produced by verapamil (Wadler & Wiernik, 1988). Although these studies and the data presented in this paper do not demonstrate a direct inhibitory effect of either forskolin or 1,9-dideoxyforskolin on P-glycoprotein-mediated efflux of drugs, the data do support a role for forskolin in partially reversing drug resistance in MDR cells. The effects of forskolin on reversing drug resistance is not a general phenomenon as forskolin does not augment cytotoxic effects in cell lines that are drug resistant to alkylating agents.[2]

Photolabeling of P-Glycoprotein. ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk photolabel the P-glycoprotein in membranes from the SKVLB cells that overexpress the protein. A 140-kDa protein, which is strongly photolabeled, is identical with that of a protein detected by rabbit antiserum that recognizes the human P-glycoprotein and to a protein immunoprecipitated by the antiserum. The absence of specific labeling in the SKOV3 cells, which do not overexpress the P-glycoprotein, is further evidence that the labeled protein is the P-glycoprotein. The reported molecular weight of the P-glycoprotein varies between 130 and 170 kDa. This may be attributed either to differences in glycosylation that may be cell-line-dependent or to differences in experimental protocols (Greenberger et al., 1988). Drugs that are known to interact with the P-glycoprotein inhibit the labeling of the 140-kDa protein with ^{125}I-6-AIPP-Fsk. These include calcium channel blockers and the cytotoxic drug vinblastine. Colchicine, another drug that the SKVLB cells are partially resistant to (Bradley et al., 1989), is also able to partially inhibit photolabeling. Therefore, the ability of these different drugs to inhibit photolabeling is consistent with ^{125}I-6-AIPP-Fsk binding at a drug-binding site on the P-glycoprotein. The data do not allow a prediction of where the binding site is on the P-glycoprotein. However, a 55-kDa peptide in SKVLB membranes is labeled by ^{125}I-6-AIPP-Fsk and is detected by the antiserum 4007. This peptide has previously been identified as a C-terminal proteolytic fragment of the P-glycoprotein (Bruggemann et al., 1989; Tanaka et al., 1990). This suggests that at least part of the forskolin-binding site is associated with the C-terminal half of the P-glycoprotein. It will be interesting to determine if ^{125}I-6-AIPP-Fsk labels the same domains that are labeled by other P-glycoprotein photolabels, such as [^{3}H]azidopine and [^{125}]iodoarylprazosin (Greenberger et al., 1990; Bruggemann et al., 1989; Safa et al., 1990).

Proteins other than the P-glycoprotein were also photolabeled in SKOV3 and SKVLB cell membranes, although only one, a 170-kDa protein present in both cell lines, sometimes showed competition of photolabeling by forskolin, 1,9-dideoxyforskolin, and some other agents. This protein does not appear to be related to the P-glycoprotein. The protein was not detected in immunoblots or immunoprecipitates of SKOV3 and SKVLB membranes when an antiserum specific for the P-glycoprotein was used. The forskolin analogues were also more specific for inhibiting photolabeling of the P-glycoprotein than the 170-kDa protein, especially at lower concentrations of the analogues.

The forskolin-binding site on the P-glycoprotein is distinct from that of adenylyl cyclase and the glucose transporter. Both ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk label the P-glycoprotein. In contrast, ^{125}I-6-AIPP-Fsk is very efficient at photolabeling adenylyl cyclase and can be used to detect adenylyl cyclase in crude membranes from bovine brain, whereas ^{125}I-7-AIPP-Fsk does not detect adenylyl cyclase in these membranes (Morris et al., 1991). Another distinction between adenylyl cyclase and the P-glycoprotein is the ability of 1,9-dideoxyforskolin to inhibit photolabeling of these proteins. 1,9-Dideoxyforskolin does not bind to adenylyl cyclase and does not inhibit photolabeling of adenylyl cyclase (Morris et al., 1991); however, 1,9-dideoxyforskolin does inhibit photolabeling of the P-glycoprotein. The photolabeling of the P-glycoprotein can also be distinguished from that of the glucose transporter. ^{125}I-7-AIPP-Fsk is more efficient than ^{125}I-6-AIPP-Fsk at photolabeling the glucose transporter (Morris et al., 1991). In contrast, both ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk photolabel the P-glycoprotein similarly. Furthermore, D-glucose inhibits ^{125}I-7-AIPP-Fsk labeling of the glucose transporter yet has no effect on the labeling of the P-glycoprotein.

The drug-binding site on the P-glycoprotein does not exhibit structural specificity and appears to bind a number of structurally unrelated drugs whose only similarity is their hydrophobic nature (Endicott & Ling, 1989). The binding of forskolin and analogues of forskolin is consistent with this lack of structural specificity. The rank order for inhibition of ^{125}I-6-AIPP-Fsk labeling is 6-HPP-Fsk > 1,9-dideoxyforskolin > forskolin. This is the same rank order for the relative lipophilicity of these compounds and is consistent with lipophilicity being important for binding at the P-glycoprotein.

[2] K.D.T. and K.B.S., unpublished results.

Table II: Transmembrane Helices 10 through 12 of the Glucose Transporter (GT), P-Glycoprotein (MDR), and Adenylyl Cyclase (AC)[a]

		residue number																					
helix		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Ar[b]	±[c]
10	GT	I	V	A	I	F	G	F	V	A	F	F	E	V	G	P	G	P	I	P	W	F	6	1
	MDR	G	W	Q	L	T	L	L	L	L	A	I	V	P	I	I	A	I	A	G	V	V	1	0
	AC	E	S	A	P	H	A	L	L	C	G	L	V	G	T	L	P	L	A	I	F	L	1	2
11	GT	A	A	I	A	V	A	G	F	S	N	W	T	S	N	F	I	V	G	M	C	F	4	0
	MDR	I	F	G	I	T	F	S	F	T	Q	A	M	M	Y	F	S	Y	A	G	C	F	7	0
	AC	M	I	L	L	A	V	L	T	T	S	Y	I	L	V	L	E	L	S	G	Y	T	2	1
12	GT	G	P	Y	V	F	I	I	F	T	V	L	L	V	L	F	F	I	F	T	Y	F	8	0
	MDR	V	L	L	V	F	S	A	V	V	F	G	A	M	A	V	G	Q	V	S	S	F	3	0
	AC	A	M	G	A	G	A	I	S	G	R	S	F	E	P	I	M	A	I	L	L	F	2	2

[a] The sequences of the transmembrane helices of adenylyl cyclase (AC), glucose transporter (GT), and the P-glycoprotein (MDR) were taken from Krupinski et al. (1989), Mueckler et al. (1985), and Chen et al. (1986), respectively. [b] The number of aromatic residues (Ar) in each helical segment was determined and includes Tyr (Y), Phe (F), and Trp (W). [c] The number of charged residues (±) in each helical segment was determined and includes Glu (E), Asp (D), His (H), Lys (K), and Arg (R).

It is interesting to note that lipophilic analogues of forskolin derivatized at the 7-position are also more potent than forskolin at the glucose transporter (Wadzinski et al., 1987). There is little structural similarity between forskolin and other agents that reverse MDR such as calcium channel blockers, calmodulin inhibitors, and other drugs (Endicott & Ling, 1989). Forskolin is a relatively hydrophobic diterpene and does not contain any aromatic groups, a feature that is observed with many of the other reversing agents. However, the increased efficiency of the aromatic derivative of forskolin, 6-HPP-Fsk, to inhibit the photolabeling of P-glycoprotein is consistent with aromatic groups playing a role in drug binding.

Model for the Forskolin-Binding Site. The three proteins that can be identified as forskolin-binding proteins on the basis of photoaffinity labeling are adenylyl cyclase, the glucose transporter, and the P-glycoprotein. The amino acid sequences of the calmodulin-sensitive adenylyl cyclase catalytic subunit, the human erythrocyte glucose transporter, and the P-glycoprotein suggest that these proteins may exhibit certain structural similarities (Krupinski et al., 1989; Mueckler et al., 1985; Chen et al., 1986). Each protein is predicted to consist of two domains, with each domain containing six transmembrane helices. Adenylyl cyclase and the P-glycoprotein contain large cytoplasmic regions connecting the two domains and a large C-terminal cytoplasmic region, while the glucose transporter has much shorter cytoplasmic regions.

It is tempting to speculate that forskolin binds to a structurally homologous site on adenylyl cyclase, the glucose transporter, and the P-glycoprotein and that the specific binding characteristics of forskolin and forskolin analogues (as well as other agents) will be determined by the specific amino acids in the transmembrane helices. Recent data have demonstrated that the forskolin-binding site on the glucose transporter is localized at least in part to a 5-kDa fragment, containing the tenth transmembrane helix, in the C-terminal half of the molecule (Wadzinski et al., 1990). We have compared the sequences of the C-terminal helices, 6 through 12, of adenylyl cyclase, the glucose transporter, and the P-glycoprotein in order to determine similarities between these proteins. There is a striking difference in the number of aromatic amino acids in the transmembrane helices 10, 11, and 12 (Table II). The glucose transporter and the P-glycoprotein contain a much larger number of aromatic amino acids in transmembrane helices 10 through 12 than does adenylyl cyclase. For example, the glucose transporter contains a total of 18, the P-glycoprotein contains 11, and the adenylyl cyclase contains only 5 aromatic residues in helical segments 10 through 12. The fact that aromatic derivatives of forskolin have an increased potency at the P-glycoprotein and the glucose transporter suggests that the aromatic group on forskolin may be interacting at a lipophilic binding site on these proteins that could be partially defined by transmembrane helices 10 through 12. In contrast, aromatic derivatives of forskolin that are derivatized at the 7-position of forskolin have a reduced potency at adenylyl cyclase. This is consistent with a lack of aromatic residues in transmembrane helices 10 through 12 of adenylyl cyclase.

It is interesting to note that the P-glycoprotein has a higher number of aromatic residues throughout all of the transmembrane segments than either adenylyl cyclase or the glucose transporter. This may be one reason why there is such little structural specificity for the binding of lipophilic drugs to the P-glycoprotein. The presence of aromatic amino acids in the N-terminal half of the P-glycoprotein (helices 1 through 6) may provide a binding domain for drugs. In this regard, azidopine does incorporate into the N-terminal half of the molecule (Qian et al., 1990; Bruggemann et al., 1989). In contrast, the binding of forskolin to the glucose transporter appears to be more exclusively associated with the C-terminal half of the molecule (Wadzinski et al., 1990), where there is a higher number of aromatic amino acid residues.

The forskolin-binding site on adenylyl cyclase is the most specific binding site of all these proteins and will only bind compounds that have a forskolin-like structure; i.e., forskolin binding is not inhibited by structurally unrelated molecules. Transmembrane helices 10, 11, and 12 of adenylyl cyclase contain fewer aromatic and more charged amino acid residues than the glucose transporter and the P-glycoprotein. This is consistent with the observation that water-soluble derivatives of forskolin, which are relatively potent at adenylyl cyclase, are not potent at inhibiting the glucose transporter (Joost et al., 1988). The lack of aromatic residues and the presence of the charged residues in the transmembrane helices of adenylyl cyclase may be restricting the binding site such that only forskolin-like compounds can bind with specificity.

Summary. The ability of forskolin and analogues of forskolin to partially reverse drug resistance in MDR cell lines and to photolabel the P-glycoprotein suggests that forskolin can bind to the P-glycoprotein in a manner analogous to other reversing agents. As such, it will be extremely interesting to determine if forskolin or forskolin-derived compounds can play a role in reversing clinical drug resistance. Forskolin interactions with the P-glycoprotein are analogous to the binding of forskolin at adenylyl cyclase and the glucose transporter. It has been suggested that forskolin may be binding at a structurally homologous binding site on several membrane transport proteins (Laurenza et al., 1989). This model can be expanded to suggest that the binding site will be composed

of transmembrane helices and the specific characteristics of the binding site will be determined by the amino acid residues in the transmembrane helices. Therefore, it will be interesting to determine the exact location of forskolin-binding sites in structurally related proteins and to determine if forskolin can interact directly with other membrane transport proteins, such as voltage-gated calcium, potassium, and sodium channels, as well as ligand-gated ion channels.

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