Partial Inhibition of Multidrug Resistance by Safingol Is Independent of Modulation of P-glycoprotein Substrate Activities and Correlated with Inhibition of Protein Kinase C*

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Safingol is a lysosphingolipid protein kinase C (PKC) inhibitor that competitively interacts at the regulatory phorbol binding domain of PKC. We investigated the effects of safingol on antineoplastic drug sensitivity and PKC activity of MCF-7 tumor cell lines. Safingol treatment of 32P-labeled MCF-7 WT and MCF-7 DOXR cells inhibited phosphorylation of the myristoylated alanine-rich protein kinase C substrate in both cell lines, suggesting inhibition of cellular PKC. However, only in MCF-7 DOXR cells did safingol treatment increase accumulation of [3H]vinblastine and enhance toxicity of Vinca alkaloids and anthracyclines. Drug accumulation changes in MCF-7 DOXR cells treated with safingol were accompanied by inhibition of basal and phorbol 12,13-dibutyrate-stimulated phosphorylation of P-glycoprotein (P-gp). Expression of P-gp and levels of mdr1 message in MCF-7 DOXR cells were not altered by safingol treatment alone or in combination with vinblastine. Treatment of MCF-7 DOXR cell membranes with safingol did not inhibit [3H]vinblastine binding or [3H]azidopine photoaffinity labeling of P-gp. Furthermore, safingol did not stimulate P-gp ATPase activity in membranes prepared from MCF-7 DOXR cells. We conclude that enhanced drug accumulation and sensitivity in MCF-7 DOXR cells treated with safingol are correlated with inhibition of P-gp drug binding through direct interaction with P-glycoprotein.

Exposure of cancer cells to a single natural product antineoplastic drug can give rise to cells that also exhibit cross-resistance to other unrelated natural cytotoxins. This phenotype is known as multidrug resistance and is characterized by the reduced intracellular accumulation of these drugs, due to drug efflux by P-glycoprotein (P-gp),1 a 150–180-kDa plasma membrane transport ATPase encoded by the human mdr1 gene (reviewed in Refs. 1 and 2). A number of agents have been shown to inhibit multidrug resistance (3). Most modulators of multidrug resistance inhibit specific binding of drugs to P-gp in membranes derived from MDR cells (4–7) and photoaffinity labeling of P-gp by photoactive drug analogs (4, 8, 9). These results suggest that competitive inhibition of P-gp drug binding is a mechanism by which P-gp function and multidrug resistance may be inhibited.

P-gp is subject to phosphorylation (10, 11) and changes in P-gp phosphorylation have been associated with inhibition of P-gp drug transport by modulators of the MDR phenotype (12, 13). Although other kinases have been shown to phosphorylate P-gp in vitro (11, 14), there is considerable experimental evidence that suggests that phosphorylation of P-gp by protein kinase C (PKC) may play a role in regulating multidrug resistance.

PKC is a family of serine/threonine kinases, composed of calcium-dependent and calcium-independent isotypes, which are dependent upon phospholipid for activation (15). In vitro, P-gp is phosphorylated by PKC (13, 16) at serine residues (13, 17) primarily in the linker region of P-gp (18, 19). Increases in PKC activity have been noted in many MDR cell lines (20–22) and in some instances have been associated with changes in the expression of specific PKC isoforms (23–25). Treatment of MDR cells with PKC activators has been reported to increase phosphorylation of the P-gp (13, 17, 25, 26) and enhance P-gp function (12, 20, 24, 26, 27). Conversely, treatment of MDR cells with PKC inhibitors has been shown to decrease drug transport activity and phosphorylation of P-gp (13, 26, 28). Most PKC inhibitors that modulate P-gp function have also been reported to inhibit labeling of P-gp by the photoactive calcium channel blocker [3H]azidopine, suggesting that these compounds also competitively inhibit drug binding to P-gp (26, 29, 30). In studies that use PKC inhibitors that also inhibit drug binding to P-gp, the following question is raised: Does inhibition of P-glycoprotein function by PKC Inhibitors bound by P-gp result from inhibition of drug binding or from inhibition of P-glycoprotein phosphorylation by PKC?

Recently, the calcium channel blocker SR33557 was shown to increase cellular levels of sphingosine leading to inhibition of protein kinase C and drug resistance (31). SR33557 did not inhibit P-gp drug binding or photolabeling of P-gp by [3H]azidopine, and the photoactive analog [3H]SR33557 did not photolabel P-gp. These results suggested that SR33557 did not directly interact with P-gp and also raised the possibility that lysosphingolipids could modulate MDR without competing for drug binding to P-gp (31).

The PKC inhibitor safingol is a saturated analog of sphingosine, the naturally occurring lysosphingolipid. PKC inhibition

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1 The abbreviations used are: P-gp, P-glycoprotein; safingol, (2S,3S,5S,6S)-2-amino-1,3-octadecanediol; MARCKS, myristoylated alanine-rich protein kinase C substrate; MDR, multidrug-resistant; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyl tetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C.
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by lysosphingolipids results from competitive interaction with the regulatory diacylglycerol/phorbol binding domain of PKC (32). Lysosphingolipids have been shown to inhibit growth (33) and differentiation of cancer cells (34, 35), gastric cancer cell invasion (36), and tumor promotion (37) by inhibiting PKC activity. In the present study we have investigated effects of safingol on phosphorylation of the myristoylated alanine-rich protein kinase C substrate (MARCKS), a prominent cellular substrate of PKC, to demonstrate inhibition of PKC activity by safingol in drug-sensitive and MDR cells. The antitumor activity of safingol, when given alone and in combination with other antineoplastic drugs, was characterized, and the effects of safingol on phosphorylation and drug binding of P-gp were studied. The results indicate that safingol inhibits protein kinase C and partially inhibits the MDR phenotype of MCF-7 DOXR cells but does not exhibit properties of a substrate for P-gp or alter P-gp expression.

EXPERIMENTAL PROCEDURES

Materials

Safingol, purified rat brain PKC, and recombinant human PKC a were provided by Sphinx Pharmaceuticals Corp. Tissue culture media, balanced salt solutions, and prestained high molecular weight standards were purchased from Life Technologies, Inc. (Grand Island, NY). Tissue culture plates and flasks were purchased from Costar (Cambridge, MA). [32P]Vinbliniste (9 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [3P]Jhorphosphate and [3P]JATP were obtained from DuPont NEN. [3H]Azidopine (52 Ci/mmol) and Western blotting reagents (ECL detection and peroxidase-conjugated antibodies) were from Amersham Corp. Protein A-Sepharose 4B was purchased from Pharmacia LKB Biotech Inc. C239 monoclonal antibody to P-gp was from Signet Laboratories (Dedham, MA). Normal rabbit serum was purchased from Vector Laboratories (Burlingame, CA). Electrophoresis reagents, polyvinylidene difluoride membranes, molecular weight standards, protein assay kits, and standard (bovine serum albumin, BSA) were purchased from Bio-Rad. Fetal calf serum, antineoplastic drugs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenylmethylsulfonyl fluoride, NaF, leupeptin, aprotinin, pepstatin A, fatty acid-free BSA, and V8 protease were acquired from Sigma. HAWP filters (0.45 micron pore size) were obtained from Millipore Corp. (Bedford, MA). Drug-sensitive MCF-7 WT and MDR MCF-7 DOXR, which were selected by doxorubicin, were provided by Dr. Ken Cowan (National Cancer Institute). Purified myelin basic protein and a polyclonal antibody to the MARCKS protein were provided by Rick Dobrowsky (Duke University Medical Center) and Dr. Perry Blackshear (Duke University Medical Center), respectively. AS15 polyclonal antibody to human P-gp was provided by Dr. Melvin Center (Kansas State University). All other materials were reagent grade and were purchased from commercial sources.

Methods

PKC Activity and Phorbol Binding Assays—PKC activity was assayed by quantitating the incorporation of [3P]JATP into myelin basic protein. The PKC reaction mixture contained in a total volume of 200 µ1 20 mM Tris-HCl, pH 7.5, 100 µg CaCl2, 10 mM MgCl2, 10 µM [3P]JATP, 30 µg/ml phosphatidylserine, 1 µg/ml dioleoylglycerol, PKC enzyme and 2 µl of ethanol or safingol dissolved in ethanol. The assays were run for 10 min at 37 °C and terminated by precipitation of proteins by glass filters, and incorporation was quantified by densitometry.

Cell Culture—Cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO2 in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal calf serum (media) in 96-well plates and allowed to adhere overnight. Drug sensitivity was measured by exposing the cells to graded concentrations of cytotoxic drugs in a final volume of 200 µl. The cell density was approximately 1.7 × 105 cells/cm2 with a media volumecell/cell ratio of 3.6 × 104 µl/cell (0.625 µl/cm2) under these conditions. At 72 h viable cells in each treatment group were estimated in a colorimetric assay that measures the formazan reduction product of MTT, which is produced by mitochondrial activity of viable cells. The reduction product was dissolved in a mixture of isopropanol, 0.1 M HCl, Triton X-100 (90:10:0.4), and absorbance was quantitated using a plate reader spectrophotometer. Control experiments showed that the reduction of MTT was proportional to cell number.

To evaluate the effect of agents on antineoplastic drug toxicity, cells were treated with 50 µM of media containing either vehicle, safingol, or verapamil for 30 min. The cells were then treated with 40 µM of media containing graded concentrations of antineoplastic drugs and 10 µM of media containing vehicle, safingol, or verapamil to maintain final concentrations of 3–5 µM safingol or 5 µM verapamil. Percentages were calculated relative to cell groups treated with solvent alone, and IC50 values were calculated as above.

Delivery of Safingol to Cells with BSA—Stock solutions of safingol were prepared by diluting 10–20 mM ethanol to obtain stock solutions of safingol into Iscove’s modified Dulbecco’s medium containing 0.1–0.5 mM BSA as described (38, 39) for serum-free delivery to cells. [3H]Vinblastine Accumulation Assay—Cells (1.5 × 105/well) were plated in 6-well plates (9.5 cm2/well) and cultured to a density of approximately 6.5 × 105/well (6.8 × 105 cells/cm2). Accumulation of [3H]Vinblastine was measured at 2 h as described previously (25) with the following modifications: the first assay was performed on cell lines transfected with a vector expressing a modified Dulbecco’s medium containing 100 µM fatty acid-free BSA, and the cells were incubated 30 min with resistance modifiers before the addition of [3H]Vinblastine (final concentration 100 nM; specific activity 4 Ci/mmol). An aliquot of the detergent lysate was counted in a scintillation counter, and protein determinations were performed by the method of Bradford (40). The data are expressed as pmol of [3H]Vinblastine/mg of protein. In parallel studies cytotoxicity was evaluated by exclusion of trypan blue and by measuring MTT reduction of cells treated with safingol in the presence and absence of vinblastine for 2.5 h.

In Situ Phosphorylation Studies and Immunoprecipitation of MARCKS and P-glycoprotein—Cells were cultured in 6-well plates, washed, and treated with drugs in phosphate-free RPMI 1640 containing 10 mM HEPES, pH 7.3, 100 µM BSA and labeled with 0.2 µCi of [3P]Jorthophosphate/ml as described in the figure legends. The samples were lysed in detergent buffer, and aliquots were immunoprecipitated using antibodies to MARCKS or P-gp as described previously (25). Incorporation of label into protein was quantitated by SDS-PAGE analysis and by scintillation counting of supernatant and trichloroacetic acid-precipitated labeled protein, respectively.

Phosphopeptide Analysis—P-gp bands identified by autoradiography were excised from gels, and Cerenkov counts were determined. The bands were digested with 2.5 µg of S. aureus V8 protease and analyzed by SDS-PAGE as described (41). Phosphopeptides were identified by autophosphorylation.

Preparation of Membrane Vesicles—For drug binding assays and ATPase studies membrane vesicles from MCF-7 cells were prepared by nitrogen cavitation (6). To prepare membranes for immunoblotsting cells were broken with a Dounce homogenizer (5). After nuclei and unbroken cells were removed by centrifugation at 1000 × g for 10 min membranes were isolated on discontinuous sucrose gradients as described (6).

Vinblastine Binding Assays and [3H]Azidopine Photoaffinity Labeling of P-gp—Membrane vesicles containing 50 µg of protein were incubated at room temperature for 20 min with vehicle, verapamil, or safingol in 10 mM Tris-HCl pH 7.5, 0.25 mM sucrose, 5 mM MgCl2, 3 mM ATP, and 100 nM [3H]Vinblastine (specific activity 9 Ci/mmol) in a final volume of 1.0 ml. Membranes were collected on filters (0.45 µm pore size) soaked in 1% BSA and washed twice with 4 ml of assay buffer. Nonspecific binding of [3H]Vinblastine was measured in the presence of 100 µM unlabeled vinblastine and was subtracted from the total binding to calculate specific binding.

Cells (5 × 105/assay) or membranes (100 µg/assay) were incubated in the presence of 30% calf serum in 1 ml of 30 mM sodium phosphate buffer, pH 7.5, containing 0.5 µM [3H]Azidopine and incubating an additional 20 min. The samples were photolabeled on ice by UV irradiation for 10 min, diluted in 2 × sample buffer (42), and analyzed by SDS-PAGE. Labeling of P-gp bands was detected by autoradiography and quantitated by densitometry.

P-gp ATPase Activity—ATPase activity of MCF-7 DOXR cells was measured by determining vanadate-sensitive liberation of inorganic phosphate from ATP as described (43, 44). Briefly, 10 µg of MCF-7 DOXR membranes were incubated in 100 µl of 50 mM Tris-HCl, pH 7.5, 5 mM
**RESULTS**

Effects of Safingol on in Vitro and in Situ PKC Activity—
Safingol (Fig. 1) inhibits enzymatic activity and [3H]phorbol dibutyrate binding of purified rat brain PKC with similar IC\textsubscript{50} values (37.5 and 31 \mu M, respectively). Safingol inhibits activity of human PKC \alpha, the major overexpressed isoenzyme in MCF-7 DOXR\textsuperscript{B} cells (25) with an IC\textsubscript{50} value of 40 (±8) \mu M (n = 3). These findings are consistent with a model in which inhibition of PKC activity by safingol results from its interaction with the regulatory domain of PKC.

To determine whether safingol could inhibit PKC activity in cells, the effect of safingol treatment on basal phosphorylation of MARCKS in MCF-7 WT and MCF-7 DOXR\textsuperscript{A} cells was evaluated. Antibodies to MARCKS immunoprecipitated an 87-kDa protein from MCF-7 WT (Fig. 2A) and MCF-7 DOXR\textsuperscript{A} cells (Fig. 2B) labeled with \textsuperscript{32}P. Safingol was shown to inhibit MARCKS phosphorylation in a concentration-dependent manner in both cell types, demonstrating that safingol treatment inhibits PKC activity of both drug-sensitive and MCF-7 DOXR\textsuperscript{A} cells.

Effects of Safingol on Basal Phosphorylation of P-gp in MCF-7 DOXR\textsuperscript{A} Cells—To determine if inhibition of cellular PKC activity in safingol-treated MCF-7 DOXR\textsuperscript{A} cells was correlated with decreased phosphorylation of P-gp, P-gp and MARCKS were immunoprecipitated from the same \textsuperscript{32}P-labeled MCF-7 DOXR\textsuperscript{A} cell extracts and analyzed by SDS-PAGE in parallel. A comparison of the concentration-dependence of safingol inhibition of P-gp phosphorylation (Fig. 3A) with that of MARCKS (Fig. 2B) showed corresponding decreases in phosphorylation of both P-gp and MARCKS. SDS-PAGE analysis of general protein phosphorylation in cells labeled and treated with safingol showed that overall protein phosphorylation was not inhibited (not shown) providing further evidence for inhibition of phosphorylation of specific cellular substrates of PKC by safingol rather than inhibition of overall \textsuperscript{32}P labeling. Western blotting demonstrated that equal amounts of P-gp were immunoprecipitated from MCF-7 DOXR\textsuperscript{A} cells treated with safingol for 2.5 h (Fig. 3B). This result verified that safingol treatment did not alter immunoprecipitation yields and indicated that the alterations of phosphorylated P-gp were due to decreased phosphorylation.

Effects of Safingol on Phorbol Ester Stimulated Phosphorylation of P-gp and Analysis of P-gp Phosphopeptides—To verify that safingol was inhibiting PKC-mediated phosphorylation of P-gp, PKC activity was stimulated with 0.1 \mu M PDBu in \textsuperscript{32}P-labeled MCF-7 DOXR\textsuperscript{B} cells pretreated with the indicated concentrations of safingol and labeled with \textsuperscript{32}Porthophosphate for 2.5 h in 1 ml of phosphate-free RPMI 1640 containing 0.1 \mu M BSA. The MARCKS protein was immunoprecipitated with a polyclonal antibody from detergent lysates, resolved by SDS-PAGE using 6 or 7.5% acrylamide gels. Film was exposed for 30 h, with an intensifying screen in panel A and overnight in panel B. The positions of molecular weight standards are shown on the left, and the MARCKS protein is identified with an arrow. Similar results were obtained in two (MCF-7 WT) or three (MCF-7 DOXR\textsuperscript{A}) additional experiments.

P-gp, PKC activity was stimulated with 0.1 \mu M PDBu in \textsuperscript{32}P-labeled MCF-7 DOXR\textsuperscript{B} cells pretreated for 90 s with vehicle (BSA) or 50 \mu M safingol. P-gp phosphorylation was increased upon PDBu stimulation (Fig. 4A) consistent with PKC phosphorylation of P-gp, and safingol treatment inhibited the PDBu dependent increase in P-gp phosphorylation. A phosphopeptide map of V8 protease digests of these P-gp bands (Fig. 4B) showed that PDBu stimulation of PKC in MCF-7 DOXR\textsuperscript{B} cells increased the phosphorylation of three major phosphopeptide bands with molecular weights of 36, 26, and approximately 8 kDa. Safingol treatment inhibited PDBu stimulated phosphorylation of all three phosphopeptides equally. This finding is consistent with a model in which safingol inhibits PKC phosphorylation of specific sites on P-gp. In contrast to results seen...
with 2.5 h of safingol exposure, cells treated with safingol alone for 10 min did not exhibit appreciable decreases in basal phosphorylation of P-gp or the phosphopeptides. Thus, the ability to detect decreases in phosphorylation of previously labeled P-gp is dependent upon turnover of phosphorylated P-gp in addition to inhibition of PKC.

Effects of Safingol on Drug Accumulation in Drug-sensitive and MCF-7 DOXR Cells—To determine if inhibition of PKC by safingol was associated with changes in drug accumulation, [3H]vinblastine accumulation was measured in MCF-7 DOXR and MCF-7 WT cells. Basal accumulation of vinblastine in drug-sensitive cells was 59.2 (± 11) pmol/mg of protein. Treatment of MCF-7 WT with 10 μM verapamil or 5-50 μM safingol did not significantly increase drug accumulation (n = 2, p > 0.1, data not shown). Safingol treatment increased vinblastine accumulation 1.3, 2.0, 3.5, and 5.6-fold in MDR cells treated with 20, 30, 40, and 50 μM safingol, respectively, from a basal level of 3.8 (± 0.5) pmol/mg protein (Fig. 5). These increases were shown to be significant in analysis of variance (p ≤ 0.0001). The positive control, 10 μM verapamil, increased vinblastine accumulation 7.3-fold above baseline (p ≤ 0.0001) but did not increase vinblastine accumulation to levels observed in MCF-7 WT. These results demonstrated a partial reversal of the drug accumulation defect of these MDR cells by safingol and verapamil. Under these conditions, cells treated with 50 μM safingol had 93 ± 2% of the untreated control MTT reduction, and 97 ± 6% percent of the cells excluded trypan blue, indicating that the changes in P-gp phosphorylation and drug accumulation did not result from cytotoxicity.
Safingol Inhibits PKC and MDR

To verify that these increases in vinblastine accumulation were related to inhibition of PKC, MCF-7 DOXR cells were treated with PDBu 5 min prior to addition of safingol and [3H]vinblastine. Safingol has much lower affinity for PKC than PDBu; thus, it is difficult for this drug to displace PDBu from the regulatory domain. PDBu treatment was found to decrease vinblastine accumulation 70 (± 15)% in cells treated with 50 μM safingol but only 10–15% in vehicle- and verapamil-treated cells (n = 3). The partial reversal of safingol-mediated increases in drug accumulation in response to prior activation of PKC by PDBu implicates PKC in these increases. The correlation of increased drug accumulation and inhibition of MARCKS (Fig. 2B) and P-gp phosphorylation (Fig. 3A) in MCF-7 DOXR cells treated with safingol suggests an association between inhibition of PKC by safingol and enhanced accumulation of vinblastine in these MDR cells. Safingol inhibition of MARCKS phosphorylation in MCF-7 WT cells was not associated with increases in drug accumulation, a finding consistent with the idea that inhibition of P-gp phosphorylation by PKC was central to the changes in drug accumulation.

Effects of Safingol on Drug Binding by P-gp—Equilibrium binding studies have suggested that there are at least two kinetically distinguishable drug-binding sites on P-gp, one for Vinca alkaloids, verapamil, and cyclosporin A, and a second for azidopine (7). To determine if drug accumulation changes were associated with competitive inhibition of drug binding by P-gp, the effects of safingol on specific binding of [3H]vinblastine and [3H]azidopine photoaffinity labeling of P-gp in MCF-7 DOXR membranes were examined (Figs. 6 and 7, respectively).

Vehicle-treated vesicles (1% ethanol) specifically bound 32.4 pmol of [3H]vinblastine/mg of protein. Unlabeled vinblastine inhibited specific binding by 73–96% at concentrations between 1 and 100 μM. In a similar fashion verapamil inhibited specific drug binding, while safingol did not appreciably reduce drug binding even when present in a 1000-fold molar excess over [3H]vinblastine (Fig. 6). MCF-7 WT membrane vesicles did not exhibit specific binding of [3H]vinblastine (data not shown).

The major [3H]azidopine photolabeled band in membrane vesicles from MCF-7 DOXR cells corresponded with P-gp and was absent from MCF-7 WT membrane vesicles (Fig. 7). Densitometry revealed greater than 50% inhibition of photolabel-
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In the presence of 10 μM nicardipine and 50 μM verapamil, which corresponded to 20- and 100-fold molar excess over [3H]azidopine, respectively. In contrast, treatment of membranes with 50 or 100 μM safingol did not inhibit photoaffinity labeling of P-gp, although it was present in a 100–200-fold molar excess over [3H]azidopine (Fig. 7). In situ [3H]azidopine photoaffinity labeling experiments gave similar results (data not shown).

Effects of Safingol on ATPase Activity of MCF-7 DOX<sup>R</sup> Membrane Vesicles—Most compounds that directly interact with P-gp, including calcium channel blockers and natural product drugs, are known to stimulate a vanadate-sensitive ATPase attributable to P-gp and associated with drug transport (46, 47). While both verapamil and vinblastine stimulated a vanadate-sensitive ATPase activity in MCF-7 DOX<sup>R</sup> membrane vesicles, safingol was completely without effect over the same concentration range (Fig. 8). Additional experiments showed that safingol at concentrations between 10 and 100 μM did not alter verapamil or vinblastine stimulated P-gp ATPase activity (data not shown). No drug-stimulated ATPase activity was found in membrane vesicles from MCF-7 WT cells. Thus, three separate lines of evidence indicate that safingol does not directly interact with P-gp, providing further evidence that its effects on drug accumulation were not due to binding to P-gp but were associated with inhibition of PKC.

Effects of Surface Dilution Kinetics of Safingol on Concentration-dependent Cytotoxicity—In MTT assays MCF-7 DOX<sup>R</sup> cells were 600-fold resistant to doxorubicin and up to 2000-fold cross-resistant to other natural product drugs, confirming results of previous clonogenic assays (25). In contrast, safingol equally inhibited growth of MCF-7 WT and MCF-7 DOX<sup>R</sup> cells. In 72-h proliferation assays drug-sensitive MCF-7 WT had an average IC<sub>50</sub> value of 8.1 (± 2.6) μM, whereas MCF-7 DOX<sup>R</sup> cells had an average IC<sub>50</sub> value of 7.1 (± 0.8) μM. These findings raised the question of cytotoxic effects being responsible for effects on drug accumulation and phosphorylation. However, viability assessments suggested cytotoxicity was minimal in drug accumulation and phosphorylation assays as noted above.

The biological activities of amphiphatic compounds such as lysosphingolipid acids are affected by partitioning in the lipid bilayer. In vitro and in cells lysosphingolipids have been reported to exhibit surface dilution kinetics. The in vitro potency of sphingosine as a PKC inhibitor has been shown to be primarily determined by its surface concentration (i.e. its molar ratio to other lipid assay components) (48). For example, increasing the concentration of lipids in mixed micelles has been reported to decrease the in vitro PKC inhibitory activity of sphingosine (48). It has been determined that in cellular systems the ratio of the total mass of lysosphingolipid present in solution to cell number provides an estimate of effective lysosphingolipid concentration in cell membranes (38, 48). The total safingol mass was calculated by multiplying the concentration of safingol by the assay volume, and the effective concentration of safingol was calculated from a formula describing surface dilution kinetics of safingol: effective [safingol] = safingol mass (mol/cell number or [safingol] × assay volume/cell number. The ratio of safingol to cells in the 72-h MTT proliferation assays was approximately 16-fold greater than in drug accumulation assays due to decreased cell number and an increase in the ratio of assay volume to cell number in MTT proliferation versus drug accumulation assays. An equivalent ratio of safingol to cells (80–100 fmol safingol/cell) was present at 50 μM safingol in drug accumulation assays and at 3 μM in MTT proliferation assays, respectively. On the basis of these calculations one would predict that surface dilution kinetics of safingol could account for these differences in cytotoxicity. A series of surface dilution experiments were performed to evaluate this hypothesis.

The effects of varying safingol mass by decreasing cell number or assay volume on toxicity of safingol were evaluated in 72-h proliferation assays (Fig. 9A). Using the same stock solutions of safingol, increasing the ratio of safingol to cells by plating half the number of cells in the assay (3000 cells/well) was found to shift the curve to the left and increase the toxicity approximately 2-fold. Conversely, decreasing safingol mass by reducing the volume of the assay 50%, from 200 to 100 μl, was found to decrease toxicity of safingol, shift the curve to the right, and approximately double the IC<sub>50</sub> value of safingol. A comparison of MTT absorbance indicated that volume differences did not alter cell growth. When the number of cells plated was reduced by half an approximately 50% reduction in absorbance was obtained. The cytotoxicity curves in 72-h MTT assays are identical when effective concentrations of safingol (calculated from the ratio of safingol mass to cell number) are plotted (Fig. 9B). An equivalent IC<sub>50</sub> value of approximately 200 fmol of safingol/cell was found for MCF-7 DOX<sup>R</sup> cells cultured under the varied assay conditions described above. These results illustrate the validity of the surface dilution kinetic model for safingol. Additional 24-h MTT proliferation experiments verified that 50 μM safingol was a nontoxic concentration under conditions when cell density and volume were standardized to mimic the higher cell density and decreased media volume/cell ratio present in drug accumulation assays (data not shown). The variation of assay volume in 24-h exposures did not alter the final cell number. These results suggested that surface dilution kinetics were responsible for the differences in the molar concentrations of safingol that produced cytotoxicity in drug accumulation and MTT assays.

Effects of Safingol on Toxicity of Antineoplastic Drugs—The ability of safingol to sensitize cells to other drugs was evaluated at concentrations that did not appreciably inhibit growth in cells treated with safingol alone. Verapamil at 5 μM was used as a positive control. The drug vehicle alone (ethanol; 0.05% final concentration) did not inhibit cell growth, and cells treated with 5 μM safingol or verapamil alone showed 97–102% of the control growth, respectively. At concentrations of 4 and 5 μM, safingol significantly enhanced the toxicity of doxorubicin; however, no changes in toxicity were evident at lower concentrations, and only marginal activity was observed at 3 μM (not
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An assessment of multiple criteria was used to demonstrate that inhibition of PKC-mediated phosphorylation of P-gp by safingol was associated with modulation of the drug accumulation defect of MCF-7 DOXR cells, a hallmark of the MDR phenotype.

Inhibition of MARCKS phosphorylation in MCF-7 WT and MCF-7 DOXR cells by safingol demonstrated inhibition of PKC in these cells. This prominent cellular substrate of protein kinase C is known to be rapidly phosphorylated subsequent to PKC activation by growth factor stimulation in fibroblasts (50), by ligands which stimulate phagocytic inflammatory cells (51, 52) and by neurosecretory stimuli (53). Previously, phosphorylation of MARCKS was shown to correlate with enhanced expression of P-gp in MCF-7 DOXR cells (25) and with overproduction of P-gp. PKC-β1 in the rat embryo fibroblast cell line R5-PKC3 (54). Although safingol treatment of MCF-7 WT and MCF-7 DOXR decreased MARCKS phosphorylation in both cell lines, safingol treatment enhanced drug accumulation only in MDR MCF-7 cells. Furthermore, the enhanced accumulation of vinblastine and decreased phosphorylation of P-gp and MARCKS in cells treated with safingol occurred with similar concentration dependence. These findings suggest that safingol inhibition of PKC-mediated P-gp phosphorylation is associated with inhibition of P-gp function.

Safingol treatment was also shown to inhibit phosphorylation of P-gp in MDR MCF-7 cells stimulated by PDBu, suggesting that safingol inhibited PKC-mediated phosphorylation of P-gp. Partial V8 protease digestion and analysis of P-gp phosphopeptides showed that PDBu stimulated phosphorylation of three major preexisting phosphorylated sites and that safingol inhibited phosphorylation of these three sites equally. These results support the notion that PKC is the major kinase that phosphorylates P-gp in MCF-7 DOXR cells under basal conditions and that safingol inhibits P-gp phosphorylation at specific PKC phosphorylation sites. These phosphopeptides may represent individual sites of phosphorylation or overlapping pep-
TABLE I

Potentiation of antineoplastic drug toxicity in MCF-7 DOX<sup>R</sup> cells

<table>
<thead>
<tr>
<th>MCF-7 DOX&lt;sup&gt;R&lt;/sup&gt;</th>
<th>Safingol (5 μM)</th>
<th>Verapamil (5 μM)</th>
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<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Reversal</td>
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<tr>
<td>Doxorubicin</td>
<td>33.7 ± 3.2</td>
<td>5.2 ± 1.1</td>
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<tr>
<td>Daunorubicin</td>
<td>8.1 ± 1.4</td>
<td>1.6 ± 1.2</td>
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<tr>
<td>Vinblastine</td>
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<td>Vinblastine</td>
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Effects of safingol on P-gp phosphorylation under MTT assay conditions. To mimic the conditions of the MTT assays, 2.8 x 10<sup>6</sup> MCF-7 DOX<sup>R</sup> cells were plated in 148-cm<sup>2</sup> culture dishes and exposed to the indicated concentrations of drugs for 24, 48, or 72 h in a final volume of 92.5 ml. Membranes were prepared from three to six plates of monolayers containing approximately 3-4 x 10<sup>7</sup> cells. 10 μg of membrane protein was resolved by SDS-PAGE, and detection of P-gp was performed as described under “Experimental Procedures” using C219 antibody and enhanced chemiluminescence. Data shown are representative of three experiments.

In MCF-7 DOX<sup>R</sup> cells, reciprocal changes in calcium-dependent and calcium-independent PKC activity have been described (25). Decreased expression of PKC δ and ε isoforms and calcium-independent PKC activity were found in MCF-7 DOX<sup>R</sup> cells relative to MCF-7 WT cells (25). In contrast, increased expression of PKC α isoform in MDR MCF-7 cells was associated with increased calcium-dependent PKC activity and enhanced phosphorylation of MARCKS and P-gp (25). A role for the PKC α isoform as a positive regulator of P-gp function in MCF-7 DOX<sup>R</sup> cells has also been suggested by transfection studies with antisense cDNA for PKC α. Expression of PKC α antisense cDNA decreased PKC activity, P-gp phosphorylation, and P-gp function (55). Additionally, transfection of MCF-7 cells expressing mdr1 with PKC α has been shown to increase resistance to natural product drugs in association with reduced accumulation and enhanced phosphorylation of P-gp (16). Safingol was found to inhibit human PKC α activity at approximately the same concentration as it inhibited PDBu binding and enzymatic activity of rat brain PKC.

Stimulation of PKC in MCF-7 DOX<sup>R</sup> cells by phorbol dibutyrate partially reversed increased drug accumulation in cells treated with safingol. The ability to reverse lysosphingolipid-mediated changes using phorbol esters or diacylglycerol has...
previously been used to discern PKC-dependent and PKC-independent activities of α-erythro-sphingosine in platelets (49), in quiescent Swiss 3T3 cells (56), and in rat adipocytes (57). The partial reversal of safingol-mediated increases in drug accumulation in response to activation of PKC by PDBu suggests that these increases are PKC-dependent.

Other PKC inhibitors including staurosporine (29), calphostin C (26), and NA-382, a staurosporine derivative (30), have been reported to inhibit [3H]azidopine photolabeling of P-gp. For these compounds it is not clear if inhibition of P-gp function is a result of PKC inhibition, inhibition of drug binding, or inhibition of both activities. In contrast, safingol did not inhibit [3H]vinblastine binding to P-gp or [3H]azidopine photoaffinity labeling of P-gp and did not alter P-gp ATPase activity in MCF-7 DOXR membrane vesicles. These findings suggest that safingol does not directly interact with these P-gp drug binding sites as a means of enhancing vinblastine accumulation. In view of the possible dual mechanisms in the previously studied PKC inhibitors above, this finding is novel.

Currently, the number and characteristics of drug binding sites on P-gp are not well defined. Structure-activity studies of thioxanthenes (58), and reserpine analogs (59) have described a general pharmacophore for modulator binding to P-gp, which contains planar aromatic domains and a basic nitrogen side chain. Lysosphingolipids lack aromatic rings but have a positively charged nitrogen at physiologic pH, which is a characteristic of most (but not all) inhibitors of multidrug resistance (2). The hypothesis that lysosphingolipids do not directly interact with P-gp is consistent with studies using the calcium channel blocker SR33557. This MDR reversal agent did not inhibit P-gp drug binding but increased levels of sphingosine in MDR leukemia cells, leading to inhibition of cellular PKC activity (31).

Although less potent than verapamil, safingol at nontoxic concentrations was shown to partially sensitize MCF-7 DOXR cells to natural product drugs (anthracyclines and Vinca alkaloids) but not to the antimitabolite 5-FU. Results of phosphorylation studies, Western blots, and Northern blots indicated that safingol treatment decreased P-gp phosphorylation rather than stability of P-gp or message for P-gp. Growth, viability, and metabolic assessments indicated that inhibition of P-gp phosphorylation and modulation of the MDR phenotype by safingol was not associated with general toxicity. The ability of safingol to inhibit P-gp phosphorylation and drug resistance without significant growth inhibition may reflect a transitory inhibition of PKC due to metabolism of safingol into more complex sphingolipids. Thus, the moderate relative efficiency of safingol as an MDR inhibitor could result from transitory inhibition of PKC and P-gp function due to safingol metabolism. Alternatively, P-gp phosphorylation may have only a limited role in modulating drug resistance in these cells. Verapamil, which inhibits drug binding to P-gp, also only partially inhibited the MDR phenotype of MCF-7 DOXR cells, perhaps because the drug-resistant phenotype of MCF-7 DOXR cells may be multifactorial.

Overexpression of glutathione detoxification enzymes (61) and increased activity of other drug-metabolizing enzymes in this cell line have been reported (62). Safingol inhibition of PKC may alter activities of other phosphorylated drug resistance-associated enzymes such as topoisomerase II, which is known to be phosphorylated by PKC in vitro (63). Hyperphosphorylation of topoisomerase II has been associated with decreased sensitivity to topoisomerase II inhibitors in human etoposide-resistant KB cancer cells (64). However, to the best of our knowledge there are no reports correlating altered phosphorylation of topoisomerase II with activity changes in the MCF-7 DOXR cell line. The reported absence of detectable message for the multidrug resistance-associated protein, MRP in MCF-7 DOXR cells (66), suggests that this member of the ABC transport protein family, which shares homology with P-gp (65), is unlikely to contribute to the MDR phenotype of these cells. Although non-P-gp-mediated mechanisms may contribute to resistance to a given drug in MCF-7 DOXR cells, P-gp is generally thought to be the major mechanism of MDR in this cell line. Although activities of other phosphorylated drug resistance proteins unrelated to P-gp may be altered by safingol inhibition of PKC, the pattern of partial inhibition of natural product drug toxicity versus unaltered 5-FU toxicity suggests an association of decreased phosphorylation of P-gp and specificity for the MDR phenotype.

A marked difference in the molar concentrations of safingol required to effectively enhance drug accumulation and inhibit resistance was noted. The data indicate that surface dilution of safingol probably accounts for the differences in concentration dependence between drug accumulation and MTT assays. This premise is supported by the finding that when assay conditions were standardized inhibition of P-gp phosphorylation was associated with both partial inhibition of drug resistance and enhanced drug accumulation. Surface dilution kinetics of sphingosine and other long chain sphingolipid bases has been previously observed both in vitro and in situ (32–34, 39, 49).

Calmodulin antagonists, cyclosporins, hormonal analogs, calcium channel blockers, and antiarrhythmic drugs can all modulate P-gp activity by directly binding to the P-gp. The evidence presented here, however, indicates that safingol may modulate P-gp activity by a different mechanism than these previously described agents, namely via inhibition of PKC-mediated phosphorylation of P-gp.

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Partial Inhibition of Multidrug Resistance by Safingol Is Independent of Modulation of P-glycoprotein Substrate Activities and Correlated with Inhibition of Protein Kinase C

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