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Effects of Sphingosine Stereoisomers on P-Glycoprotein Phosphorylation and Vinblastine Accumulation in Multidrug-Resistant MCF-7 Cells

Clifford W. Sachs, Larry M. Ballas,† S. Wayne Mascarella,‡ Ahmad R. Safa,§ Anita H. Lewin,‡ Carson Loomis,† F. Ivy Carroll,‡ Robert M. Bell‖ and Robert L. Fine*¶*

*Division of Hematology/Oncology, Department of Medicine, Duke University and Durham VA Medical Center, Durham, NC 27705; †Sphinx Pharmaceuticals, Durham NC 27710; ‡Research Triangle Institute, Research Triangle Park, NC 27709; §Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL 60637; and ‖Department of Molecular Cancer Biology, Duke University Medical Center, Durham, NC 27710, U.S.A.

ABSTRACT. To investigate the role of protein kinase C (PKC) in the regulation of multidrug resistance and P-glycoprotein (P-gp) phosphorylation, the natural isomer of sphingosine (SPH), D-*erythro* sphingosine (De SPH), and its three unnatural stereoisomers were synthesized. The SPH isomers showed similar potencies as inhibitors of *in vitro* PKC activity and phorbol binding, with IC_{50} values of approximately 50 μM in both assays. Treatment of multidrug-resistant MCF-7^{ADR} cells with SPH stereoisomers increased vinblastine (VLB) accumulation up to 6-fold at 50 μM but did not alter VLB accumulation in drug-sensitive MCF-7 wild-type (WT) cells or accumulation of 5-fluorouracil in either cell line. Phorbol dibutyrate treatment of MCF-7^{ADR} cells increased phosphorylation of P-gp, and this increase was inhibited by prior treatment with SPH stereoisomers. Treatment of MCF-7^{ADR} cells with SPH stereoisomers decreased basal phosphorylation of the P-gp, suggesting inhibition of PKC-mediated phosphorylation of P-gp. Most drugs that are known to reverse multidrug resistance, including several PKC inhibitors, have been shown to directly interact with P-gp and inhibit drug binding. SPH stereoisomers did not inhibit specific binding of [^{3}H] VLB to MCF-7^{ADR} cell membranes or [^{3}H]azidopine photoaffinity labeling of P-gp or alter P-gp ATPase activity. These results suggest that SPH isomers are not substrates of P-gp and suggest that modulation of VLB accumulation by SPH stereoisomers is associated with inhibition of PKC-mediated phosphorylation of P-gp. BIOCHEM PHARMACOL 52;4:603–612, 1996.

KEY WORDS. P-glycoprotein; sphingosine; multidrug resistance; protein kinase C

The MDR** phenotype arises in cancer cells exposed to cytotoxic drugs derived from natural products. Resistance to the selecting drug and cross-resistance to other natural product drugs, including useful antineoplastic drugs such as *Vinca* alkaloids, antibiotics, anthracyclines, taxanes, and epipodophyllotoxins, are closely associated with the reduced intracellular accumulation of these drugs, a hallmark of the MDR phenotype, which occurs secondary to energy-dependent drug efflux. P-gp is considered the major mediator of multidrug resistance, and is thought to bind drugs and pump them out of the cell (reviewed in Refs. 1 and 2).

Numerous diverse agents have been shown to increase drug accumulation, and inhibit multidrug resistance [3] by competing with anticancer drugs for binding and transport by P-gp. This mechanism for modulation of drug accumulation and resistance has been demonstrated by inhibition of specific and saturable binding of radioactive analogs of *Vinca* alkaloids [4, 5], and certain calcium channel blockers [6, 7], or by inhibition of photoaffinity labeling of P-gp with photoactive analogs of VLB [4, 8] and [^{3}H]azidopine [9, 10]. Recently, a novel calcium channel blocker that is inherently photoactive was shown to potently inhibit multidrug resistance in the murine MDR P388/ADR cell line. This compound did not photolabel the P-gp and did not compete for [^{3}H]azidopine photoaffinity labeling sites but was shown to increase levels of SPH [11], an inhibitor of PKC [12].

PKC activity is increased markedly in many MDR cell lines relative to their drug-sensitive counterparts [13–17] and in some MDR cell lines can be attributed to selective

¶ Corresponding author: Robert L. Fine, M.D., Division of Medical Oncology, Columbia University College of Physicians and Surgeons, Black Bldg., Room 20-05, 630 W. 168th St., New York, NY 10032. Tel. (212) 305-1168; FAX (212) 305-7348.

** *Abbreviations:* MDR, multidrug-resistant; IMDM, Iscove's modified Dulbecco's medium; PDBu, phorbol 12,13-dibutyrate; P-gp, P-glycoprotein; PKC, protein kinase C; SPH, sphingosine; De SPH, D-*erythro* sphingosine; Le SPH, L-*erythro* sphingosine; Dt SPH, D-*threo* sphingosine; Lt SPH, L-*threo* sphingosine; N-AcSPH, *N*-acetyl sphingosine; VRP, verapamil; VLB, vinblastine; and 5-FU, 5 fluorouracil.

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changes in the expression of PKC isoenzymes [18–20]. For example in the MCF-7^{ADR} human breast cancer cell line used in these studies, there are reciprocal changes in calcium-dependent and calcium-independent PKC activity, relative to the drug-sensitive, parental cell line MCF-7 WT [20]. Increased calcium-dependent PKC activity in MCF-7^{ADR} cells is associated with increased expression of PKC α. In contrast, decreased expression of PKC δ and ε relative to MCF-7 WT leads to decreased calcium-independent PKC activity in MCF-7^{ADR} cells [20].

Treatment of MDR cells with PKC activators such as phorbol esters or cell permeant diacylglycerol increases phosphorylation of the P-gp [20–23], and in many MDR cell lines, including MCF-7^{ADR}, decreases drug accumulation and increases resistance [14, 18, 24]. Conversely, treatment of MDR cells with inhibitors of PKC decreases drug transport activity and phosphorylation of P-gp [22, 25]. However, several of the PKC inhibitors used to inhibit multidrug resistance, including calphostin C and staurosporine, also inhibit drug binding [26–28] to P-gp, suggesting that their modulation of the MDR phenotype may involve competition for drug binding in addition to or independent of inhibition of P-gp phosphorylation.

PKC inhibitory activity of SPH results from its interaction with the regulatory domain of PKC, where diacylglycerol and phorbol esters bind [12]. The natural isomer of sphingosine, De SPH, is found in ceramides, sphingomyelins, and glycosphingolipids of cell membranes. It can be synthesized *de novo* or generated by the action of ceramidases (reviewed in Refs. 29 and 30). SPH contains two chiral centers, C2 and C3, and has three unnatural stereoisomers. De SPH is known to interact with a number of cell signaling systems independent of PKC inhibition [31–35]; therefore, it was of interest to investigate the biological activity of the SPH stereoisomers.

This research addressed the following questions. Does SPH, a possible lipid second messenger and inhibitor of PKC, inhibit P-gp phosphorylation and function without binding to the putative binding sites on P-gp for azidopine or VLB? Are there stereospecific differences between the SPH isomers in their ability to inhibit P-gp phosphorylation or drug accumulation?

MATERIALS AND METHODS

Materials

Dioleoylglycerol and phosphatidylserine were from Avanti (Birmingham, AL). Tissue culture media, balanced salt solutions and prestained high molecular weight standards were purchased from GIBCO-BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). Tissue culture plates and flasks were purchased from Costar (Cambridge, MA). [^{3}H]VLB (10 Ci/mmol) and [^{3}H]5-FU (21 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]Azidopine (52 Ci/mmol) and western blot reagents were from Amersham (Arlington Heights, IL). [^{32}P]Orthophosphate, [γ-^{32}P]ATP and [^{3}H]PDBu were obtained from Du Pont-NEN (Boston, MA). Protein A-Sepharose 4B was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). C219 monoclonal antibody was from Centecor (Malvern, PA). Normal rabbit IgG was purchased from Vector Laboratories (Burlingame, CA). Electrophoresis reagents, molecular weight standards, protein assay dye solution, and standards were purchased from Bio-Rad (Richmond, CA). Histone type IIIs, protease inhibitors, fatty acid free BSA, and V8 protease were acquired from the Sigma Chemical Co. (St. Louis, MO). HAWP filters (0.45 μm pore size) were obtained from the Millipore Corp. (Bedford, MA). All other materials were reagent grade and were purchased from commercial sources.

Synthesis of SPH Stereoisomers

The SPH isomers were prepared by modification of previously described methods [36–38]. The synthetic material was purified by flash chromatography, dried *in vacuo* at 40° for 8 hr, and stored in a freezer under argon. The chemical identity and purity of the stereoisomers were verified by comparison of the respective 250 MHz proton NMR spectra and demonstrated to be greater than 99% pure by TLC, and by HPLC analysis of the *o*-phthalaldehyde derivatives [32].

PKC Activity and Phorbol Binding Assays

Rat brain PKC was purified as previously published [39]. PKC activity was assayed by quantitating the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type IIIs. The reaction mixture contained, in a total volume of 250 μL, 30 μg phosphatidylserine, 0.5 μg dioleoylglycerol, 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 47.5 μM EGTA, 100 μM $CaCl_2$, 10 μL DMSO or compound dissolved in DMSO, 30 μM [γ-^{32}P]ATP, and PKC enzyme. The assays were run for 10 min at 30°. Phorbol binding assays were performed as previously described [12].

Cell Culture

Drug-sensitive, MCF-7 WT, and MCF-7^{ADR} cells were provided by Dr. Ken Cowan (National Cancer Institute). The cell lines were grown in IMDM supplemented with 10% heat-inactivated fetal bovine serum. The MCF-7^{ADR} cell line was exposed to 10 μM doxorubicin, and experiments were performed on cells that had been cultured drug free for at least 2 weeks.

Drug Accumulation Assays

Drug accumulation assays were performed as previously described [20] with the following modifications. SPH stereoisomers were added to the cells as a 1:1 complex with fatty acid free BSA to minimize the cytotoxicity of SPH [40]. Stock solutions of SPH stereoisomers, VRP, N-AcSPH, and a vehicle control were prepared in IMDM containing 1 mM BSA at a concentration of 1 mM. Cells (approximately 7 ×

10^5/well) were preincubated for 30 min in 1 mL of IMDM containing 100 μM fatty acid free BSA and drugs before adding either 100 nM [^{3}H]VLB or 100 nM [^{3}H]5-FU diluted to a final specific activity of 4 Ci/mmol. Net accumulation of drugs was measured at 2 hr after washing and lysing the cells with 0.1% Triton X-100. Samples were assayed in triplicate, and the data are expressed as picomoles [^{3}H]-labeled drug/mg protein. In parallel studies, using unlabeled drugs, cells exposed to SPH stereoisomers were counted in a hemacytometer, and viability was determined to be greater than 95% by exclusion of trypan blue.

Intact Cell Phosphorylation Studies and Immunoprecipitation of P-gp

These studies were performed under culture conditions similar to those of the drug accumulation assays, using BSA to deliver the SPH isomers. MCF-7^{ADR} cells were incubated in 1 mL of phosphate-free RPMI-1640 buffered with 10 mM HEPES (pH 7.3) containing 0.2 mCi [^{32}P]orthophosphate for 2 hr before the addition of drugs and a further incubation for 2 hr at 37°. P-gp immunoprecipitated from unlabeled cells treated with drugs under similar conditions was quantitated by western blotting as previously described [41]. In some experiments, cells were washed to remove unincorporated label before adding 1 mL of medium containing 50 μM SPH isomers or vehicle (100 μM BSA, 0.2% ethanol) to the wells at timed intervals. After 90 sec, 100 nM PDBu or vehicle (0.01% DMSO) was added to the cells which were then incubated for 10 min. Incubations were terminated by lysing cells in detergent. Aliquots normalized by trichloroacetic acid-precipitated radioactivity were immunoprecipitated using C-219 monoclonal antibody to P-gp and resolved by SDS–PAGE using methods that have been published previously [20]. P-gp bands were identified by autoradiography and excised, and radioactivity was determined by Cerenkov counting.

Preparation of Membrane Vesicles and VLB Competition Binding Assays

Membrane vesicles from sensitive WT and MCF-7^{ADR} cells were prepared by nitrogen cavitation and centrifugation on discontinuous sucrose gradients as previously described [6]. Binding of [^{3}H]VLB to membranes containing P-gp was measured in a rapid filtration assay according to published methods [41]. Non-specific binding of [^{3}H]VLB was measured in the presence of 100 μM unlabeled VLB and was subtracted from the total binding to calculate specific binding.

[^{3}H]Azidopine Photoaffinity Labeling of P-gp in MDR Cells and Membranes

Membranes (100 μg/assay) or cells (5×10^5/assay) were incubated with drugs or vehicle DMSO for 30 min before adding 0.5 μM [^{3}H]azidopine and incubating for an additional 20 min. The samples were photolabeled by UV irradiation and analyzed by SDS–PAGE as previously described [9].

P-gp ATPase Activity

ATPase activity of cell membranes prepared from MCF-7 cells was measured by determining vanadate-sensitive liberation of inorganic phosphate from ATP, which was attributable to P-gp as described [42, 43].

Statistical Analysis

Student's *t*-test and ANOVA were performed on a Macintosh computer using commercially available software.

RESULTS

Effects of SPH Stereoisomers on Activity and [^{3}H]PDBu Binding of Purified Rat Brain PKC In Vitro

Figure 1 shows Fischer projections of the SPH stereoisomers. The data in Table 1 show that the SPH stereoisomers had similar *in vitro* PKC inhibitory activity. Additionally, the IC_{50} values for De SPH, Dt SPH, and Le SPH in the vesicular PKC activity assay were comparable to the IC_{50} values for displacement of phorbol, approximately 50 μM, consistent with the notion that SPH stereoisomers inhibit PKC activity through effects on the regulatory site of PKC where phorbol esters bind.

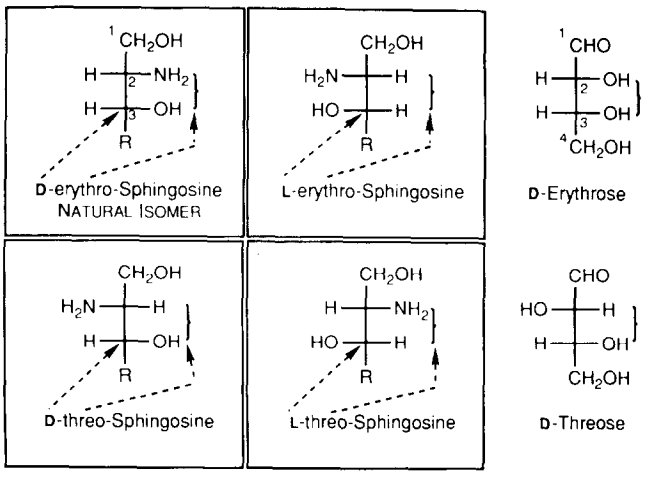

FIG. 1. Fisher projections of SPH stereoisomers. The assignment of the relative (*erythro, threo*) and absolute (D, L) stereochemical designation of the SPH stereoisomers can be derived from these figures. When both OH and NH_2 functional groups are on the same side of the carbon backbone, the isomers are described as *erythro* isomers by analogy to the sugar erythrose. Similarly, when these functional groups are on opposite sides of the backbone, the isomers are described as *threo* isomers by analogy to the sugar threose. The D and L designations are determined by the position of the OH functional group on the highest numbered carbon atom: when it is on the right of the backbone, the isomer is designated D; when it is on the left, the isomer is designated L. The SPH stereoisomers are comprised of two pairs of diastereomers (mirror images): D- and L-*erythro* SPH and D- and L-*threo* SPH.

TABLE 1. Effects of SPH stereoisomers on *in vitro* activity of partially purified rat brain PKC and specific binding of [^{3}H]PDBu

	PKC activity IC_{50} (μM)	Phorbol binding IC_{50} (μM)
De SPH	45	50
Dt SPH	50	50
Le SPH	45	50
Lt SPH	40	ND*

PKC activity and phorbol binding were assayed as described under Materials and Methods in the presence of graded concentrations of SPH stereoisomers. The concentrations that inhibited activity or binding by 50% were estimated by probit analysis. Data shown are the averages of two determinations.

* Not determined.

Effects of VRP, SPH Isomers, and N-AcSPH on Drug Accumulation

Figure 2A shows that basal accumulation of VLB at 2 hr was 4.6 (±0.7) pmol/mg protein in the MCF-7^{ADR} cell line. The SPH isomers did not alter drug accumulation at concentrations below 25 μM. At 25 μM the SPH isomers increased drug accumulation up to 2-fold, and at 50 μM they increased drug accumulation approximately 4- to 5-fold. The differences in VLB accumulation between the vehicle-treated group and groups treated with these active concentrations of the SPH isomers were highly significant as determined by the paired Student's *t*-test ($P \leq 0.005$); however, no significant differences in activity between the isomers were found. Thus, the SPH isomers had similar potencies and activities as modulators of VLB accumulation in this MDR cell line. The negative control, N-AcSPH, which does not inhibit PKC activity or phorbol binding *in*

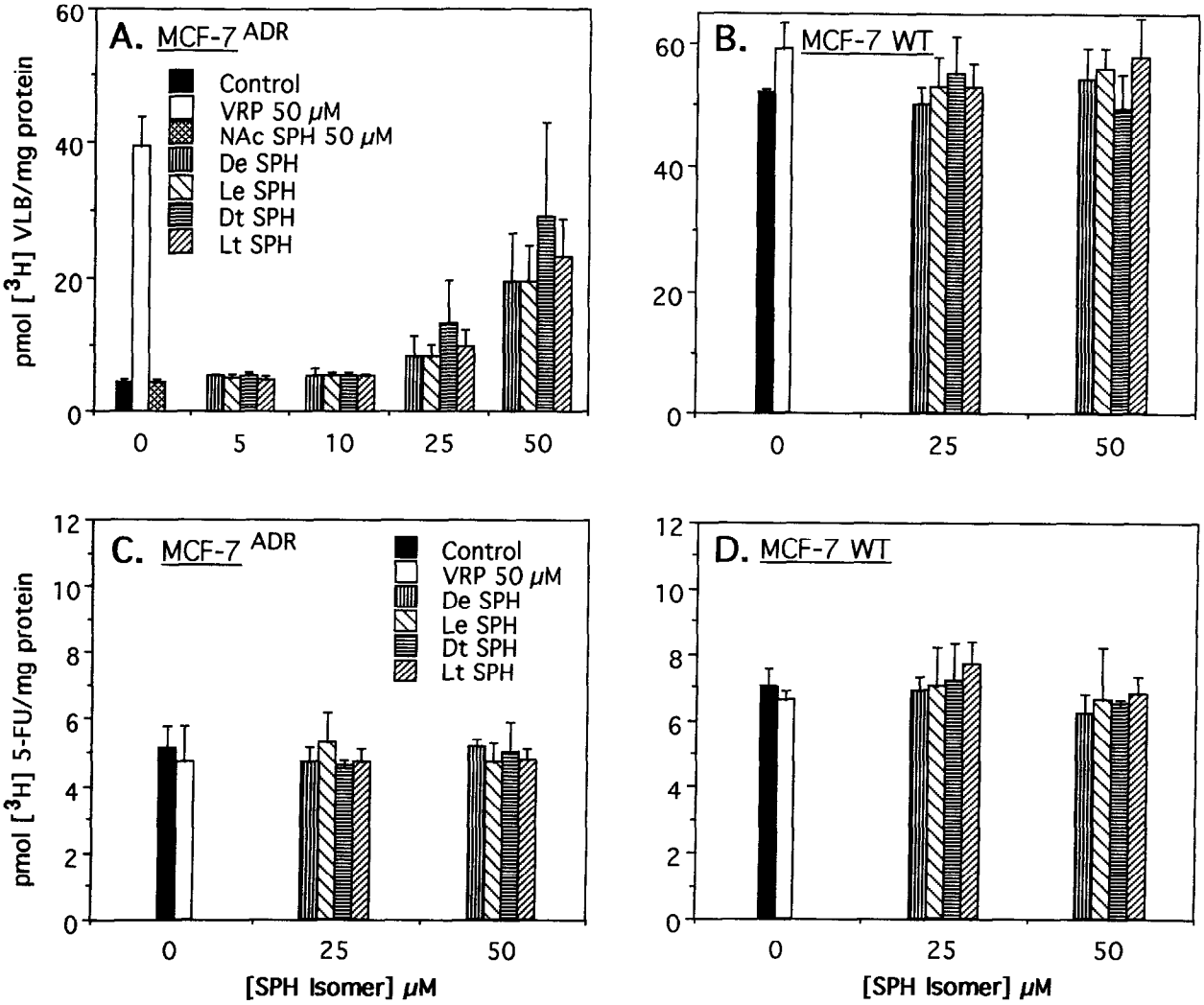

FIG. 2. Effects of VRP and SPH isomers on drug accumulation. MCF-7 cells were incubated with a vehicle control (50 μM BSA, 0.01% ethanol), 50 μM VRP, 50 μM N-AcSPH, De SPH, Le SPH, Dt SPH, or Lt SPH, at the indicated concentrations before adding either [^{3}H]VLB (A and B) or [^{3}H]5-FU (C and D) to a final concentration of 100 nM. Net accumulation of drugs was measured at 2 hr and is expressed as pmol/mg protein. Data shown are the means ± SD of two experiments performed in triplicate.

vitro, had no significant effect on drug accumulation. VRP showed much greater activity than the SPH isomers; it increased drug accumulation 8.6-fold at 50 μM. Trypan blue exclusion of cells was not affected by these drugs at these concentrations, indicating that modulation of drug accumulation was not associated with alterations of membrane integrity or cell viability. When compared to VLB accumulation by drug-sensitive MCF-7 WT cells (Fig. 2B), it is readily apparent that neither VRP nor the SPH isomers were able to reverse completely the drug accumulation defect, which was measured as an approximately 10- to 12-fold difference in VLB accumulation compared with MCF-7^{ADR} cells. A comparison of 5-FU accumulation in MDR and MCF-7 WT cells (Fig. 2, C and D) indicated no apparent drug accumulation defect. Treatment with VRP or SPH isomers did not alter 5-FU accumulation in either cell line. This result is consistent with previous observations that MDR cells are generally not cross-resistant to antimetabolites such as 5-FU and demonstrates a specificity of changes in drug accumulation to substrates of P-gp.

Effects of PDBu, SPH Isomers, VLB, and VRP on Phosphorylation of P-gp Immunoprecipitated from Intact MCF-7^{ADR} Cells

Figure 3 shows the concentration dependence (left panel) and time–course (right panel) of PDBu stimulation of P-gp phosphorylation in MCF-7^{ADR} cells. These results indicate that P-gp is rapidly and maximally phosphorylated subsequent to PKC activation by phorbol ester and that this response is elicited by nanomolar amounts of PDBu. Since PDBu is known to activate PKC, the observed enhancement of P-gp phosphorylation with PDBu stimulation was probably mediated by PKC.

Figure 4 shows that the SPH isomers at a concentration of 50 μM inhibited PDBu-stimulated phosphorylation of P-gp, consistent with the notion that SPH stereoisomers inhibit PKC-mediated P-gp phosphorylation. During this short-term incubation with SPH isomers, phosphorylation of P-gp was not changed appreciably. This latter result is consistent with preliminary time–course studies with De SPH which showed significant decreases in P-gp phosphorylation beginning at 30 min (data not shown). The results of these experiments suggest that SPH isomers inhibit activation of PKC by PDBu and subsequent phosphorylation of P-gp.

Figure 5 shows the concentration dependence of inhibition of basal P-gp phosphorylation by SPH isomers. Decreased P-gp phosphorylation was observed in cells treated with SPH isomers for 2 hr at a final concentration of 50 μM, a concentration that elicited maximal enhancement of VLB accumulation without general toxicity. Densitometry revealed only marginal decreases in P-gp phosphorylation (up to 30%) in cells treated with SPH stereoisomers at 25 μM and up to 70% inhibition of P-gp phosphorylation in cells treated with SPH isomers at 50 μM. This result suggests that PKC phosphorylates P-gp in these cells under basal conditions. Figure 5 also shows that neither VLB nor VRP treatment significantly altered P-gp phosphorylation and that by 2 hr the enhancement of P-gp phosphorylation by PDBu was attenuated noticeably.

In some cell lines, expression of P-gp can be modulated by treatment with cytotoxic drugs, PKC activators [44], and PKC inhibitors [45]. In experiments performed under identical conditions, immunoprecipitated P-gp was quantitated by western blotting. Results of these control experiments indicated that P-gp immunoprecipitation yields were unaffected by these treatments, suggesting that changes in P-gp expression did not contribute to phosphorylation changes (data not shown).

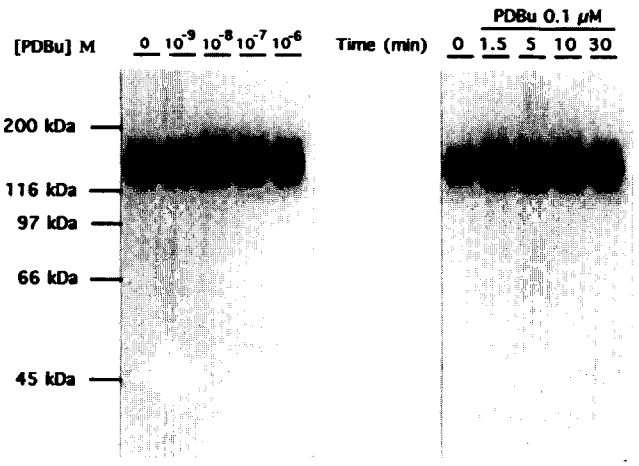

FIG. 3. Effects of PDBu treatment on P-gp phosphorylation in MCF-7^{ADR} cells. P-gp was immunoprecipitated from cells labeled with ^{32}P before adding PDBu to the final concentration indicated and further incubation for 10 min (left panel). Time–course of P-gp phosphorylation changes in response to PDBu treatment between 90 sec and 30 min (right panel). Incubations were terminated by detergent lysis to extract P-gp for immunoprecipitation. Positions of molecular weight standards are shown on the left. Data shown are representative of two experiments.

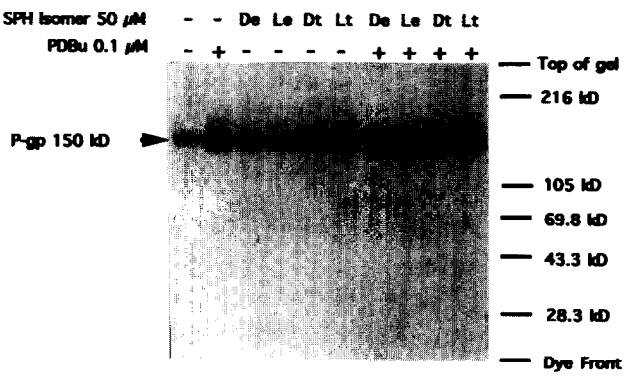

FIG. 4. Effects of SPH isomers on PDBu-stimulated phosphorylation of P-gp. MCF-7^{ADR} cells labeled with [^{32}P]orthophosphate for 4 hr were washed and treated with either vehicle (medium containing 100 μM BSA) or SPH isomers for 90 sec prior to adding PDBu (100 nM) and a further 10-min incubation. Data shown are representative of three experiments.

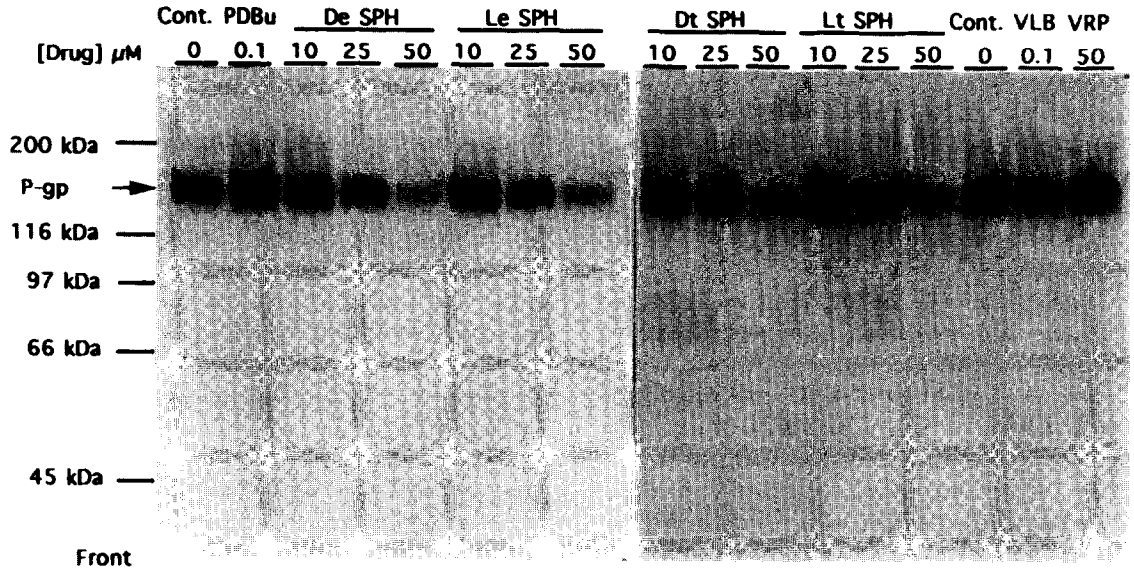

FIG. 5. Effects of PDBu, SPH stereoisomers, VLB, and VRP on basal phosphorylation of P-gp. MCF-7^{ADR} cells were labeled with [^{32}P]orthophosphate as described under Materials and Methods and treated for 2 hr with the indicated agents prior to detergent lysis and immunoprecipitation of P-gp. Data shown are from a single representative experiment run on two gels. Similar results were obtained for these agents on one to three separate occasions.

Effects of SPH Stereoisomers, VLB, and VRP on [^{3}H]Azidopine Photolabeling of P-gp

Figure 6 shows that the major protein labeled by [^{3}H]azidopine in MDR MCF-7^{ADR} cells corresponded to P-gp and was absent from membranes prepared from drug-sensitive cells. VLB most effectively inhibited [^{3}H]azidopine photolabeling of P-gp. Densitometry revealed that at 10 μM VLB, greater than 50% inhibition of photolabeling was obtained. VRP was less effective; only 44% inhibition was obtained at 50 μM as compared with greater than 90% inhibition by 50 μM VLB. In contrast, SPH isomers had no effect on photolabeling even at concentrations of 100 μM, representing a 200-fold molar excess over [^{3}H]azidopine. This result was confirmed in intact MCF-7^{ADR} cells and indicated a lack of interaction by the SPH isomers with the [^{3}H]azidopine binding site on P-gp.

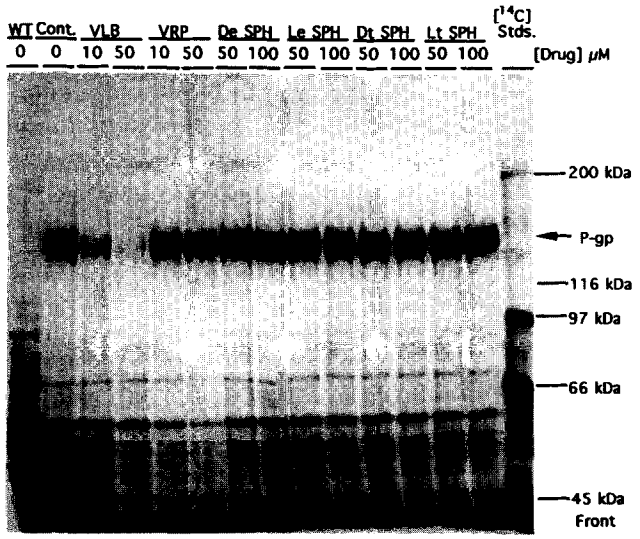

FIG. 6. Effects of SPH isomers on [^{3}H]azidopine photoaffinity labeling of P-gp in membrane vesicles of MCF-7^{ADR} cells. [^{3}H]Azidopine photolabeling of membranes prepared from sensitive (WT) MCF-7 and MCF-7^{ADR} cells was performed on membranes preincubated in the presence of a vehicle control (1% DMSO) or with the indicated concentrations of drugs. The samples were analyzed by SDS–PAGE and then stained with Coomassie Blue. Autofluorography was performed as described in Materials and Methods. Data are representative of three experiments, and similar results were observed in an experiment in which [^{3}H]azidopine photolabeling of intact MCF-7^{ADR} cells was studied.

Differential Inhibition of [^{3}H]VLB Binding by VRP and SPH Isomers

Figure 7 shows that the specific binding of [^{3}H]VLB to membrane vesicles prepared from MCF-7^{ADR} cells was 29 pmol/mg membrane protein in the presence of 100 nM [^{3}H]VLB and 3 mM ATP. This specific binding was displaced by VRP, with an IC_{50} of approximately 5 μM. The discrepancy in potency of VRP in these two different types of drug-binding assays is consistent with the notion that VRP interacts with a VLB-binding site on P-gp, which is thought to be a different site than the azidopine drug-binding site [7]. In contrast, SPH isomers did not alter specific binding of VLB, even at concentrations 20-fold greater than the IC_{50} value for VRP and in a 1000-fold molar excess over [^{3}H]VLB. No VLB specific binding was observed in membranes from drug-sensitive MCF-7 WT cells (data not shown), consistent with the association of specific binding with the presence of P-gp in membranes. The lack of inhibition of specific binding by the SPH ste-

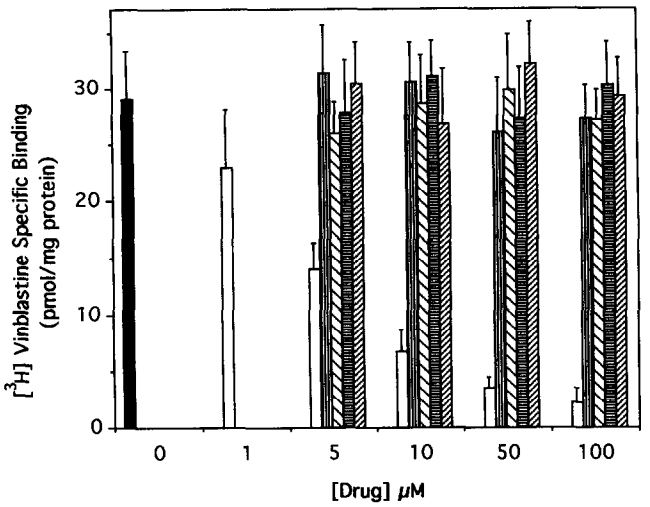

FIG. 7. Effects of SPH isomers and VRP on specific binding of [^{3}H]VLB to membranes of MCF-7^{ADR} cells. Specific binding of VLB to MDR cell membranes incubated with either a vehicle control (■), VRP (□) De SPH (▥), Le SPH (▧), Dt SPH (▤) or Lt SPH (▨) was measured in a rapid filtration assay. Specific binding was calculated from the difference between binding in the absence and presence of a 1000-fold molar excess of unlabeled VLB (concentration 100 μM). Data shown are the means ± SD of a representative experiment performed in triplicate, which was repeated with good agreement.

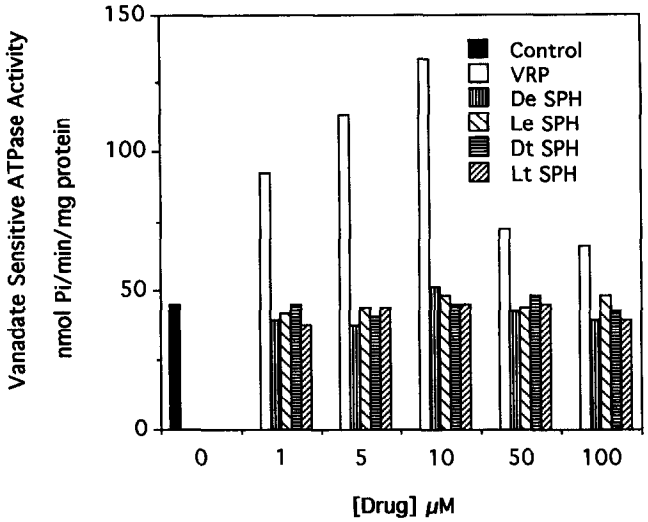

FIG. 8. Effects of VRP and SPH isomers on the ATPase activity of P-gp in MCF-7^{ADR} cell membranes. Ten micrograms of MCF-7^{ADR} cell membrane protein in ATPase assay buffer was treated with a vehicle control (ethanol 1%), or VRP, De SPH, Le SPH, Dt SPH, or Lt SPH diluted 100-fold from ethanol stock solutions to the indicated concentrations. Vanadate-sensitive ATPase activity was measured for 20 min as described under Materials and Methods. Data shown are representative of two experiments performed in duplicate.

reoisomers indicates that they do not interact with the VLB binding site on P-gp as a means of altering VLB accumulation.

Effects of VRP and SPH Isomers on P-gp ATPase Activity

Compounds that are substrates of P-gp stimulate a vanadate-sensitive ATPase attributable to P-gp and associated with drug transport. Figure 8 shows that 5–10 μM VRP approximately doubled vanadate-sensitive ATPase activity in MCF-7^{ADR} membranes. In contrast, the SPH stereoisomers did not alter P-gp ATPase activity. No drug-stimulated ATPase activity was found in membrane vesicles from MCF-7 WT cells. This result confirms that the SPH stereoisomers do not exhibit properties of P-gp substrates that alter P-gp ATPase activity.

DISCUSSION

In this report, chemically synthesized SPH stereoisomers were shown to inhibit phorbol binding, PKC activity *in vitro*, and P-gp phosphorylation and, in MCF-7^{ADR} cells, to enhance VLB accumulation with similar potency.

The SPH isomers inhibited phosphorylation of P-gp in cells in which PKC was stimulated by PDBu, demonstrating inhibitory effects of SPH isomers on activation of PKC. Phosphorylation of P-gp was not inhibited by SPH isomers in the short incubations used in these experiments; however, inhibition of basal phosphorylation in longer 2-hr exposures to SPH stereoisomers was observed. This decrease in basal phosphorylation of P-gp with longer exposures may reflect a combination of inhibition of PKC-mediated phosphorylation, dephosphorylation of labeled P-gp, and further labeling of P-gp in untreated cells over time. Contributions of these processes to the change in phosphorylation are minimized in short-term incubations. Dephosphorylation of P-gp has been associated with activity of type 1 and 2A phosphatases [46]. The observation that treatment with SPH isomers decreased basal P-gp phosphorylation in MCF-7^{ADR} cells is consistent with the idea that PKC may also be responsible for basal phosphorylation of P-gp in these cells.

The major *in vitro* PKC and PKA phosphorylation sites of both the human P-gp [47] and the murine *mdr*1b P-gp [48] are serine residues found in the linker region of P-gp, which joins the homologous halves of this protein. Previously, increased expression of PKC α isoenzyme in MCF-7^{ADR} cells was associated with increased calcium-dependent PKC activity, and enhanced phosphorylation of MARCKS and P-gp [20]. Transfection of MCF-7 cells expressing *MDR*1 with PKC α was also shown to increase resistance to natural product drugs in association with reduced accumulation and enhanced phosphorylation of P-gp [49], whereas transfection and expression of antisense cDNA for PKC α decreased PKC activity, P-gp phosphorylation, and P-gp function [50]. Recently, drug-binding affinity and ATPase activity of baculovirus expressed P-gp was shown to be modulated by coexpression with PKC α [51]. In this system, mutation of the linker region serine-671 to asparagine was

shown to abolish the enhancement by PKC α of drug-stimulated ATPase activity [51], indicating a critical role for this site in the positive regulation of P-gp function by PKC α [51].

SPH stereoisomers were shown to decrease basal P-gp phosphorylation and enhance VLB accumulation in MCF-7^{ADR} cells with similar concentration dependence. Like most PKC inhibitors, the SPH stereoisomers could only partially reverse the drug accumulation defect of the MDR cell line [15, 22, 25, 52]. Unlike most inhibitors of multidrug resistance, or previously tested PKC inhibitors that modulate drug accumulation, SPH stereoisomers did not inhibit drug binding. PKC inhibitors such as calphostin C, staurosporine, and its analogs have been shown to inhibit phosphorylation of P-gp in MDR cells [15, 25, 27] and to decrease drug efflux [25, 27] and resistance [15, 52]. However, the role of inhibition of PKC-mediated phosphorylation of P-gp in the modulation of multidrug resistance could not be determined with certainty in these previous studies because inhibition of P-gp function could be attributable to phosphorylation changes in P-gp, resulting from inhibition of PKC, or inhibition of drug binding to P-gp, or inhibition of both activities. Equilibrium binding studies indicate that there are at least two kinetically distinguishable drug-binding sites on P-gp, one for *Vinca* alkaloids, VRP, and cyclosporin A, and a second for azidopine [7]. Our results are in agreement with this finding in that VRP was more effective in inhibiting VLB binding than azidopine photoaffinity labeling. The SPH isomers did not inhibit [^{3}H]VLB binding in MDR cell membranes or [^{3}H]azidopine photoaffinity labeling of P-gp, indicating that they do not directly interact with the P-gp drug binding site(s). The absence of alteration of ATPase activity suggests that SPH isomers probably do not interact at uncharacterized sites on P-gp. These results confirm and extend previous observations with the SPH analog safingol which inhibits PKC activity and the MDR phenotype independent of modulation of P-gp substrate activities [41]. The finding that the calcium channel blocker SR 3357 increased levels of SPH in MDR cells, causing inhibition of cellular PKC activity and drug resistance, but did not inhibit azidopine photoaffinity labeling of P-gp [11] is consistent with the premise that SPH and its stereoisomers do not directly interact with P-gp.

SPH is rapidly taken up by cells within minutes [53] where it can be metabolized, resulting in loss of PKC inhibitory activity by acylation with fatty acids to form ceramides [54]; or via phosphorylation by SPH kinase to SPH phosphate [54, 55]. The half-life of added SPH seems to vary with cell type. In studies using platelets that contain SPH kinase, greater than 50% of administered [4-^{3}H]De SPH was degraded within 1 min and greater than 90% was degraded within 1 hr [53]. SPH is reported to have a much longer half-life of 6 hr in HL-60 cells that metabolize SPH by acylation, forming ceramides [56]. Recently, the stereoisomers of SPH have been shown to inhibit SPH kinase *in vitro* and in platelets [55]. In the present study, we found that SPH stereoisomers enhanced VLB accumulation by MDR cells and inhibited the phosphorylation of P-gp and *in vitro* PKC activity with similar concentration dependence. Differences resulting from the metabolism of SPH stereoisomers may become more evident with longer exposures.

One mechanism that has been elucidated for the modulation of P-gp by most agents is by direct binding to the P-gp. The present work suggests that the SPH isomers may modulate the MDR phenotype by a second mechanism that is associated with inhibition of PKC-mediated phosphorylation of P-gp.

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