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SDZ PSC 833, the Cyclosporine A Analogue and Multidrug Resistance Modulator, Activates Ceramide Synthesis and Increases Vinblastine Sensitivity in Drug-sensitive and Drug-resistant Cancer Cells[1]

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ABSTRACT

Resistance to chemotherapy is the major cause of cancer treatment failure. Insight into the mechanism of action of agents that modulate multidrug resistance (MDR) is instrumental for the design of more effective treatment modalities. Here we show, using KB-V-1 MDR human epidermoid carcinoma cells and [^{3}H]palmitic acid as metabolic tracer, that the MDR modulator SDZ PSC 833 (PSC 833) activates ceramide synthesis. In a short time course experiment, ceramide was generated as early as 15 min (40% increase) after the addition of PSC 833 (5.0 μM), and by 3 h, [^{3}H]ceramide was >3-fold that of control cells. A 24-h dose-response experiment showed that at 1.0 and 10 μM PSC 833, ceramide levels were 2.5- and 13.6-fold higher, respectively, than in untreated cells. Concomitant with the increase in cellular ceramide was a progressive decrease in cell survival, suggesting that ceramide elicited a cytotoxic response. Analysis of DNA in cells treated with PSC 833 showed oligonucleosomal DNA fragmentation, characteristic of apoptosis. The inclusion of fumonisin B_1, a ceramide synthase inhibitor, blocked PSC 833-induced ceramide generation. Assessment of ceramide mass by TLC lipid charring confirmed that PSC 833 markedly enhanced ceramide synthesis, not only in KB-V-1 cells but also in wild-type KB-3–1 cells. The capacity of PSC 833 to reverse drug resistance was demonstrated with vinblastine. Whereas each agent at a concentration of 1.0 μM reduced cell survival by ~20%, when PSC 833 and vinblastine were coadministered, cell viability fell to zero. In parallel experiments measuring ceramide metabolism, it was shown that the PSC 833/vinblastine combination synergistically increased cellular ceramide levels. Vinblastine toxicity, also intensified by PSC 833 in wild-type KB-3-1 cells, was as well accompanied by enhanced ceramide formation. These data demonstrate that PSC 833 has mechanisms of action in addition to P-glycoprotein chemotherapy efflux pumping.

INTRODUCTION

The evolvement of MDR[3] is a major roadblock to effective chemotherapy. Overexpression of P-gp is one of the most consistent alterations in MDR cells (1, 2); however, the mechanisms of action and control points of P-gp are largely unknown. Studies have revealed that P-gp, a M_r 170,000 membrane protein, functions in part via its capacity to act as an efflux pump for chemotherapeutic drugs that enter the cell (3). The antineoplastic agents involved in MDR are diverse and include anthracyclines, such as doxorubicin, *Vinca* alkaloids such as vinblastine, and taxanes, including taxol. In addition to the association of P-gp, MDR is also characterized by an array of separate biochemical changes including altered membrane structure and fluidity, enhanced glutathione *S*-transferase activity, and down-regulation of topoisomerase II (4, 5). Ovarian cancers and melanomas represent malignancies in which prevalence of the MDR phenotype has prevented significant cure with current chemotherapeutic regimens, and a high degree of resistance to chemotherapy is also associated with cancers of the liver, colon, kidney, and pancreas.

Circumvention of MDR via resensitizing cells to chemotherapy drugs carries major clinical importance. A battery of chemically diverse agents has been shown to modulate MDR. These agents render otherwise resistant cells sensitive to chemotherapeutic drugs. Included here are the calcium channel blockers verapamil and SR33557 (6, 7), the immunosuppressant cyclosporine A (8), the triphenylethylene antiestrogen tamoxifen (9, 10), GF 120918, an acridonecarboxamide (11), LY335979, a novel cyclopropyldibenzosuberane (12), and VX-710 (13). The mechanism of MDR reversal is believed to be via direct binding of modulator to cellular P-gp with subsequent inhibition of pump activity (1, 2). However, several points suggest that the classical MDR modulators also have mechanisms of action divorced from P-gp. Verapamil can induce redistribution of doxorubicin from cytoplasm to nucleus (14), and IFN-α, which is not a P-gp substrate, can enhance anthracycline cytotoxicity (15). Some MDR modulators such as quinine, which has a weak effect on cellular doxorubicin accumulation in drug-resistant cells, is able to restore doxorubicin sensitivity (16). Tamoxifen, a P-gp substrate, can retard ceramide glycosylation in various MDR cancer cells (17, 18), a property that is noteworthy in view of the biological responses that are now known to be influenced by ceramide. Ceramide, a mediator of cellular apoptosis (19, 20), is generated in cells by signaling through CD95 Fas/Apo-1 (21), tumor necrosis factor-α (22), anti-IgM (20), ionizing radiation (23), and chemotherapeutic drugs (24, 25). Apoptosis is believed to be a cellular response crucial for chemotherapy cytotoxicity (26); therefore, agents that impact ceramide metabolism have relevance in cancer therapeutics.

Cyclosporine A and PSC 833, derivatives of cyclosporine, are powerful MDR modulators (8, 27, 28). PSC 833, which is more potent than cyclosporine A and nonimmunosuppressive, is effective in reversing resistance to doxorubicin and vincristine (28). Binding of PSC 833 to P-gp has been demonstrated (29), and this interaction is believed to be the basis of chemotherapy resistance modulation. Several recent reports, however, suggest that PSC 833 has alternate mechanisms of action (28, 30, 31). In the present work, we demonstrate a novel activity of PSC 833. Here we reveal, using MDR cells, that single-agent PSC 833 elicits ceramide generation and that the increase in ceramide is accompanied by a decrease in cell survival. In addition, we show that combination PSC 833/vinblastine results in a synergistic increase in cellular ceramide and enhanced chemosensitization. We also show that this response is not limited to P-gp-expressing, drug-resistant cancer cells. Because ceramide plays an important role in signaling apoptosis, we suggest that the potency of PSC 833 is not solely associated with P-gp binding but is partially related to ceramide metabolism.

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[3] The abbreviations used are: MDR, multidrug resistance; PSC 833, SDZ PSC 833; P-gp, P-glycoprotein; FBS, fetal bovine serum; FB_1, fumonisin B_1; LSC, liquid scintillation counting.

MATERIALS AND METHODS

Materials. PSC 833 was a gift from Novartis Pharmaceuticals (East Hanover, NJ). The human MDR oral epidermoid carcinoma cell line, KB-V-1, and KB-3–1, the wild-type counterpart, were obtained from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD). Culture medium was a product of Life Technologies, Inc. (Grand Island, NY), and FBS was from HyClone (Logan, UT). FB_1 was purchased from Biomol (Plymouth Meeting, PA). Glucosylceramide (Gaucher's spleen) was purchased from Matreya, Inc. (Pleasant Gap, PA), and ceramide and sphingomyelin (brain-derived) were from Avanti Polar Lipids (Alabaster, AL). [9,10-^{3}H]Palmitic acid (50 Ci/mmol) and EN^{3}HANCE were from Dupont NEN (Boston, MA), and water-compatible LSC fluid (EcoLume) was from ICN Biomedicals (Costa Mesa, CA). TLC plates (Silica gel G) were purchased from Analtech (Newark, DE). Vinblastine (sulfate salt) was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. KB-V-1 cells were cultured in high-glucose DMEM (4.5 g/l) containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 584 mg/l L-glutamine, and 1.0 μg/ml vinblastine. KB-3-1 cells were cultured in the same medium minus vinblastine. Cells were grown in humidified, 5% CO_2 tissue culture incubator at 37°C and subcultured weekly using 0.05% trypsin/0.53 mM EDTA solution. For the experiments, cells were subcultured into 6-well or 96-well plates or 10-cm dishes, and the FBS content of the medium was lowered to 5%. Vinblastine was omitted when KB-V-1 cells were used. Stock solutions of PSC 833 (10 mM) were prepared in ethanol in 1-dram glass vials and stored at −20°C. Culture medium containing PSC 833 was prepared just prior to use. Ethanol vehicle was present in controls.

Cell Radiolabeling and Lipid Analysis. [^{3}H]Palmitic acid (1.0 μCi/ml culture medium) was used as the lipid precursor to trace cellular metabolism of ceramide, sphingomyelin, and glucosylceramide. After radiolabeling for the specified times, 0.1-ml aliquots of media were removed and analyzed by LSC to determine cellular uptake of the tritiated fatty acid. After removal of media, cell monolayers were rinsed twice with cold PBS. Ice-cold methanol containing 2% acetic acid was added, and cells were scraped free of the substratum (plastic scraper) for lipid extraction using chloroform and water in 1-dram glass vials as described (17). After centrifugation, the resulting organic lower phase of the biphasic extraction was withdrawn, transferred to a glass vial, and evaporated to dryness under a stream of nitrogen.

[^{3}H]Ceramide was resolved from other labeled lipids by TLC using a solvent system containing chloroform:acetic acid (90:10, v/v), and [^{3}H]sphingomyelin was resolved by TLC in chloroform:methanol:acetic acid:water (60:30:7:3, v/v/v/v). Ceramide was also analyzed by an alternate method that consisted of first subjecting an aliquot of the total lipid extract to mild alkaline hydrolysis (0.1 N KOH in methanol, 1 h at 37°C), followed by reextraction (17). The resulting ceramide was then resolved by TLC in a solvent system containing hexane:diethyl ether:formic acid (50:50:1, v/v/v). Both methods of ceramide analysis yielded similar results. Radioactivity in glucosylceramide was analyzed by TLC resolution of total lipids using a solvent system containing chloroform:methanol:ammonium hydroxide (80:20:2, v/v/v). Migration of glucosylceramide was compared with commercial glucocerebroside, and lipids, after iodine vapor visualization, were scraped from the TLC plate for tritium quantitation by LSC (17, 18).

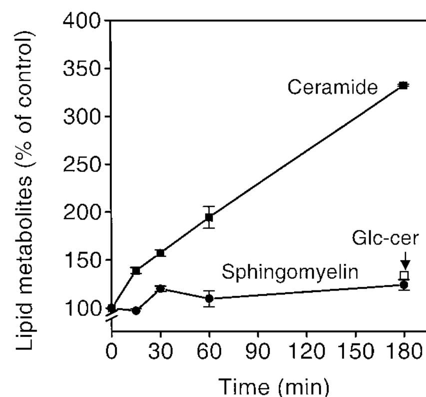

Fig. 2. Influence of time in the presence of PSC 833 on ceramide and sphingomyelin metabolism in KB-V-1 cells. Cultures, at ~75% confluence were incubated in the absence or presence of PSC 833 (5.0 μM) in medium containing [^{3}H]palmitic acid for the times indicated. Total cellular lipids were extracted, and lipid metabolites were quantitated as described in "Materials and Methods." Each point represents the mean from three separate cultures; *bars,* SD. *Glc-cer,* glucosylceramide, indicated by the *arrow*. Glc-cer data are as follows: control, 7343 cpm ± 306; PSC 833, 9203 cpm ± 465.

To char unlabeled cellular ceramide for mass comparisons, after development of the chromatogram in the appropriate solvent system, the TLC plates were dried, sprayed with 30% (v/v) sulfuric acid, and heated in an oven for 20 min at 180°C.

Cytotoxicity Assays. KB-V-1 cells, counted by hemocytometer, were seeded into 96-well plates (2500 cells/well) in 0.1 ml of medium containing 5% FBS. We did not use perimeter wells of the 96-well plates for cells; perimeter wells contained 0.2 ml of water. Cells were cultured for 24 h before addition of PSC 833. PSC 833 was diluted into 5% FBS-containing medium and added to each well in a volume of 0.1 ml. Cells were incubated at 37°C for the times indicated. Cell viability was determined using the Promega Cell Titer 96 Aqueous cell proliferation assay kit. Each experimental point was performed in six replicates. Promega solution (20 μl, not the suggested 40 μl) was aliquoted to each well, and cells were placed at 37°C for 1–2 h or until an absorbance of 0.9–1.1 was obtained as the highest reading. Absorbance at 490 nm was recorded using an ELISA plate reader (Molecular Devices, San Diego, CA).

Determination of DNA Fragmentation. Cells were seeded in 10-cm dishes in medium containing 5% FBS. After 24 h, cells were treated with ethanol (control) or 5.0 μM PSC 833 for a total of 48 h. Cells were then harvested by trypsin-EDTA, isolated by centrifugation, and incubated with digestion buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, 0.5% SDS, and 0.3 mg/ml proteinase K, pH 8.0) at 45°C for 18 h. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and precipitated in one-third volume 7.5 M ammonium acetate and 2 volumes 100% ethanol at −20°C overnight. The preparation was centrifuged for 20 min at 10,000 × g at 4°C. RNA was digested in buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 0.1% SDS, and 100 units/ml RNase at 37°C for 2 h. Reextracted DNA (2.0 μg) was analyzed by electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris-acetate and 1.0 mM EDTA, pH 8.3). DNA fragments were visualized with ethidium bromide under UV light.

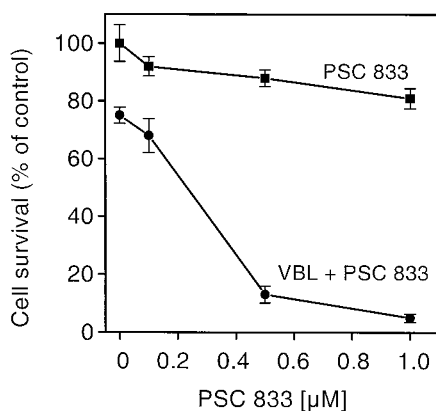

Fig. 1. PSC 833 sensitizes KB-V-1 cells to vinblastine. Cells were seeded in 96-well plates and treated the following day with vehicle (control), PSC 833 as indicated, or PSC 833 plus 1.0 μM vinblastine. Cell viability was determined 3 days later using cell proliferation reagents from Promega. Each point represents the mean (n = 6); *bars,* SD.

RESULTS

We have used the MDR cell line KB-V-1 to study biochemical responses of PSC 833 that may be related to restoration of chemosensitivity. Preliminary experiments were conducted to analyze vinblastine resistance and assess the MDR modulatory properties of PSC 833 in KB-V-1 cells. The data in Fig. 1 show that PSC 833 (0.1–1.0 μM) caused only slight toxicity (15% cell death, 1.0 μM). Exposure of cells to high-dose vinblastine (1.0 μM) was also mildly cytotoxic (25% cell death). When vinblastine was kept constant and PSC 833

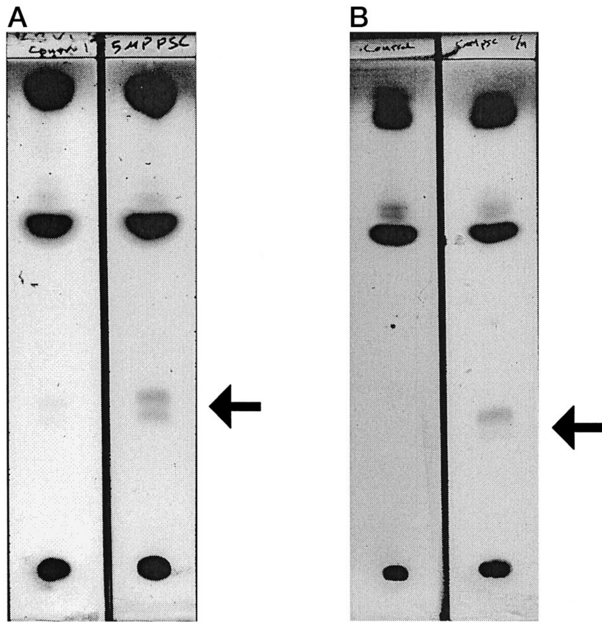

Fig. 3. Increase in ceramide mass in wild-type and drug-resistant cells exposed to PSC 833. Cultures of KB-V-1 cells (*A*) and KB-3–1 cells (*B*) were incubated without or with PSC 833 (5.0 μM) for 24 h. Total cellular lipids were extracted, and 200 and 150 μg of lipid by weight, from KB-V-1 and KB-3–1 cells, respectively, was applied to TLC plates. Ceramide was resolved in a solvent system containing chloroform:acetic acid (90:10, v/v), and lipids were charred as described in "Materials and Methods." *Arrow,* ceramide.

was increased, cell survival dropped precipitously (Fig. 1, *lower curve*). Whereas single agents were only slightly toxic, combination drug treatment brought cell viability to near zero.

Several MDR modulators that we have previously evaluated inhibit glycolipid metabolism, specifically the formation of glucocerebrosides such as glucosylceramide (17). To determine whether PSC 833 inhibited glycolipid metabolism, cultures were exposed to PSC 833 for short time periods in the presence of [^{3}H]palmitic acid. Analysis revealed that PSC 833, after 180 min, did not inhibit but increased the formation of glucosylceramide by ~25% (Fig. 2, *arrow*). Ceramide, the immediate precursor of glucosylceramide, also increased in response to PSC 833 in a time-dependent manner (Fig. 2). As early as 15 min after PSC 833 addition, [^{3}H]ceramide synthesis was 40% higher compared with untreated cells, and after 60 and 180 min, [^{3}H]ceramide nearly doubled and more than tripled, respectively. Further analysis revealed, in contrast with ceramide, that PSC 833 did not influence sphingomyelin metabolism (Fig. 2). Sampling the culture medium at each time point showed that PSC 833 had no effect on cellular uptake of [^{3}H]palmitic acid. After 180 min, palmitic acid incorporation was 45 and 46% in control and PSC 833-treated cultures, respectively.

To determine whether radiolabeling with fatty acid was reflective of a mass increase in ceramide and whether wild-type, drug-sensitive cells would respond, KB-V-1 and KB-3–1 cells were incubated with PSC 833 for 24 h, and cellular lipids were assessed quantitatively. A TLC char of total cell lipid shows that PSC 833 treatment caused a marked increase in the mass of ceramide in KB-V-1 cells (Fig. 3*A*) and in drug-sensitive KB-3–1 cells (Fig. 3*B*). Densitometric analysis revealed that treated KB-V-1 cells contained 10-fold higher ceramide compared with controls. The doublet shown in Fig. 3*A, right lane,* is characteristic of ceramide migration in this solvent system, wherein the lower spot represents ceramide species containing mostly palmitoyl groups and the upper ceramide spot is comprised predominantly of longer chains, such as lignoceroyl and nervonoyl groups. Curiously, although KB-3-1 cells responded positively, the ceramide increase was limited to long-chain species, as shown by the upper band in Fig. 3*B*. Alkaline hydrolysis of an aliquot of total lipid did not diminish the intensity of the ceramide spots on TLC. This indicates that there are no contaminating ester-linked lipids migrating with ceramide.

Finding that higher concentrations of PSC 833 greatly enhanced cellular ceramide levels (Fig. 3) prompted experiments to determine whether the MDR modulator had cytotoxic properties. Results of a dose-response experiment demonstrated that as PSC 833 was increased, cellular ceramide increased proportionally (Fig. 4*A*). Sphingomyelin metabolism, also depicted in Fig. 4, was not altered in response to PSC 833. The increase in ceramide formation was mirrored by a decrease in cell viability (Fig. 4*B*). At 5 μM PSC 833, [^{3}H]ceramide levels were 9-fold greater than control, and cell survival fell to 60% (Fig. 4). Similar toxicity has been reported recently in MCF-7 human breast cancer cells exposed to PSC 833 (32). This experiment also shows that KB-V-1 cells generate glucosylceramide from ceramide that is formed in response to PSC 833 (Fig. 4*A*), as demonstrated by the 4-fold increase in glucosylceramide at a concentration of 5 μM PSC 833. The cytotoxic principle of PSC 833 was further examined by surveying DNA integrity. Exposure of KB-V-1 cells to PSC 833 elicited oligonucleosomal DNA fragmentation (Fig. 5, *Lane 2*). This pattern of DNA laddering is characteristic of apoptosis. In contrast, control cells incubated without PSC 833 did not display DNA fragmentation (Fig. 5, *Lane 1*).

The data in Figs. 2 and 4 show that PSC 833 markedly increased ceramide formation, whereas sphingomyelin metabolism remained unchanged. This suggests that PSC 833 activates ceramide formation through synthesis rather than via hydrolysis of sphingomyelin by a sphingomyelinase. To more closely evaluate the role of ceramide synthesis in PSC 833-induced ceramide formation, a ceramide synthase inhibitor, FB_1, was used. FB_1 abolished PSC 833-induced cer-

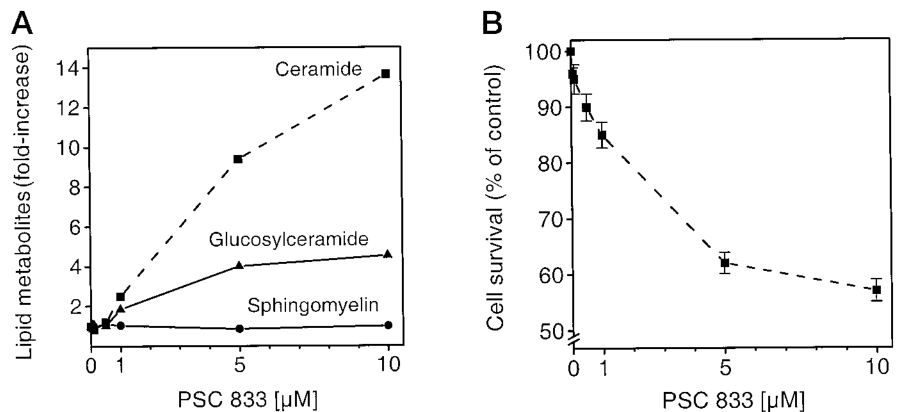

Fig. 4. The influence of PSC 833 on ceramide metabolism and cell viability. In *A,* KB-V-1 cells, in 6-well plates at 60% confluence, were treated without or with PSC 833 at the concentrations indicated for 48 h in medium containing [^{3}H]palmitic acid. Lipids were extracted, and [^{3}H]ceramide, [^{3}H]glycosylceramide, and [^{3}H]sphingomyelin were analyzed by TLC and LSC. Each point represents mean ± SD from three separate cultures. In *B,* parallel experiments were conducted without radioactivity in 96-well plates, and cell viability was determined using the Promega cell proliferation assay method. Each point represents the mean (n = 6); *bars,* SD. The percentage of cell survival was calculated from the absorbance units in control *versus* PSC 833-treated cultures.

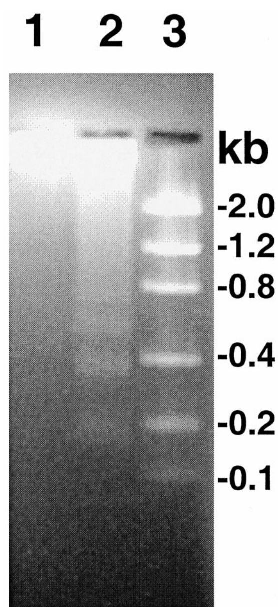

Fig. 5. The influence of PSC 833 treatment on DNA integrity in KB-V-1 cells. Cultures, in 10-cm dishes, were exposed to PSC 833 (5.0 μM) for 48 h. Cells were harvested using trypsin-EDTA, and DNA was extracted and analyzed by electrophoresis on 2% agarose gels as described in "Materials and Methods." *Lane 1,* untreated controls; *Lane 2,* PSC 833; *Lane 3,* DNA standard mass ladder.

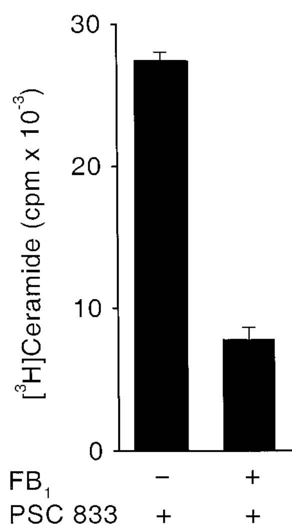

Fig. 6. FB_1, a ceramide synthase inhibitor, blocks PSC 833-induced ceramide production. KB-V-1 cells were exposed to PSC 833 (5.0 μM) in the absence or presence of FB_1 (100 μM) for 2 h in medium containing [^{3}H]palmitic acid. Cellular [^{3}H]ceramide in the total lipid extract was quantitated by TLC and LSC. Drugs had no influence on the cellular uptake of radiolabeled fatty acid. *Bars,* SD.

amide formation (Fig. 6). In cultures containing both PSC 833 and FB_1, the levels of [^{3}H]ceramide were reduced from 27,000 cpm (PSC 833 only) to 7,500 cpm (Fig. 6). Sphingomyelin metabolism was also assessed in cells that were prelabeled with [^{3}H]palmitic acid prior to being treated with PSC 833. In prelabeled cultures that were reincubated in medium devoid of [^{3}H]palmitic acid, levels of [^{3}H]sphingomyelin in control and in PSC 833-treated cells remained similar throughout the 180-min treatment period (Fig. 7). Therefore, PSC 833 was without influence on the decay rate of cellular sphingomyelin.

Because a P-gp-related mechanism of action has been ascribed for PSC 833, it was of interest to determine whether ceramide metabolism would be influenced during the process of MDR modulation with two drugs, similar to the enhanced chemosensitivity shown in Fig. 1. Using low-dose vinblastine and PSC 833, separately and in combination, cells were treated for 3 days, and levels of intracellular [^{3}H]-ceramide were determined. PSC 833 elicited a 50% increase in ceramide, whereas vinblastine was without major influence (4% over control, Fig. 8). Exposing cells simultaneously to PSC 833 and vinblastine, in contrast, resulted in ceramide levels 370% above control. The magnitude of the response is indicative of a synergistic mechanism underlying the changes in ceramide metabolism. This poses the idea that increases in cellular ceramide may be part of the chemosenstization process in drug resistance modulation.

The chromatogram shown in Fig. 3*B*, demonstrating ceramide production in drug-sensitive cells exposed to PSC 833, prompted us to examine whether PSC 833 would enhance chemotherapy sensitivity in a P-gp-deficient model. KB-3-1 cells are sensitive to vinblastine, and under our culture conditions, the EC_{50} was ~1.5 nM. The experiment in Fig. 9 shows that PSC 833 not only enhances vinblastine toxicity but as well enhances ceramide production when administered with vinblastine. Fig. 9*A* shows that whereas PSC 833 alone was not

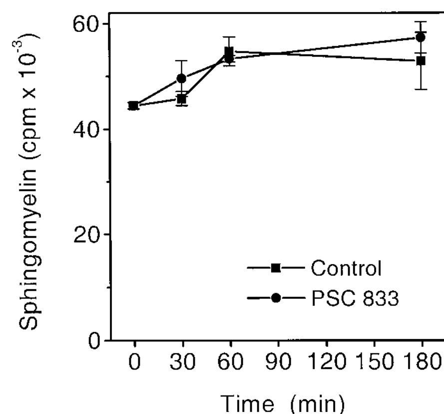

Fig. 7. The influence of PSC 833 on sphingomyelin metabolism in KB-V-1 cells. KB-V-1 cells in six-well plates were grown for 24 h in medium containing [^{3}H]palmitic acid (1.0 μCi/ml 5% FBS-containing medium). After removal of labeling medium, cultures were rinsed three times in aged medium (medium minus [^{3}H]palmitate, conditioned at 37°C and CO_2-equilibrated for 24 h) and then treated in the absence or presence of PSC 833 (5.0 μM) for the times shown. PSC 833 was dissolved and administered in aged medium. After the indicated time intervals, lipids were extracted from control and treated cells, and [^{3}H]sphingomyelin was analyzed by TLC and LSC. *Bars,* SD.

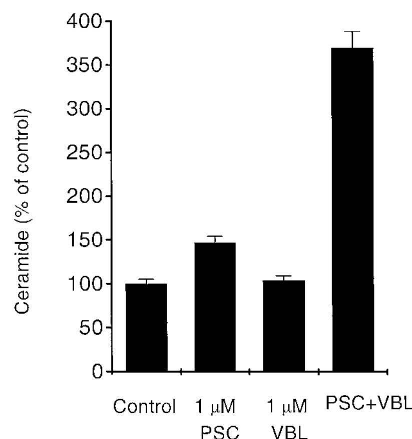

Fig. 8. PSC 833 and vinblastine are synergistic for synthesis of ceramide in drug-resistant KB-V-1 cells. Cultures in six-well plates at 50% confluence were given fresh medium containing vehicle (control), PSC 833, vinblastine, or a mixture of PSC 833 and vinblastine for 3 days. The media contained [^{3}H]palmitic acid. The culture conditions were similar to those of Fig. 1, *lower curve*. Lipids were extracted and analyzed for ceramide by TLC and LSC. Experimental points represent the means from triplicate cultures; *bars,* SD.

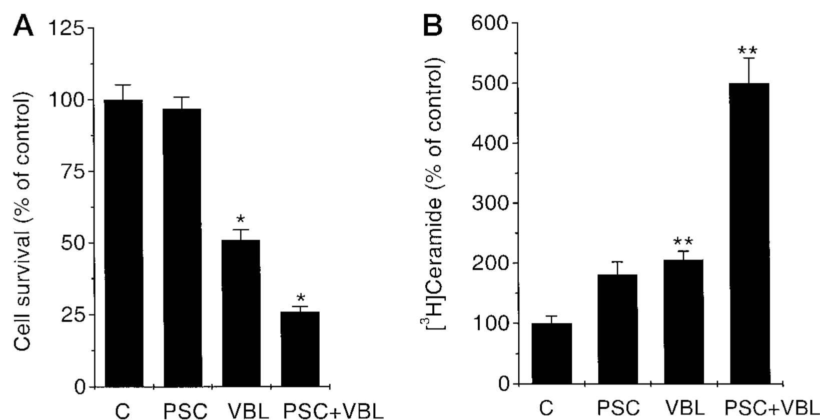

Fig. 9. Influence of PSC 833 on vinblastine sensitivity and ceramide generation in wild-type, drug-sensitive KB-3-1 cells. In *A,* for drug sensitivity assays, KB-3-1 cells were seeded in 96-well plates at 2000 cells/well and treated the following day. Treatments, for a total of 3 days, comprised vehicle (ethanol control), PSC 833 (2.5 μM), vinblastine (*VBL*; 1.5 nM), or PSC 833 plus vinblastine. Cell survival was determined spectrophotometrically using reagents from Promega. Each experimental point represents the mean (n = 6); *bars,* SD.*, $P < 0.0001$. In *B,* the influence of drugs on ceramide metabolism in wild-type cells was conducted in six-well plates with addition of drugs when cells were 50% confluent. Cultures were exposed for 3 days in medium containing [^{3}H]palmitic acid (1.0 μCi/ml) to vehicle (control), PSC 833 (2.5 μM), vinblastine (1.5 nM), or PSC 833 plus vinblastine. **, P = 0.005. Total cellular lipids were extracted, and tritiated ceramide was analyzed as described in "Materials and Methods". *Bars,* SD.

cytotoxic, and vinblastine imparted 50% kill, the PSC 833/vinblastine combination brought cell survival to 25%. This, compared with vinblastine alone, accounts for a 50% increase in cytotoxicity. Similar to the results using KB-V-1 cells, Fig. 9*B* shows that the PSC 833/vinblastine combination promoted enhanced ceramide production in KB-3-1 cells. In comparing cell viability (Fig. 9*A*) with ceramide metabolism (Fig. 9*B*), the most cytotoxic regimen was that which produced the largest increase in ceramide.

DISCUSSION

We have shown previously that drug resistance modulators such as tamoxifen, verapamil, and cyclosporine A retard ceramide metabolism at the juncture of glycosylation to glucocerebrosides (17); however, this report demonstrates a downstream influence on ceramide metabolism, *i.e.,* on ceramide synthesis by the MDR modulator, PSC 833. As such PSC 833, although similar in structure to cyclosporine A, has a radically different influence. Agents that modify ceramide metabolism are of potential importance for targeting pathways of cellular apoptosis.

The MDR modulatory properties of PSC 833 have been studied in murine and human tumor cell lines (28), multicellular tumor spheroids (33), and in mouse models (30, 34). PSC 833 is presently in clinical trials for multiple myeloma (35) and leukemia (36). Although studies have confirmed strong reactivity of PSC 833 with P-gp, other studies suggest that PSC 833 has non-P-gp-related activity. PSC 833 has been shown to be partially active in modulating daunorubicin resistance in P-gp-deficient models (31), and studies on MDR modulation by MRK-16, a P-gp monoclonal antibody, suggest that the molecular mechanisms of drug resistance reversal differ between PSC 833 and cyclosporine A (37). In a recent study using drug-sensitive wild-type MCF-7 cells, we showed that PSC 833 activates ceramide formation, whereas cyclosporine A was without influence (32). MCF-7 cell survival fell to 50% with 5 μM PSC 833 (32), similar to the 60% survival observed in P-gp-rich KB-V-1 cells (Fig. 4*B*). The facility to activate ceramide formation in MCF-7 (32) and in KB-3-1 wild-type cells (Fig. 3*B*), as well as in MDR cells such as KB-V-1, provides evidence for a P-gp-independent mechanism of ceramide generation. The present studies (Fig. 9) also complement the ideas of Merlin *et al.* (31) in that PSC 833 partially modulated anthracycline resistance in cells with poor P-gp expression. Because PSC 833 markedly increases cellular ceramide levels (32, 38) and ceramide has been shown to play a role in mediating cytotoxicity (19–25), we propose that the potency of PSC 833 is in part linked to an influence on ceramide metabolism. It is not known whether PSC 833 would modulate resistance to cisplatin via a ceramide mechanism of action. It is our contention, however, that drugs such as the anthracyclines, which singly promote ceramide generation (24, 25, 38, 39), and vinblastine, which elevates ceramide levels in leukemia cells,[4] must be given in conjunction with PSC 833 to achieve heightened toxicity.

The data of Fig. 4 highlight parallels between the elicitation of ceramide formation by PSC 833 and a decrease in cell survival. Similar results in evidence of ceramide toxicity have been reported in cells in response to ceramide supplementation, sphingomyelinase treatment, and exposure to ionizing radiation, chemotherapy, and biological response modifiers (20–25, 38–41). Although treatment with PSC 833 resulted in marked ceramide increases, cell viability was maintained at ~50% (Fig. 4). Several factors may be involved in cellular response to ceramide, thereby making it difficult to assign a threshold dose that would elicit toxicity, particularly in different cell lines. Toxicity may also be dependent on cellular capacity to metabolize ceramide. MDR cells convert ceramide to glucosylceramide at an accelerated rate, compared with drug-sensitive cancer cells (42, 43).

PSC 833 cytotoxicity observed in the present study in KB-V-1 cells and in MCF-7 cells (32) may be associated only with higher doses. Cell viability fell to 60 and 50% in KB-V-1 and in MCF-7 cells (32), respectively, at a concentration of 5 μM. The cytotoxic response was less at 2.5 μM, and in KB-3–1 cells toxicity was negligible (Fig. 9). Toxicity has not been observed in a Taxol-resistant human breast cancer cell line (33) or in KG1a, a human myeloblastic cell (44). Because of the wide range in sensitivity, the data also suggest that sensitivity to PSC 833 is cell type specific.

The mechanism of PSC 833-activated cellular ceramide formation appears to be via a synthetic route as opposed to degradation of sphingomyelin by sphingomyelinase (19–21, 23, 39). Exposure of cells to FB_1, an inhibitor of ceramide synthase, blocked the induction of ceramide formation by PSC 833 (Fig. 6). Similar results were obtained in P388 murine leukemia cells wherein FB_1 depressed ceramide elevation in response to daunorubicin treatment (24). Furthermore, results of a short time course experiment (Fig. 2) strongly support the idea that sphingomyelin hydrolysis does not contribute to the ceramide increase caused by PSC 833. As shown, the levels of sphingomyelin remained nearly constant, whereas the increase in ceramide was immediate and continued to increase throughout the 180-min time course. It could be argued, because radiolabeled palmitic acid and PSC 833 were added simultaneously, that depletion of sphingomyelin (to produce ceramide) could not be measured due to constant synthesis of sphingomyelin via palmitate incorporation. However, inhibition of PSC 833-induced ceramide formation by FB_1 and results from prelabeling experiments argue strongly against a sphingomyelinase pathway. When KB-V-1 cells were prelabeled with [^{3}H]palmitic acid and washed and reincubated in label-free medium, sphingomyelin decay curves were

[4] Unpublished data.

identical in control and PSC 833-treated cells (Fig. 7). Our results with PSC 833 in KB-V-1 cells also differ from Bezombes *et al.* (44), who ascertained that a neutral sphingomyelinase was activated in U937 cells treated with PSC 833 and tumor necrosis factor-α.

The dynamic nature of the interaction of PSC 833 with vinblastine, the common influence on glycolipid metabolism of MDR modulators such as tamoxifen, verapamil, and cyclosporine A (17), and the ability of not only *Vinca* alkaloids but anthracyclines to promote ceramide formation (25), complement work demonstrating the importance of interactions between combinations of drugs and chemosensitizers. In P388 leukemia cells, drugs and drug resistance modulators, such as vinblastine, mefloquine, and tamoxifen, have mutually cooperative interactions to bring about enhanced cell sensitization (45). Similar results have been seen using median effect analysis to identify synergistic combinations of agents for reversal of P-gp-mediated drug resistance (46). A recent report by Cai *et al.*, (41) emphasized the association of sphingomyelin/ceramide metabolic pathways with resistance of human cancer cells to tumor necrosis factor-α-mediated cytotoxicity. We (17, 18, 42, 43) and others (41) have shown that alterations in glycolipid and ceramide metabolism are associated with cancer cell drug resistance. Targeting these aspects of lipid metabolism can lead to a more mechanistic approach for the treatment of cancer.

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