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

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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 [3H]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, [3H]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 B1, 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 MDR1 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 Mr 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 triphenylethylenantiestrogens tamoxifen (9, 10), GF 120918, an acridonecarboxamide (11), LY335979, a novel cyclopropyldibenzozauberane (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.
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). FB1 was purchased from Chemol (Plymouth Meeting, PA). Glucosylceramide (Gancher’s spleen) was purchased from Matrex, 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'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 Sigma Chemical Co. (St. Louis, MO).

Cell Culture. KB-V-1 cells were cultured in high-glucose DMEM (4.5 g/l D-glucose) containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 584 mg/ml 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% CO2 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. [3H]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 a alternate method that consisted of first subjecting the total lipid extract to mild alkaline hydrolysis (0.1 N KOH in methanol, 1 h at 37°C), followed by reconstitution (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 compared with commercial glucocerebrosides, and lipids, after iodine vapor visualization, were scraped from the TLC plate for tritium quantitation by LSC (17, 18).

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 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 protease 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 two volumes 100% ethanol at −20°C overnight. The preparation was centrifuged for 20 min at 10,000 x 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. Exertacted 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.

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...
glycosylceramide, and sphingomyelin were analyzed in total cell lipid extracts. A TLC char of total cell lipid extracts showed that PSC 833 treatment caused a marked increase in the mass of ceramide in KB-V-1 cells (Fig. 3A) and in drug-sensitive KB-3–1 cells (Fig. 3B). Densitometric analysis revealed that treated KB-V-1 cells contained 10-fold higher ceramide compared with controls. The doublet shown in Fig. 3A, 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. 3B. 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). 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, FB1, was used. FB1 abolished PSC 833-induced cer-
amide formation (Fig. 6). In cultures containing both PSC 833 and FB1, the levels of [3H]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 [3H]palmitic acid prior to being treated with PSC 833. In prelabeled cultures that were reincubated in medium devoid of [3H]palmitic acid, levels of [3H]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 [3H]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. 3B, 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 EC50 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. 9A shows that whereas PSC 833 alone was not
cytotoxic, and vinblasticine imparted 50% kill, the PSC 833/vinblastin
combination brought cell survival to 25%. This, compared with vin-
blastin alone, accounts for a 50% increase in cytotoxicity. Similar to
the results using KB-V-1 cells, Fig. 9B shows that the PSC 833/ vinblas-
tine (VBL; 1.5 nM), vinblastin (1.5 nM), or PSC 833 plus vinblasticin.

**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,
**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), vinblas-
tine (VBL; 1.5 nM), or PSC 833 plus vinblasticin. 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

PSC 833 (2.5 μM), ceramide was analyzed as described in "Materials and Methods". Bars, SD.

PSC 833 activates ceramide synthesis by PSC 833 and a decrease in cell survival. Similar results
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. 4B). The facility to
activate ceramide formation in MCF-7 (32) and in KB-3-1 wild-type
cells (Fig. 3B), 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 anthraclycin 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 anthraclycines, which singly promote
ceramide generation (24, 25, 38, 39), and vinblasticin, which elevates
ceramide levels in leukemia cells, must be given in conjunction with
PSC 833 to achieve heightened toxicity.

The data of Fig. 4 highlight parallels between the elicitation of ceram-
dide 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 ceramid, 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 FB1, 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 FB1 depressed cer-
amide elevation in response to daunorubicin treatment (24). Further-
more, 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

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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, melphalan, 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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