The multidrug resistance modulator SDZ PSC 833 is a potent activator of cellular ceramide formation

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Abstract In this study we demonstrate that the multidrug resistance (MDR) modulator PSC 833 is a potent agonist of ceramide metabolism. When added with [³H]serine or [³H]palmitic acid to the culture medium of MCF-7 cells, PSC 833, in a dose-responsive fashion (1–10 μM), increased the levels of [³H]ceramide as much as 16-fold over control. The actual increase in ceramide mass was verified by thin-layer chromatographic chars. Cellular sphingomyelin radioactivity did not decrease during treatment, indicating that PSC 833 does not elicit ceramide formation through a sphingomyelinase pathway. Inclusion of fumonisin B_1, an inhibitor of ceramide synthase, blocked formation of ceramide by PSC 833. The results of cell proliferation assays demonstrated a clear correlation between PSC 833 elicitation of ceramide formation and increased cytotoxicity. The MDR modulator and chemical cousin of PSC 833, cyclosporin A, had little impact on cellular ceramide formation. At a concentration of 2.5 μM, cyclosporin A and PSC 833 treatment increased ceramide formation by 20% and 7.5-fold, respectively. These results reveal a new action of PSC 833 which may contribute to its potency as a drug resistance modulator.
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Key words: Ceramide; Multidrug resistance; SDZ PSC 833; Cancer; Cyclosporin

1. Introduction

Multidrug resistance (MDR) is a major cause of cancer treatment failure. Overexpression of P-glycoprotein (P-gp), a 170 kDa plasma membrane protein that functions as a drug efflux pump for chemotherapeutic agents, is one of the most consistent alterations of the MDR phenotype [1,2]. P-gp action lowers the effective intracellular drug concentration thereby limiting the accumulation of therapeutic agents. Many of the antineoplastic agents involved in MDR are quite diverse and represent an important segment of the chemotherapy armamentarium. The drugs include anthracyclines such as doxorubicin and daunorubicin, etoposide, paclitaxel (taxol), and vinca alkaloids such as vinblastine and vincristine.

Numerous agents have been studied in an effort to overcome MDR; however, a major challenge in cancer chemotherapy today is to understand the molecular mechanisms by which MDR modulators, e.g. tamoxifen, PSC 833, verapamil, reverse drug resistance. Works have shown that MDR modulators bind directly to P-gp and thus interfere with binding and export of anticancer agents [3–5]. This appears to be the most prominent mechanism for restoring cytotoxicity in an otherwise resistant setting. Our recent studies with MDR modulators demonstrate that these agents also have an impact on glycolipid metabolism [6,7], namely inhibition of ceramide glycosylation which results in a lowering of cellular glucocerebroside (glucosylceramide) levels. Because of the involvement of ceramide in the potentiation of apoptosis [8] and the interest in apoptosis as a mechanism important for cytotoxic response to anticancer agents [9], we sought to determine whether some MDR modulators also influence ceramide metabolism downstream from the glycosylation step.

Two cyclosporin derivatives, cyclosporin A and PSC 833, have been shown to be potent MDR reversing agents [10–12]. Direct interaction between PSC 833 and P-gp has been demonstrated [13], and it is this binding that is believed to be the basis for modulation of chemotherapy resistance. Our studies here demonstrate that PSC 833 has a profound effect on ceramide formation, whereas the chemical analog, cyclosporin A, is without influence. Further, the elevation of ceramide, in response to PSC 833 treatment, correlated with an increase in cell death. These data suggest that the MDR modulator PSC 833 has a P-gp independent mechanism of action which may account for its potency.

2. Materials and methods

2.1. Materials

MCF-7 (human breast cancer) cells were provided by Dr. Kenneth Cowan and Dr. Merrill Goldsmith, National Cancer Institute, Bethesda, MD. Cyclosporin A and PSC 833, [3′-keto-Bmt-1]-[Val-2]-cyclosporin, were gifts from Novartis, East Hanover, NJ. [9,10-³H]Palmitic acid (50 Ci/mmol) was from Dupont NEN, Boston, MA, and [³H]serine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, St. Louis, MO. Ceramide and sphingomyelin (brain-derived) were purchased from Avanti Polar Lipids, Alabaster, AL. TLC plates (Silica gel G) were from Analtech, Newark, DE, and FBS was purchased from Hyclone, Logan, UT. RPMI-1640 medium was purchased from Gibco-BRL, Grand Island, NY. Tissue culture plasticware was from Corning, Cambridge, MA, and water-compatible liquid scintillation cocktail, Ecolume, was purchased from ICN Biomedicals, Costa Mesa, CA.

2.2. Cell culture

Cells were grown in RPMI 1640 medium containing 10% FBS (v/v), 100 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/l L-glutamine and subcultured as described [7]. The FBS content of the medium was lowered to 5% for all experiments. Stock solutions of PSC 833 (10 mM) were prepared in ethanol in 1-dram glass vials with Teflon-lined screw caps and stored at −20°C. Culture medium containing PSC 833 was prepared just prior to use. Ethanol vehicle was present in all controls.

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2.3. Cell radiolabeling and lipid analysis

Cultures, during log phase growth, were treated with PSC 833 and supplemented with [³H]palmitic acid (1.0 μCi/ml medium) or [³H]serine (2.0 μCi/ml) for the times specified. Experiments were terminated by removing the medium, rinsing the monolayers three times with cold phosphate buffered saline, and scraping the cells in ice-cold methanol containing 2% acetic acid. Lipid extraction [14] was continued in 1-dram glass vials with screw caps. After vortex mixing and centrifugation the lower phase of the biphasic extraction containing total cellular lipids was removed and taken to dryness under a stream of nitrogen. Lipids were dissolved in chloroform/methanol (2:1, v/v), usually 50–100 μl, for analysis by TLC.

Ceramide was resolved from total lipids by TLC in a solvent system containing chloroform/acetic acid (90:10, v/v), and sphingomyelin was resolved in a solvent system containing chloroform/methanol/acetic acid/water (50:30:7:4, v/v). Glucosylceramide was separated from other lipids by TLC in chloroform/methanol/ammonium hydroxide (70:20:4, v/v). Commercial lipid standards were co-applied to the TLC plate origins, and lipid identification was done by comparing migration after iodine staining. Areas of the chromatogram containing the radiolabeled lipid were scraped into plastic mini scintillation vials containing 0.5 ml water followed by 4.5 ml Ecolume, and tritium analysis was by LSC.

To char unlabeled cellular lipids for mass analysis, following development of the chromatogram in the appropriate solvent system, the plates were dried, sprayed with 30% sulfuric acid, and heated in an oven for 20 min at 180°C. For mass comparisons of ceramide in control and PSC 833 treated cells, aliquots of 100–200 μg total lipid (by weight) were adequate. To assess ceramide by an alternative method, total cell lipids were base-hydrolyzed (mild alkaline hydrolysis) in 0.1 N KOH in methanol for 1 h at 37°C. Lipids were re-extracted [14] and analyzed by TLC either in the above described solvent system or in a system containing hexane/diethyl ether/acetic acid (50:50:1, v/v).

2.4. Cytotoxicity assay

Cells (2000/well/0.1 ml medium) were seeded into 96-well plates and after 24 h, treatment medium (0.1 ml) containing PSC 833 was added. After 4 days, cell viability was determined spectrophotometrically (λ 490, Molecular Devices, Emax microplate reader, Sunnyville, CA) using the Promega Titer 96 Aqueous cell proliferation assay kit. This assay is based on the capacity of viable cells to convert a tetrazolium derivative to a water-soluble formazan that absorbs light at 490 nm.

3. Results

In previous work we demonstrated that modulators of MDR influence ceramide metabolism by retarding the glycosylation step that forms glucocerebrosides [6,7]. PSC 833 was however different from tamoxifen, verapamil, and cyclosporin A in that glucocerebrosides such as glucosylceramide increased in response to PSC 833 treatment [15]. This finding led us to investigate the influence of PSC 833 on the metabolism of ceramide, the immediate precursor of glucocerebrosides. The data in Table 1 show that PSC 833 treatment of
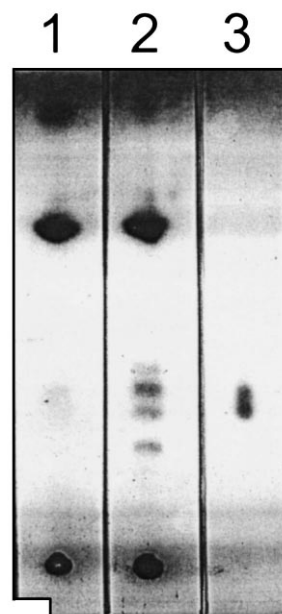

Fig. 1. Thin-layer chromatographic char of lipids isolated from cells treated with PSC 833. MCF-7 cells were treated with PSC 833 (5.0 μM) for 24 h. The total lipid extract (200 μg aliquot) was applied to TLC plates for ceramide resolution. Visualization was by charring. Lane 1, control cells; lane 2, PSC 833 treatment; lane 3, commercial ceramide standard.

MCF-7 cells elicited a 16-fold increase (over control) in levels of [³H]ceramide after a 48 h exposure. Based on total lipid tritium per culture, ceramide accounted for 0.45 and 7.3% of total lipid radioactivity in control and PSC 833 treated cells, respectively. Results are also presented from experiments using [³H]serine, a more specific sphingolipid precursor that does not radiolabel the aliphatic chains. As shown, PSC 833 (5.0 μM) elicited a > 6-fold increase in cellular ceramide after only 24 h (Table 1). PSC 833, as previously reported for adriamycin resistant breast cancer cells [15], increased glucosylceramide in MCF-7 cells by 2.5-fold (Table 1). Radioactivity in sphingomyelin, which has been shown by many investigators to be the source of agonist-induced ceramide formation, was not depleted in response to PSC 833. On the contrary, radioactivity in cellular sphingomyelin increased by 50% (Table 1).

Although [³H]palmitic acid has reached steady state after a 48 h incubation and tritium levels would be reflective of actual lipid mass changes, we analyzed cellular lipids by TLC char-

Table 1
The influence of PSC 833 on lipid metabolism in MCF-7 cells

Lipid	Cell incubation conditions	
	− PSC 833	+ PSC 833
Ceramide	2270 ± 270[a]	36410 ± 603
Ceramide[b]	4637 ± 49	29848 ± 1707
Glucosylceramide	8957 ± 364	22046 ± 280
Sphingomyelin	41560 ± 3219	62918 ± 7009

MCF-7 cells were cultured for 48 h in the presence of [³H]palmitic acid and PSC 833 (10 μM), after which time total cell lipids were extracted [14] and [³H]ceramide, [³H]glucosylceramide, and [³H]sphingomyelin were quantitated by TLC and LSC. Data represent the mean ± S.D. Experiments were repeated two to three times with similar results.
[a]Numbers represent cpm in the specified lipid class per 500000 cpm total lipid tritium.
[b]These data were obtained from cells labeled for 24 h with [³H]serine and co-cultured in the absence or presence of PSC 833 (5.0 μM).
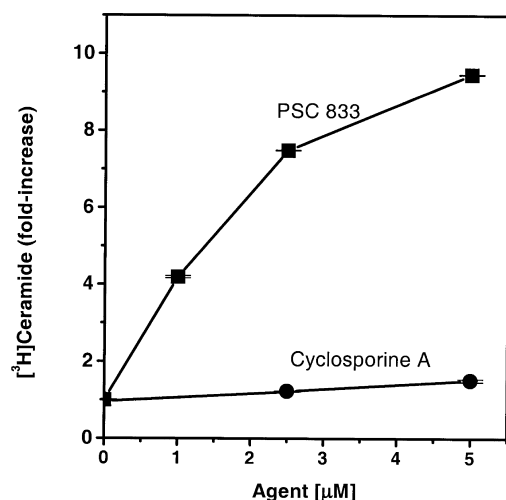
Fig. 2. Influence of PSC 833 concentration on ceramide formation in MCF-7 cells. Cells were treated for 48 h with PSC 833 at the concentrations indicated. The culture medium contained [^{3}H]palmitic acid (1.0 μCi/ml). [^{3}H]Ceramide was analyzed in the total lipid extract by TLC and LSC. PSC 833 at all concentrations shown did not influence the cellular uptake of [^{3}H]palmitic acid. Data points represent the average of duplicate cultures; variability was < 10%.

ring. The chromatogram (Fig. 1) shows the changes in ceramide mass after a 24 h exposure of cells to PSC 833. In control cells (Fig. 1, lane 1), ceramide was barely detectable. In contrast, cellular ceramide mass increased markedly in cells that were treated with PSC 833 (Fig. 1, lane 2). The two central spots (lane 2), which correspond to the migration of the commercial standard doublet (Fig. 1, lane 3), represent ceramide molecular species differing in their aliphatic constituents. The lower-most spot (lane 2) has been identified as monoacylglycerol. The upper-most spot was also present in the commercial

brain derived ceramide standard; however, at the concentration applied to lane 3, it is not visible. Subjecting a sample of total lipid to mild alkaline hydrolysis followed by TLC analysis gave identical ceramide results as the char shown in Fig. 1. These alkaline conditions hydrolyze ester-linked acyl groups, removing possible contaminates, while leaving the amide linkage of ceramide intact.

To determine whether ceramide generation occurs in a dose responsive manner, cells were exposed to increasing concentrations of PSC 833. As shown in Fig. 2, cellular [^{3}H]ceramide increased with increasing PSC 833. At a concentration of 1.0 μM, PSC 833 enhanced cellular [^{3}H]ceramide content by 4-fold, and at 5.0 μM PSC 833, cellular [^{3}H]ceramide content was > 9-fold that of control. In sharp contrast, treatment of cells with cyclosporin A (2.5 and 5.0 μM), which is very similar in structure to PSC 833, elicited a 0.23- and 0.51-fold increase, respectively, in [^{3}H]ceramide (Fig. 2).

Ceramide has been shown to be toxic in numerous cell systems [8,16,17], and we have shown that PSC 833 profoundly elevates cellular ceramide levels. Taking this into account it was reasoned that the PSC 833/ceramide response should impact cell viability. The data of Fig. 3 show that PSC 833 was cytotoxic, and cytotoxicity was positively correlated with increasing drug concentration. At 10 μM PSC 833, cell survival was reduced to 20%. The IC$_{50}$ (amount of drug that causes 50% cell death) for PSC 833 was approximately 5.0 μM, and at this concentration cell ceramide levels were 9-fold greater than control (Fig. 2). A comparison of Figs. 2
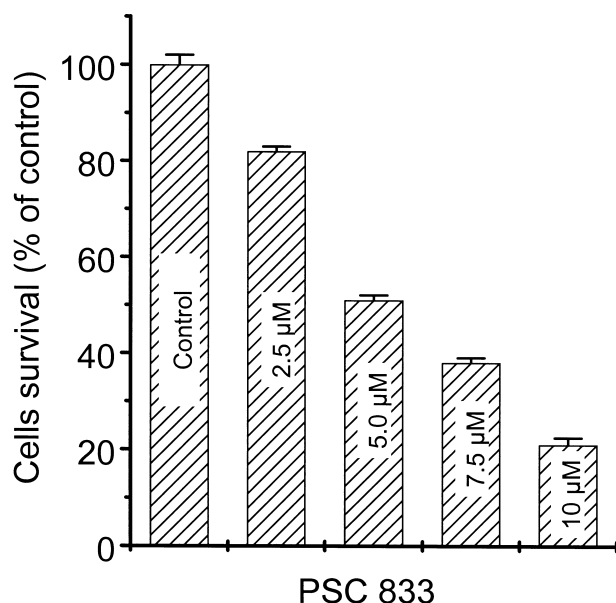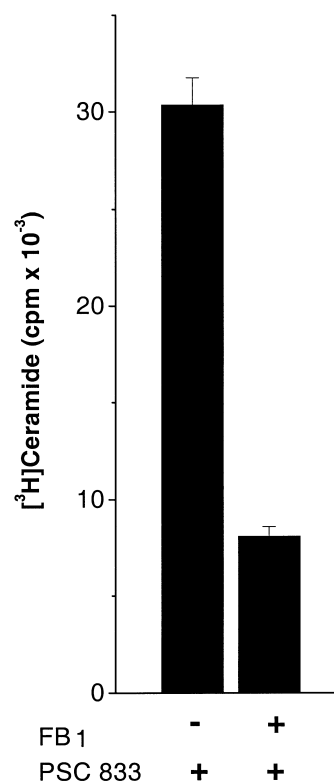

Fig. 3. Influence of PSC 833 concentration on cell viability. MCF-7 cells, seeded at 2000 cells/well in 96-well plates, were treated for 4 days with PSC 833 at the concentrations indicated. Cell viability was determined spectrophotometrically as described in Section 2. Each data point represents the average ± S.D. of six replicate wells.

Fig. 4. Effect of fumonisin B$_1$ on ceramide formation by PSC 833. MCF-7 cells in 6 cm tissue culture dishes were treated when approximately 70% confluent with PSC 833 (5.0 μM) and given [^{3}H]palmitic acid in the absence or presence of FB$_1$ (50 μM) for 2 h. FB$_1$ was dissolved in water. Cellular lipids were analyzed for [^{3}H]ceramide content by TLC and LSC. Drugs had no influence on the uptake of palmitic acid.

and 3 shows a strong association between increasing cellular ceramide levels (Fig. 2) and a decrease in cell survival (Fig. 3). These data suggest that PSC 833 is cytotoxic through a ceramide related event.

Table 1 shows that PSC 833, while having a marked effect on ceramide formation, had little influence on sphingomyelin metabolism. To determine if PSC 833 is affecting ceramide metabolism via synthesis, the influence of the ceramide synthase inhibitor, FB_1, was evaluated. Pretreating MCF-7 cells with FB_1 severely inhibited the promotion of ceramide formation by PSC 833 (Fig. 4). When PSC 833 and FB_1 were present simultaneously, levels of [^{3}H]ceramide were reduced from approximately 30 000 cpm (PSC 833 only) to 8000 cpm.

4. Discussion

The mechanism of action of the MDR modulator PSC 833 is believed to involve inhibition of drug efflux by P-gp [13,18,19]. However, the specific sites of action and the mechanisms by which MDR modulators reverse chemotherapy resistance continue to be a topic of interest. Here we show for the first time, using a cell line that is P-gp deficient, that PSC 833 has a profound impact on ceramide formation. These experiments employed PSC 833 as the sole drug; therefore, the complexities of combination chemotherapy were not involved in the biochemistry of the ceramide response. The increase in cell ceramide caused by PSC 833 paralleled a decrease in cell survival. This suggests that the action of PSC 833 is, in part, related to the effects of this drug on the metabolism of ceramide. Cyclosporin A, which structurally is very similar to PSC 833, by contrast was a weak agonist of ceramide formation (Fig. 2).

Ceramide is an important effector of apoptosis and a second messenger of cellular differentiation [8,20]. Thus, agents that elicit ceramide formation are potentially important in cell regulatory processes. Ceramide generation can be promoted by a myriad of agents including anti-IgM [16], ionizing radiation [21], and Fas/Apo-1 [22]. In most instances, ceramide formation is triggered via enzymatic degradation of sphingomyelin [8,16,21,22]. Our results differ in that ceramide is not generated, in response to PSC 833, at the expense of sphingomyelin (Table 1). These data suggest that PSC 833 impacts ceramide synthesis. To further test this hypothesis, FB_1, a ceramide synthase inhibitor, was employed. The data showed that PSC 833 elicited ceramide generation was blocked by FB_1. This strongly argues against a sphingomyelinase pathway of ceramide genesis in the PSC 833 response.

There exist questions regarding the differential mechanisms of actions and potencies of cyclosporin A and PSC 833 as MDR modulators. PSC 833 has been shown to be more potent in reversing MDR [11,12] and whereas both PSC 833 and cyclosporin A bind to P-gp, only the latter is transported [13]. In comparing cyclosporin A and PSC 833, we show that cyclosporin A had little influence on cellular ceramide formation. The two MDR modulators differ only slightly in structure [13]; therefore, the ceramide response must be closely related to the chemistry of these agents. The question arises whether PSC 833 exerts influence on ceramide metabolism

through interaction with P-gp. Although we are only beginning to address this issue, MCF-7 wild-type cells are P-gp negative. We have confirmed this by Western blot analysis using the P-gp rich MCF-7-adriamycin resistant cell line as a positive control (data not shown). This implies that the influence of PSC 833 on ceramide synthesis is independent of P-gp. Overall, the results presented herein strongly support a mechanism of action of PSC 833 that is divorced from P-gp. The involvement of ceramide in cellular response to anticancer agents and MDR modulators merits further study.

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