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13. ABSTRACT (Maximum 200 Words) Our previous studies showed that PSC 833, the multidrug resistance modulator, increases cellular ceramide levels. Our studies in the first grant year demonstrated that PSC 833 increases ceramide levels via serine palmitoyl-transferase (SPT) activation. However, the effects of PSC 833 on other <i>de novo</i> enzymes, the scope of PSC 833/ceramide response in human breast cancer cells, and the extent to which other anticancer drugs impact SPT remain unknown. To this end, during the past year I have mastered several research techniques, analyzed experimental results, and prepared a manuscript for publication. We have demonstrated that PSC 833 activates SPT independently of SPT gene transcription, MAP kinase, and PKC. The effect of PSC 833 on SPT activation is closely related to its molecular structure. The research has also shown that PSC 833 induces ceramide generation in a broad spectrum of human breast cancer cell lines. Furthermore, we have demonstrated that SPT is the target enzyme in ceramide generation that is induced by other well known chemotherapeutic drugs, including etoposide, taxol, and retinoids. This is a significant finding which provides a novel approach to breast cancer treatment opening the door for new drug design targeting ceramide metabolism.				
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INTRODUCTION

Multidrug resistance (MDR) is a major cause of treatment failure in breast cancer. The purpose of this research is to improve the efficacy of breast cancer treatment. While P-glycoprotein (P-gp) may be the most understood mechanism of MDR (1-4), other important mechanisms have been identified in recent years, such as cellular increases in glucosylceramide, and changes in the activities of glutathione S-transferase and topoisomerase (5-10). Numerous agents have been studied in an effort to overcome MDR (11-14). A major challenge in breast cancer chemotherapy today is to understand the molecular mechanisms by which MDR modulators, e.g., tamoxifen, PSC 833, reverse drug resistance (13,14). While studies have shown MDR modulators bind directly to P-gp and thus interfere with binding and export of anticancer agents, it is increasingly apparent that some chemotherapeutic agents stimulate ceramide generation in cancer cells, and this leads to apoptosis (15,16). Adriamycin, the most widely used single agent for treatment of breast cancer, also activates ceramide formation (17). Our recent studies have shown that PSC 833 markedly enhances cellular ceramide formation and is synergistic with adriamycin (14,17,18). Because the effect of PSC 833 on ceramide formation is correlated with an increase in cell death and reversal of multidrug resistance in breast cancer cells (14,18), knowledge of the biochemical pathways involved is essential. Ceramide generation may be increased by either the hydrolysis of membrane-resident sphingomyelin or by de novo synthesis at the level of the endoplasmic reticulum (19-24). Our studies in the first grant year demonstrated that PSC 833 elicits the generation of ceramide via the de novo synthesis pathway, presumably through serine palmitoyltransferase (SPT) and not the widely speculated ceramide synthase pathway. Therefore, we have focused our studies (listed in the Statement of Work) in the second grant year on characterizing SPT activity in breast cancer. We will assess the biochemical mechanism of enzyme activation using several human breast cancer cell lines, exposed not only to PSC 833 but to well known chemotherapeutic drugs like 4-HPR (N-[4-Hydroxyphenyl]retinamide), daunorubicin, etoposide, and taxol. These experiments will clarify the role of SPT activation in chemotherapeutic drug induced apoptosis in breast cancer, and provide a novel approach for anticancer drug design strategy, based upon targeting enzymes of sphingolipid metabolism.

BODY

This section provides a description of research accomplishments associated with tasks listed in our Statement of Work.

1. **C₆-Ceramide is Cytotoxic to Human Breast Cancer Cell Lines.**

C₆-Ceramide is cytotoxic to the human breast cancer cell line, MDA-MB 468 (EC₅₀ = 1.9 μM). This supports the activity of ceramide as a second messenger in chemotherapy induced cytotoxicity in human breast cancer.

2. **Characterization of SPT Activation by PSC 833:**

a. **PSC 833 increases SPT activity independently of SPT gene transcription.**

To understand the regulation of SPT activation following PSC 833 treatment, we evaluated the mRNA levels of LCB1 and LCB2 subunits of SPT, using RT-PCR. No increases in mRNA levels for either SPT subunit were evident by gel electrophoresis. The RNA synthesis inhibitor, actinomycin, did not inhibit ceramide generation induced by PSC 833 treatment. These results show that activation of SPT by PSC 833 is independent of SPT gene transcription.

b. **Activation of SPT by PSC 833 is independent of PKC and MAP kinase signal transduction pathways.**

Previous reports demonstrated that the PKC activator, TPA, activated PKC and induced ceramide generation and apoptosis in LNCaP cells. To determine whether PSC 833 induces ceramide generation and apoptosis through PKC activation, we treated MDA-MB 468 cells with PKC inhibitor, GF 106203x. The results showed that GF 106203x did not block the action of PSC 833 on ceramide generation, suggesting that PSC 833-induced SPT activation is independent of PKC signal transduction pathways. It has also been reported that the anti-tumoral action of cannabinoids is related to sustained ceramide accumulation and MAP kinase (extracellular signal-regulated kinase) activity. Therefore, we wanted to clarify if MAP kinase activation is related to SPT activation. Our results demonstrate that the MAP kinase inhibitor, PD 98059, does not inhibit ceramide generation induced by PSC 833, suggesting that PSC 833-induced SPT activation is independent of MAP kinase signal transduction pathways.

c. **Effect of PSC 833 on SPT activity is closely related to molecular structure.**

PSC 833 is very similar in structure to cyclosporine A; however, cyclosporine A has no influence on SPT activity as determined recently. Because there are only two minor functional group differences in the two "cyclosporines", it is evident that structure-activity relationships for activation of SPT are quite stringent. This knowledge is essential for drug development.

3. **PSC 833 Does Not Stimulate SPT Activity Directly:**

Preexposure of microsomes (complete cell-free enzyme assay system minus substrate) to PSC 833 (10 μM) for 10 min at 37°C, followed by substrate addition and incubation for 10 min, failed to stimulate SPT activity compared to controls (minus PSC 833). This shows that enzyme activation is not direct. However, stimulation of SPT can be achieved in intact cells upon exposure to PSC 833, followed by cell-free assay.

4. **Sphingolipid Generation Initiated by PSC 833 in Different Human Breast Cancer Cell Lines, and SPT Activation Initiated by Different Chemotherapeutic Drugs:**

a. **MDA-MB 468 is a P-gp negative human breast cancer cell line.**

Because no data are available on the P-gp status of MDA-MB 468 cells, we performed immunofluorescent staining to determine the P-gp content. The results showed that MDA-MB 468 cells were negative for P-gp.

b. **PSC 833 elicits ceramide generation in a number of human breast cancer cell lines, independent of estrogen receptor (ER) and P-gp status.**

To determine the scope of the response of PSC 833 on human breast cancer cells, we analyzed ceramide generation following PSC 833 exposure in several human breast cell lines: BT-20, HS-578T, MDA-MB 231, MCF-7, MCF-7/Adr, and T-47D. The results showed that PSC 833-increased ceramide generation across the board, and this was independent of ER and P-gp status.

c. **Chemotherapeutic drugs that elicit formation of ceramide activate synthesis upstream of ceramide synthase.**

To determine whether chemotherapeutic drugs that elicit de novo ceramide synthesis activate enzymes upstream of ceramide synthase, we analyzed sphinganine, the product of SPT, following drug exposure (6 hr). The results showed that 4-HPR, daunorubicin, Taxol, and etoposide all increased sphinganine levels, revealing a common target in the mechanism of action of several well known chemotherapeutic drugs.

d. **SPT and not palmitoyl coenzyme A synthetase or ceramide synthase is the target enzyme activated by chemotherapeutic drugs eliciting ceramide synthesis - a cell-free study.**

To determine whether de novo enzyme activation could be initiated by pre-exposure to chemotherapeutic drugs, human breast cancer cells (MDA-MB 468) were incubated with either 4-HPR, daunorubicin, Taxol, or etoposide, and microsomes were isolated for cell-free enzyme evaluation. The results show that only SPT is activated. Because SPT is a prime target, this provides a novel approach for chemotherapeutic drug design.

KEY RESEARCH ACCOMPLISHMENTS

- Mastered cell culture techniques for various human breast cancer cell lines;
- Mastered RT-PCR technique for SPT subunits LCB₁ and LCB₂ mRNA detection;
- Mastered immunofluorescent staining techniques for P-gp in MDA-MB 468 cells;
- Improved manuscript preparation skills;
- Determined that c₆-ceramide is cytotoxic to human breast cancer cells;
- Determined that PSC 833 increases SPT activity independently of SPT gene transcription;
- Determined that activation of SPT by PSC 833 treatment is independent of PKC and map kinase signal transduction pathways;
- Demonstrated that effect of PSC 833 on SPT activity is closely related to molecular structure;
- Determined that PSC 833 does not stimulate SPT directly;
- Determined that PSC 833 elicits ceramide generation in a broad spectrum of human breast cancer cell lines, independently of ER and P-gp status;

- Demonstrated that SPT, and not palmitoyl coenzyme a synthetase or ceramide synthase, is the target enzyme activated by several well known chemotherapeutic drugs.

REPORTABLE OUTCOMES

- a. Wang H, Giuliano AE, Cabot MC. Ceramide synthesis through serine palmitoyl-transferase by anticancer drugs in human breast cancer cells. *Cancer Res*, submitted, 2001.
- b. Liu YY, Wang H, Litvak DA, Giuliano AE, Cabot MC. Targeting ceramide - a therapeutic strategy for cancer treatment. Abstract presentation at the 5th Joint Conference of the American Association for Cancer Research and Japanese Cancer Association. Maui, Hawaii: February 12-16, 2001.

CONCLUSIONS

During the past year, our studies have demonstrated that PSC 833 activates SPT independently of SPT gene transcription and independently of MAP kinase, and PKC signal transduction pathways. The effect of PSC 833 on SPT activation is closely related with molecular structure. PSC 833 also induces ceramide generation in a broad spectrum of human breast cancer cell lines, independently of either ER or P-gp status. Furthermore, and most importantly we have demonstrated that SPT is the target enzyme of several well known chemotherapeutic drugs. This is a significant finding that greatly extends our original aims. This research provides a novel approach for new anticancer drug design targeting ceramide metabolism.

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APPENDICES

- a. Manuscript submitted to *Cancer Research* (Manuscript No. CAN-2567-1; September 17, 2001).
- b. Abstract Presentation at the 5th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association.

Molecular Biology and New Therapeutic Strategies

Targeting Ceramide – A Therapeutic Strategy for Cancer Treatment. Yong Y. Liu, Hongtao Wang, David A. Litvak, Armando E. Giuliano, and Myles C. Cabot. John Wayne Cancer Institute, 2200 Santa Monica Blvd, Santa Monica, CA 90404

Apoptotic signaling through ceramide determines the cytotoxic response to a number of chemotherapeutic agents, including paclitaxel (taxol), irinotecan (CPT-11), and the novel diphtheria toxin conjugate DT₃₈₈-GM-CSF. In addition, upregulated glycosylation of ceramide is responsible for resistance to chemotherapy in some types of cancer cells [Liu, Y.Y., Han, T.Y., Giuliano A.E., and M.C. Cabot (1999) J. Biol. Chem. 274, 1140-46]. Here we show that targeting ceramide metabolism, by pharmacologic or genetic intervention, is an effective strategy to either enhance sensitivity to chemotherapy or temper or completely reverse resistance to chemotherapy in cancer cells. The pharmacologic approach utilizes agents to augment chemotherapy-induced ceramide levels or to hinder subsequent ceramide metabolism. The endpoint of this approach is a synergistic increase in ceramide and a heightened cytotoxic response. Examples here include anthracyclines, 4-HPR (fenretinide), DT₃₈₈-GM-CSF, and CPT-11, to activate ceramide formation, combined with drugs to augment or retard ceramide metabolism such as C₆-ceramide (a ceramide analog), SDZ PSC 833 (a cyclosporin A analog), tamoxifen, mifepristone, and PPMP (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol). Combinations of these agents can enhance cell kill by as much as 2-3 logs. The genetic approach involves blocking the enzyme GCS (glucosylceramide synthase), which catalyzes the glycosylation of ceramide, rendering it non-cytotoxic. We introduced human GCS antisense cDNA into MCF-7 adriamycin-resistant breast cancer cells. Transfecting these cells with antisense GCS, rendered them 40-, 100-, and 200-fold more sensitive to adriamycin, vinblastine, and taxol, respectively. This work shows that targeting ceramide metabolism, pharmacologically or through gene therapy, effectively kills cancer cells *in vitro* and may be a viable clinical strategy in the treatment of cancer patients.

Molecular Biology and New Therapeutic Strategies: Cancer Research in the 21st Century

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**Ceramide Synthesis through Activation of Serine Palmitoyltransferase by
Anticancer Drugs in Breast Cancer Cells¹**

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Running Title: Activation of SPT by Anticancer Drugs

Keywords: SDZ PSC 833, serine palmitoyltransferase, ceramide, breast cancer cells,
chemotherapy.

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³ The abbreviations used are: P-gp, P-glycoprotein; FB₁, Fumonisin B₁; SPT, serine palmitoyltransferase; PSC 833, SDZ PSC 833; LSC, liquid scintillation counting; TLC, thin-layer chromatography; FBS, fetal bovine serum; 4-HPR, N-(4-hydroxyphenyl)retinamide, fenretinide.

ABSTRACT

Multidrug resistance (MDR) is the major cause of cancer treatment failure. SDZ PSC 833 (PSC 833), a P-glycoprotein-targeted MDR modulator, sensitizes cancer cells to chemotherapy, but additionally markedly increases cellular ceramide levels. Because ceramide is a constituent of chemotherapy-induced apoptosis, knowledge of the lipid pathways influenced by PSC 833 is of relevance therapeutically. Here we show in MDA-MB 468 human breast cancer cells that PSC 833 elicits ceramide generation through activation of the *de novo* synthesis pathway and not via sphingomyelin hydrolysis. In intact cells, ceramide generation increased 3-fold 1 h after addition of PSC 833 (5.0 μ M). Cyclosporine A, a close chemical cousin of PSC 833, was not active. Sphinganine generation also increased in response to PSC 833, and this could be blocked by adding L-cycloserine, a serine palmitoyltransferase (SPT) inhibitor, to the culture medium. Exposure of cells to PSC 833 (30 min – 4 h; 1 – 10 μ M) followed by isolation of microsomes for *in vitro* assay, enhanced SPT by as much as 60%, whereas palmitoyl CoA synthetase and ceramide synthase activities were not altered. SPT activity was also enhanced, as measured *in vitro*, by pretreating cells with either Taxol, daunorubicin, 4-HPR, or etoposide; however, activation was half that attained by PSC 833. PSC 833 also activated ceramide generation in other breast cancer cell lines including BT-20, MDA-MB 231, Hs 578T, T-47D, and MCF-7. In conclusion, PSC 833 increases cellular ceramide levels by activation of SPT, the rate-limiting enzyme in the *de novo* pathway. As such, in addition to blocking P-

glycoprotein, PSC 833 may be of utility therapeutically to enhance apoptosis when used in conjunction with modulators of ceramide metabolism.

INTRODUCTION

Multidrug resistance is the major cause of cancer treatment failure (1,2). Overexpression of P-gp³ (P-glycoprotein), a 170 kDa transmembrane protein that functions as a drug efflux pump, is one of the most consistent alterations of the MDR phenotype (3). Numerous agents have been studied in an effort to overcome P-gp-mediated MDR, tamoxifen, PSC 833 (Valspodar), verapamil, cyclosporine A, and VX-710, among them (4-9). The MDR mediators bind directly to P-gp and thereby interfere with cellular export of anticancer drugs. This appears to be the most prominent mechanism for restoring cytotoxicity in an otherwise drug resistant setting.

Ceramide, the lipid backbone of sphingomyelin and glycolipids, is an important second messenger of apoptosis (10,11). Many chemotherapeutic agents stimulate the production of ceramide, an upstream signal of apoptosis (12-15), and it is now becoming apparent that initiation of programmed cell death may have greater therapeutic value than antiproliferative routes. Our previous studies show that the P-gp drug, PSC 833, also activates ceramide generation, and that the effect of PSC 833 on ceramide metabolism correlates with an increase in cell death and a dampening of MDR in breast cancer cells (16-18). These results suggest that restoration of drug sensitivity, essentially the role of PSC 833, is associated with ceramide formation.

Ceramide levels may be increased by hydrolysis of membrane-resident sphingomyelin by sphingomyelinase or by *de novo* synthesis at the level of the

endoplasmic reticulum (19-23). Previous studies on the involvement of ceramide in the activation of apoptotic pathways elicited by $\text{TNF}\alpha$, Fas, and ionizing radiation show that intracellular ceramide elevation results from sphingomyelin hydrolysis (24-26). However, a recent report showed that ceramide increases in response to PSC 833 treatment were not accompanied by depletion of sphingomyelin (16,17). Several enzymes, such as palmitoyl coenzyme A synthetase, SPT, and ceramide synthase, contribute to catalyze *de novo* formation of ceramide (21-23). Although our previous data showed that the ceramide synthase inhibitor, FB_1 , blocked ceramide generation induced by PSC 833 (17), details of the enzyme activation pathway remained unknown. Here, we focused our studies on assessing the target of PSC 833, and we have demonstrated that this P-gp modulator activates SPT, the rate-limiting enzyme in the *de novo* ceramide synthesis pathway (see Fig. 1), in a cell line devoid of P-gp. We also show that other cancer drugs, among them taxanes, retinoid analogs, and etoposide, share this activity in common.

MATERIALS AND METHODS

Materials - PSC 833 was a gift from Novartis Pharma AG (Basel, Switzerland). The human breast cancer cell lines MDA-MB 468, MDA-MB 231, T-47D, BT-20, and Hs 578T were purchased from the American Type Culture Collection (Rockville, MD). The human breast carcinoma cell line MCF-7 was obtained from Dr. Merrill E. Goldsmith (National Cancer Institute, Bethesda, MD). Culture media were products of Life Technologies, Inc. (Grand Island, NY), and FBS was from HyClone (Logan, UT). Taxol, daunorubicin, and etoposide were from Sigma (St. Louis, MO). 4-HPR was kindly

provided by R. W. Johnson Pharmaceuticals (Spring House, PA). FB₁ and L-cycloserine were purchased from Biomol (Plymouth Meeting, PA). Ceramide and sphingomyelin (brain derived) were from Avanti Polar Lipids (Alabaster, AL). Sphinganine (D-erythro-dihydrosphingosine in pure form) was from Matreya (Pleasant Gap, PA). [9,10-³H(N)]Palmitic acid (50 Ci/mmol) was from Dupont/NEN (Boston, MA). [5,6-³H]Sphinganine (60 Ci/mmol), and L-[³H(G)]serine (20 Ci/mmol), were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Silica Gel G TLC plates were purchased from Analtech (Newark, DE). Other chemicals were from Sigma (St. Louis, MO).

Cell Culture - MDA-MB 468, MDA-MB 231, and MCF-7 cells were cultured in RPMI-1640 medium containing 10% FBS, and 584 mg/liter L-glutamine. T-47D cells were cultured in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES (pH 7.3), 1.0 mM sodium pyruvate, and 7 µg/ml bovine insulin. Hs 578T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 4.5 g/liter glucose, and 10 µg/ml bovine insulin. BT-20 cells were cultured in minimum essential medium Eagle's with 2 mM L-glutamine and Earle's balanced salt solution, adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. All cell culture media contained 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified 5% CO₂ tissue culture incubator at 37 °C and subcultured using 0.05% trypsin/0.53 mM EDTA solution. For the experiments, cells were subcultured into 6-well or 96-well plates, or 6-cm or 10-cm dishes, and the FBS content of the medium was lowered to 5%. Stock solutions of PSC 833 (10 mM) were prepared in ethanol in 1-dram glass vials and stored at -20 °C.

Culture media containing PSC 833 or other drugs were prepared just prior to use.

Ethanol vehicle was present in controls.

Metabolic Labeling and Analysis of Cellular Lipids - After radiolabeling (1.0 μCi [^3H]palmitic acid /ml culture medium) for the specified times, 0.1 ml aliquots of media were removed and analyzed by LSC to determine cellular uptake of fatty acid. The culture medium was aspirated, and monolayers were rinsed twice with ice-cold PBS. Ice-cold methanol containing 2% acetic acid was added, and cells were scraped free of the substratum (plastic scraper) for lipid extraction in 1-dram glass vials as described (13,14). 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. [^3H]Ceramide was resolved from other radiolabeled lipids by TLC using a solvent system containing chloroform/acetic acid (90:10, v/v). [^3H]Sphinganine was resolved by TLC in chloroform/methanol/ammonium hydroxide (70:20:4, v/v/v), and [^3H]sphingomyelin was resolved by TLC in chloroform/methanol/acetic acid/water (60:30:7:3, v/v/v/v). After iodine vapor visualization, the lipids of interested were scraped from the TLC plate for tritium quantitation by LSC using Ecolume (13).

Isolation of Microsomal Membranes – Cultures, at 80% confluence in 10-cm dishes were placed on ice, rinsed twice with ice-cold PBS, and scraped into 0.5 ml homogenization buffer (20 mM HEPES, pH 7.4, 5 mM DTT, 5 mM EDTA, 2 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin). Cell suspensions were sonicated over ice for 60 seconds (20% output, alternating 15-second sonication and 20-second pause) using a Micro Ultrasonic Cell Disrupter from Konte (Vineland, NJ). Lysates were centrifuged at 10 000 x g for 10 min. The postnuclear supernatant was isolated and centrifuged at 100 000 x

g for 60 min at 4 °C. The microsomal membrane pellet was resuspended in 100 µl homogenization buffer by sonication for 5 seconds and frozen at -80 °C (27).

Serine Palmitoyltransferase Assays - Enzymatic activity was determined by measuring the incorporation of [³H]serine into 3-ketosphinganine. Each tube (0.1 ml final volume) contained 0.1 M HEPES, pH 8.3, 2.5 mM EDTA, 50 µM pyridoxal phosphate, 5 mM DTT, 1.0 mM L-serine, and 100 µg microsomal protein. After preincubation at 37 °C for 10 min, the reaction was initiated by simultaneous addition of palmitoyl CoA (0.2 mM) and 1.0 µCi [³H]serine. Control tubes contained either boiled microsomes or no protein. The reaction was incubated in 37 °C for 7 min, and terminated by addition of 0.2 ml 0.5 N NH₄OH. Organic-soluble products were isolated by addition of 3 ml chloroform/methanol (2:1), 25 µg sphingosine carrier, and 2.0 ml 0.5N NH₄OH. The washed organic phase was isolated, and 1.0 ml was dried under a stream of nitrogen and analyzed by LSC (27).

Ceramide Synthase Assays - For assaying ceramide synthesis (28), [³H]sphinganine was used as radiolabeled precursor. The reaction mixtures contained 25 mM HEPES, pH 7.4, 2 mM MgCl₂, 0.5 mM DTT, 10 µM sphinganine, and 100 µg microsomal protein. Sphinganine was dried under nitrogen from a stock solution in chloroform/methanol (2:1) and dissolved with sonication in the reaction mixture prior to addition of microsomal protein. The total reaction volume was 0.1 ml. Assays were initiated by simultaneous addition of palmitoyl CoA (0.1 mM) and 0.5 µCi [³H]sphinganine followed by incubation at 37 °C for 40 min with gentle shaking. Controls were as above. The reaction was terminated by lipid extraction and dihydroceramide was isolated and quantitated by TLC and LSC.

Palmitoyl-CoA Synthetase Assays – We used a slight modification of a method previously described (29). Microsomal protein (100 μ g) was added to a buffer mixture containing 200 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 8 mM MgCl₂, 2 mM EDTA, 20 mM NaF, 0.1% Triton X-100, and 10 μ M palmitic acid. Reactions were initiated by simultaneous addition of acetyl CoA (0.1 mM) and 1.0 μ Ci [³H]palmitic acid. The reaction, 0.5 ml total volume, was incubated at 37 °C for 10 min with gentle shaking and terminated by the addition of 1.5 ml of isopropanol/heptane/2 M H₂SO₄ (40:10:1, v/v/v). After addition of 0.65 ml H₂O and 1.5 ml heptane containing 5 mg/ml palmitic acid, the mixtures were vortexed and the organic phase was removed. The aqueous phase, containing palmitoyl CoA formed during reaction, was washed three times with 2 ml heptane containing 5 mg/ml palmitic acid, and 0.2 ml were analyzed by LCS. In control experiments either the microsomes or acetyl CoA was omitted.

RESULTS

The main pathways for cellular production of ceramide are shown in Fig. 1. L-cycloserine and FB₁ are inhibitors of *de novo* enzymes. Our study was conducted to determine the avenue by which PSC 833 enhances ceramide production in breast cancer cells. The influence of PSC 833 on ceramide metabolism in MDA-MB 468 breast cancer cells is shown in Fig. 2. Intracellular ceramide increased as early as 15 min after the addition of drug, and by 1 h ceramide levels had risen 3-fold over control. The effect of PSC 833 on ceramide metabolism was also dose-dependent and plateaued at approximately 5 μ M (Fig. 2, inset).

Results obtained using SPT and ceramide synthase inhibitors indicated that PSC 833 targets the *de novo* ceramide pathway upstream of ceramide synthase. As shown in Fig. 3A, PSC 833 alone, activated formation of sphinganine, the ceramide precursor, by 50%. Exposure of cells to FB₁, a ceramide synthase inhibitor, caused sphinganine build-up 5-fold over control. When FB₁ was added to block conversion of sphinganine to ceramide, sphinganine increased nearly 10-fold in response to PSC 833 addition; however, when L-cycloserine was added (refer to Fig. 1) sphinganine formation was halted in response to PSC 833. Under the same conditions, PSC 833 increased ceramide 2.5-fold over control (Fig. 3B). FB₁ treatment decreased cellular ceramide formation by ~ 40%, and FB₁ also decreased the formation of ceramide in response to PSC 833 treatment. Inhibition of SPT by inclusion of L-cycloserine reduced the amount of ceramide generated, and nearly halted PSC 833-induced ceramide formation.

The contributions of *de novo* synthesis and sphingomyelin hydrolysis to the production of ceramide by PSC 833 were compared by cellular differential radiolabeling. Twenty-four h prelabeling of cells with [³H]palmitic acid followed by a wash and PSC 833 treatment yielded no increase in intracellular [³H]ceramide (Fig. 4, left). However, when [³H]palmitic acid and PSC 833 were added simultaneously, intracellular ceramide increased 5-fold (Fig. 4, middle). By the same token, combining both radiolabeling techniques, 24 h prelabeling followed by simultaneous addition of PSC 833 with a 2 h pulse of fresh [³H]palmitic acid likewise yielded a 5-fold increase in [³H]ceramide (Fig. 4, right). Therefore, equilibrium radiolabeling of sphingomyelin did not enhance ceramide formed as a result of PSC 833 exposure, demonstrating that sphingomyelin decay is not contributory to ceramide production.

A comparison of PSC 833 with cyclosporine A, a close structural analog, shows that only the former promotes an increase in cellular ceramide levels (Fig. 5). PSC 833 also promoted the production of sphinganine, approximately 50% over control (Fig. 5), which again indicates the involvement of *de novo* enzymes in the mechanism of action of PSC 833, as opposed to hydrolysis of sphingomyelin by sphingomyelinase (see Fig. 4).

Cell-free experiments were carried out to assess the effect of PSC 833 on the different enzymes of ceramide synthesis. MDA-MB 468 cells were exposed to PSC 833 prior to harvesting and isolation of microsomes for *in vitro* assays. Of the major enzymes in the *de novo* synthesis pathway (refer to Fig. 1), only SPT activity was enhanced by PSC 833 (Table 1). Sphinganine formation was 57 ± 0.2 and 91 ± 7.1 p mol/mg protein/min in control and PSC 833 treated cells, respectively, accounting for a 60% increase in enzyme activity. Palmitoyl-CoA synthase and ceramide synthase activities were not influenced by exposing cells to PSC 833 prior to cell-free assaying. Stimulation of SPT by PSC 833 pretreatment of MDA-MB 468 cells was both time- and dose-dependent as measured *in vitro* (Fig. 6). SPT activation was biphasic with regard to time (Fig. 5A) with an early peak at 15 min (17% increase) followed by prolonged and greater activation thereafter (50% increase by 2 h), and activity was enhanced over a concentration range of 1.0 – 10 μ M (Fig. 4B).

To determine the scope of the PSC 833 response, we evaluated other human breast cancer cell lines selected from a spectrum including estrogen receptor positive and negative cells. As shown in Fig. 7, all cell lines tested responded to PSC 833. The range of activation of ceramide synthesis was from 3- to nearly 7-fold, with the

exception of BT-20 cells which showed the poorest response, 72% over control. Data from preliminary experiments show that BT-20 cells are refractory to PSC 833 cytotoxicity, as tested over a concentration range of 1-5 μ M (data not shown).

We next compared PSC 833 with other anticancer drugs for effects on ceramide generation and on the enzymes of ceramide synthesis. When added to cultures of MDA-MB 468 cells together with [3 H]palmitic acid, all drugs accelerated the generation of [3 H]ceramide, with 4-HPR being the most potent, nearly 300% of control (Table 2). Daunorubicin was the least effective in enhancing ceramide production. Both Taxol and etoposide enhanced the production of [3 H]sphinganine during the time-frame studied, suggesting SPT activation, and the inclusion of FB₁ to block ceramide synthase showed that daunorubicin and 4-HPR also enhanced [3 H]sphinganine formation in intact cells (Table 2). Cell-free enzyme assays conducted after exposure of cells to the various chemotherapy drugs, were carried out to determine whether SPT was targeted, similar with the mechanism of action of PSC 833. Taxol, 4-HPR, and etoposide enhanced SPT activity to a similar extent, 24-29% over control (Table 3), and daunorubicin showed the least effect. Neither palmitoyl CoA synthase nor ceramide synthase activities were significantly altered by pretreating cells with the various chemotherapy drugs. Of interest, cyclosporine A, which is very similar in structure to PSC 833, was totally devoid of activity (see also Fig. 5).

DISCUSSION

PSC 833, a second generation P-gp antagonist developed to combat MDR, is being evaluated in patients with advanced cancers including acute myeloid leukemia

and refractory ovarian carcinoma (30-32). Studies amply demonstrate that PSC 833 retards drug efflux (5,33) and our group has determined that PSC 833 activates the formation of cellular ceramide, independently of P-gp (18). In as much as ceramide has been linked with apoptosis pathways enforced by chemotherapy drugs (reviewed in 11), it is reasonable to hypothesize that the cytotoxic principle of PSC 833 is in part associated with ceramide. Results from other laboratories mirror this idea. In acute myeloid leukemia it is thought that PSC 833 acts independently of P-gp to enhance apoptosis through sphingomyelin/ceramide-linked events (34). In work with prostate cancer cell lines, it was concluded that PSC 833 alone or in combination with estramustine, etoposide, ketoconazole, suramin, or vinorelbine, exerted anticancer effects by an avenue divorced from pump interaction (35). Whether strictly P-gp-directed or otherwise, PSC 833 and similar chemotherapy resistance modulators hold promise as co-drugs in cancer treatment. Knowledge of mechanisms and targets is essential for furthering therapeutics in this area.

Our preliminary results using RT-PCR showed that no gross changes occurred in the levels of mRNAs coding for the SPT subunits in response to PSC 833 treatment (data not shown). This suggests that a posttranscriptional avenue of enzyme activation is likely. Further evidence for a nontranscriptional effect of PSC 833 on SPT is the rapid 15 min activation time (Figs. 2, 6). Although P-gp is not required for the PSC 833 ceramide response (18), influences on lipid transport and substrate availability provoked by PSC 833 could play a role in enhanced ceramide formation in intact cells. MDR3 P-gp can function as a phosphatidylcholine translocase (36), and similarly, PSC 833 has been shown to influence sphingolipid translocation in CHO cells (37). Such physical

events may be in operation at the ER/Golgi level; however, the activation of SPT in cell-free incubations using exogenously added radiolabeled substrate (Fig. 6) would argue against this activity.

It is of interest that several well-known chemotherapy drugs also elicited ceramide formation through SPT. This may be a common target of utility in the cytotoxic response to anticancer agents. That cyclosporine A was devoid of activity compared to PSC 833, is noteworthy, as there are only slight differences in the chemical structures: the β -ketoamide in cyclosporine A is a β -hydroxyamide in PSC 833, and PSC 833 has an isopropyl group replacing one of the ethyl groups.

Results from this work provide strong evidence that PSC 833 activates SPT, the rate-limiting enzyme in the *de novo* pathway of sphingolipid biosynthesis. The time frame for activation of SPT by PSC 833, using microsomes isolated from pretreated cells, was biphasic, similar to previous findings with 4-HPR in neuroblastoma (15) and etoposide in Molt-4 cells (23). Enhancement of ceramide formation by PSC 833 was also clearly not cell type-specific, as we demonstrated that all breast cancer cells tested exhibited response, albeit the BT-20 response was minimal by comparison.

Knowledge of sentinel cellular targets of agents like PSC 833 and other anticancer drugs is necessary for the design of more effective treatment. In this specific instance, drugs that target ceramide metabolism have been shown to be effective in the elimination of cancer cells (11,38). The efficacy of targeting ceramide synthesis or degradation pharmacologically to enhance the cytotoxic effects of several clinically relevant drugs has also been demonstrated (reviewed in 11). Targeting ceramide metabolism holds promise as a clinical strategy for treatment of cancer.

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TABLES

Table 1. *Effect of cellular exposure to PSC 833 on the enzymes of de novo ceramide synthesis measured in vitro*

Following PSC 833 exposure (10 μ M) for 4 h, cultures of MDA-MB 468 cells were harvested, microsomes were isolated, and enzyme activities were determined as described in Materials and Methods. Data represent the mean \pm SD of triplicate samples.

Treatment	Enzyme activity ^a (pmol/mg protein/min)		
	Pal-CoA syn	SPT	Cer syn
Control	476 \pm 12	57 \pm 0.2	157 \pm 3.3
PSC 833	451 \pm 13	91 \pm 7.1 ^b	155 \pm 4.6

^a Pal-CoA syn, palmitoyl-CoA synthetase, palmitoyl-CoA synthesized; SPT, sphinganine synthesized; Cer syn, ceramide synthase, ceramide synthesized.

^b statistically significant from control, $p < 0.01$.

Table 2. Influence of chemotherapy drugs on ceramide metabolism in intact MDA-MB

468 cells

Cells in 6-well plates were exposed for 6 h to either Taxol (1.0 μ M), daunorubicin (10 μ M), 4-HPR (10 μ M), or etoposide (10 μ M) with simultaneous addition of [3 H]palmitic acid. Cellular lipids were extracted and analyzed as detailed in Materials and Methods.

Drugs	Ceramide formed (% of control)	Sphinganine formed ^a (% of control)	
		Agents alone	Agents + FB ₁
Taxol	189 \pm 2.4 ^{*c}	128 \pm 0.2 [*]	ND ^b
Daunorubicin	136 \pm 0.8 [*]	100 \pm 13.4	117 \pm 3.4 [*]
4-HPR	298 \pm 6.7 [*]	97 \pm 2.0	144 \pm 3.7 [*]
Etoposide	161 \pm 3.9 [*]	118 \pm 8.8 [*]	ND

^a Sphinganine formation was determined in the absence and presence of FB₁ in order to block conversion of sphinganine to ceramide.

^b ND, not determined

^c statistically significant compared to control, * p < 0.01.

Table 3. Influence of pre-exposure of cells to chemotherapy drugs on enzymes of *de novo* ceramide synthesis measured *in vitro*

Cultures of MDA-MB 468 cells were exposed to the drugs indicated in Table 2 (cyclosporine A, 10 μ M), without radiolabel present, harvested for isolation of microsomes, and cell-free enzyme assays were conducted.

Drugs	Enzyme activity ^a (% of control)		
	Pal-CoA syn	SPT	Cer syn
Taxol	97 \pm 0.7	129 \pm 1.6 ^{*c}	99 \pm 2.5
Daunorubicin	102 \pm 1.5	114 \pm 4.7	94 \pm 5.6
4-HPR	99 \pm 6.4	124 \pm 2.4 [*]	104 \pm 5.3
Etoposide	107 \pm 6.2	128 \pm 4.7 [*]	106 \pm 3.5
Cyclosporine A	ND ^b	101 \pm 2.7	ND

^a Enzymes and products as footnoted in Table 1; percent of control based on pmol product/mg protein/min.

^b ND, not determined.

^c statistically significant compared to control, * $p < 0.01$.

FIGURE LEGENDS

Fig. 1. Schematic of ceramide metabolism and the impact of FB₁ and L-cycloserine on the *de novo* elements. SM, sphingomyelin; SMase, sphingomyelinase; SMSyn, sphingomyelin synthase.

Fig. 2. Time course and dose response effect of PSC 833 on ceramide generation in MDA-MB 468 cells. MDA-MB 468 cells were seeded in 6-well plates, and experiments initiated at ~ 80% confluence. For time course study, [³H]palmitic acid (1.0 μCi/ml medium) and PSC 833 (10 μM) were added simultaneously for times indicated. For dose response study, cells were treated with PSC 833 at the concentrations indicated for 1 h before the addition of [³H]palmitic acid (1.0 μCi/ml medium) for an additional 3 h. Total cell lipids were extracted, and ceramide was quantitated by TLC and LSC. Data represent the mean ± SD of triplicate samples.

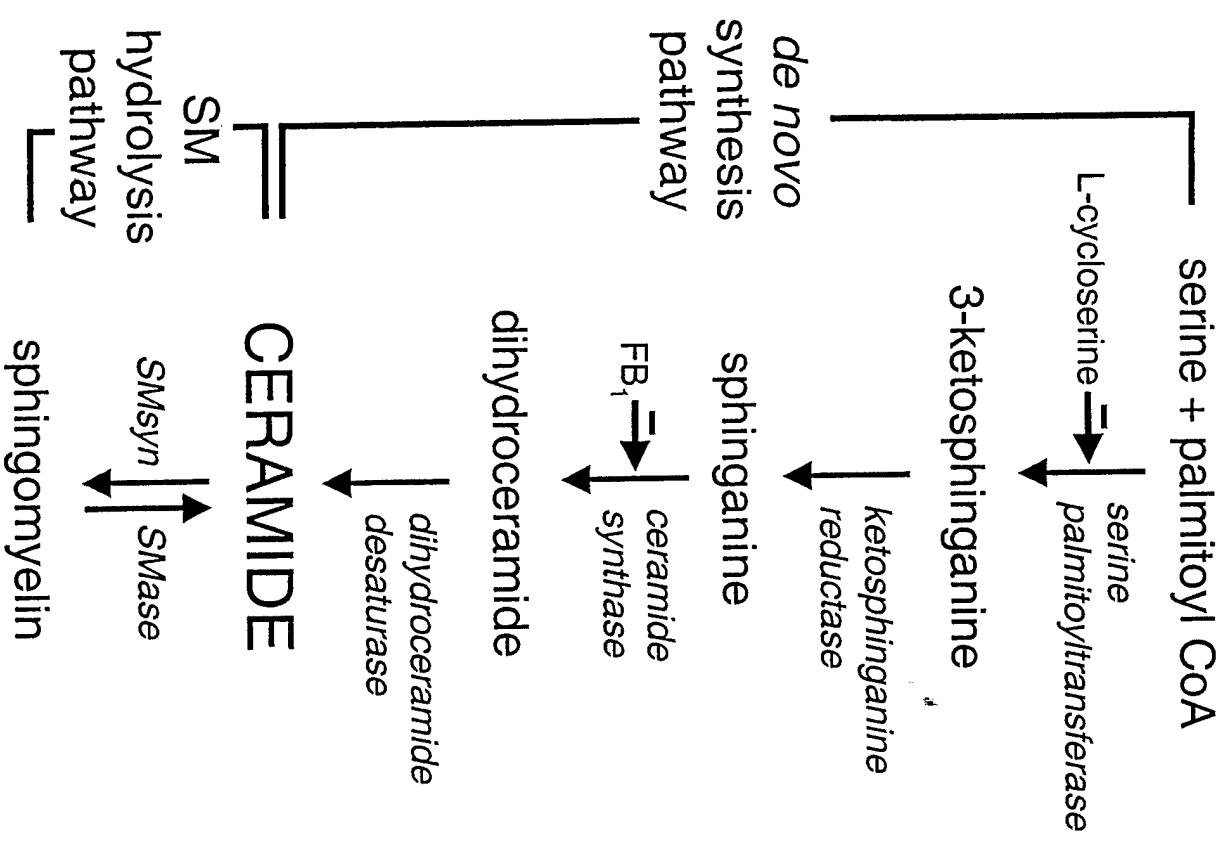
Fig. 3. Effect of *de novo* enzyme inhibitors FB₁ and L-cycloserine on PSC 833-induced sphinganine and ceramide formation. *A*, sphinganine formation. *B*, ceramide formation. At ~ 80% confluence, MDA-MB 468 cells were treated with either 50 μM FB₁ or 2 mM L-cycloserine for 30 min before the addition of PSC 833 (10 μM) and [³H]palmitic acid (1.0 μCi/ml medium) for 2 h. Cellular lipids were extracted, and ceramide was analyzed by TLC and LSC. Data are expressed as cpm specified lipid/500,000 cpm total lipid tritium, and represent the mean ± SD of triplicate samples.

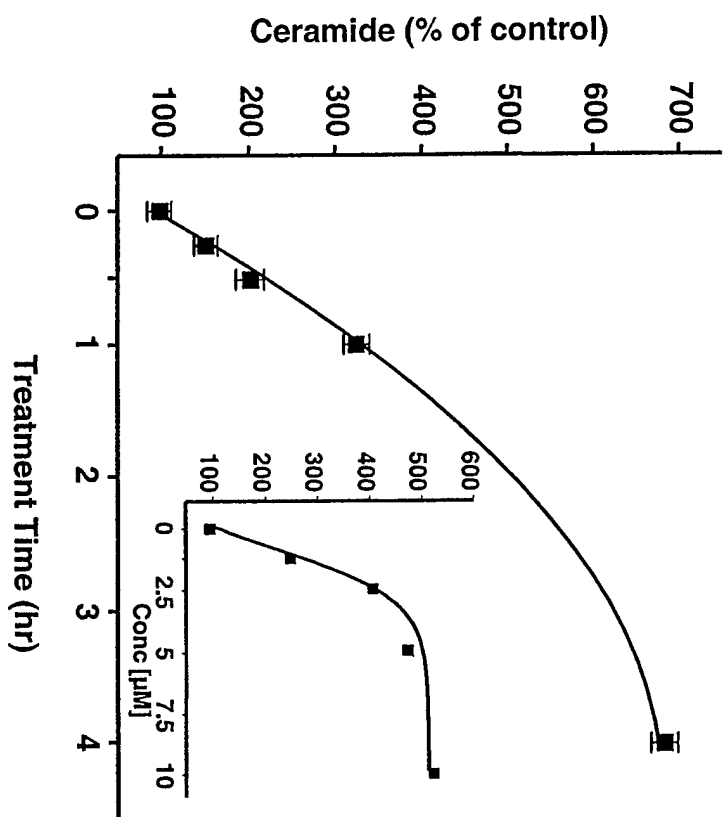
Fig. 4. Analysis of ceramide generated via *de novo* synthesis and sphingomyelin hydrolysis. To measure ceramide generated by sphingomyelin hydrolysis, cells were prelabeled with [³H]palmitic acid (1.0 μCi/ml medium) for 24h. After washing and a 3-h chase in medium containing 5% FBS, cells were treated with 10 μM PSC 833 for 2 h. To measure ceramide generated *de novo*, cells were treated with 10 μM PSC 833 and supplemented with [³H]palmitic acid (1.0 μCi/ml medium) simultaneously for 2 h. To measure total ceramide generated from both *de novo* synthesis and sphingomyelin hydrolysis, after 24 h prelabeling and wash/chase, cells treated with 10 μM PSC 833 and also supplemented with [³H]palmitic acid (1.0 μCi/ml medium) for 2 h. After treatment, cellular lipids were extracted, and ceramide was analyzed by TLC and LSC. Data are expressed as cpm in ceramide/500,000 cpm total lipid tritium, and represent the mean ± SD of triplicate samples.

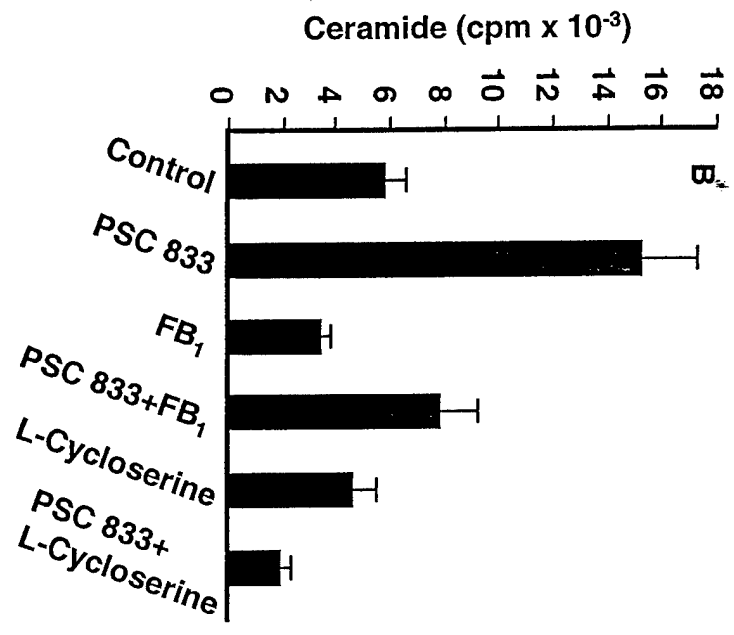
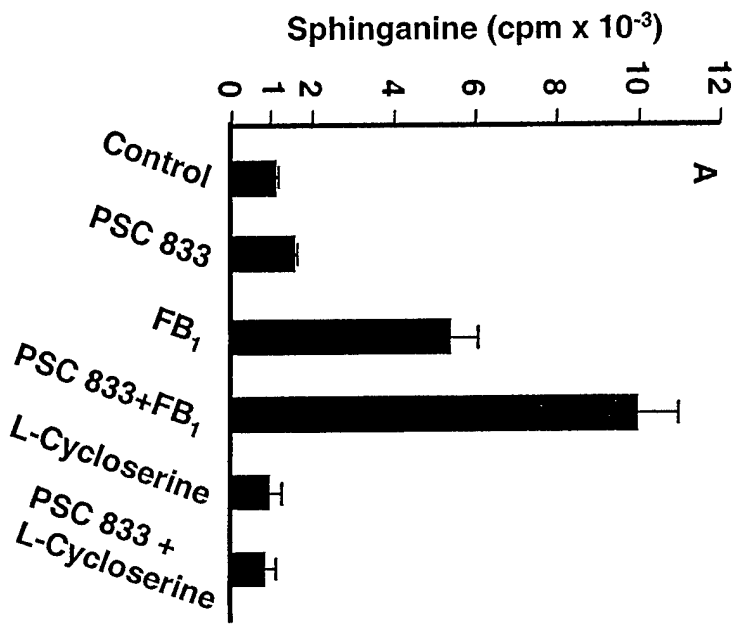
Fig. 5. Comparison of the effects of cyclosporine A and PSC 833 on ceramide and sphinganine generation in MDA-MB 468 cells. Cells were treated with either cyclosporine A (10 μM) or PSC 833 (10 μM) and simultaneously radiolabeled with [³H]palmitic acid for 4 h. Cellular lipids were extracted, and ceramide and sphinganine were analyzed by TLC and LSC. Data represent the mean ± SD of triplicate samples.

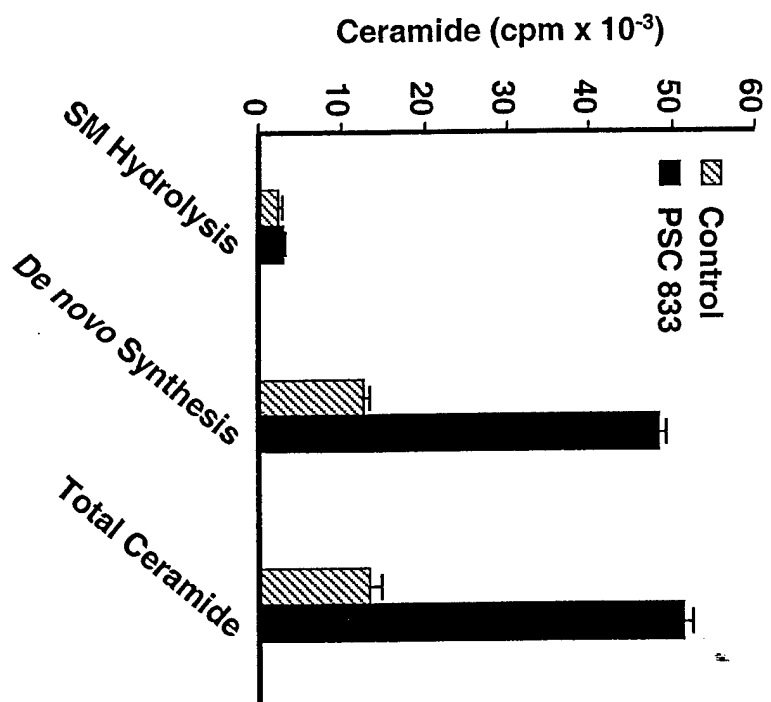
Fig. 6. Time course and dose response effects of PSC 833 on cellular SPT activity measured *in vitro*. *A*, time course study. MDA-MB 468 cultures were pretreated with 10 μ M PSC 833 for the indicated times. *B*, dose response study. Cultures were treated with PSC 833 at the concentrations indicated for 2 h. Following treatment, cells were harvested, microsomes were isolated, and SPT activity was determined. Data represent the mean \pm SD of duplicate samples, and calculations are based on pmol sphinganine formed/mg protein/min.

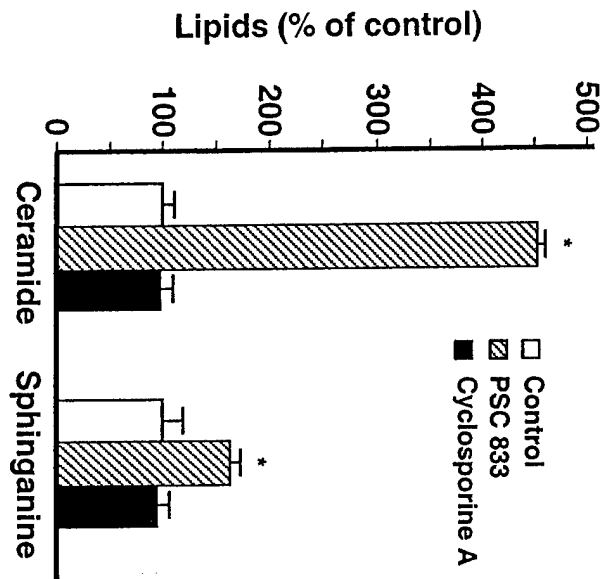
Fig. 7. Effects of PSC 833 on ceramide generation in various human breast cancer cell lines. Experiments were performed at \sim 80% cell confluence. The cells were treated with 5 μ M PSC 833 for 1 h, before the addition of [3 H]palmitic acid (1.0 μ Ci/ml medium) for additional 3 h. Total lipids were extracted, and ceramide was quantitated by TLC and LSC. Data represent the mean \pm SD of triplicate samples.

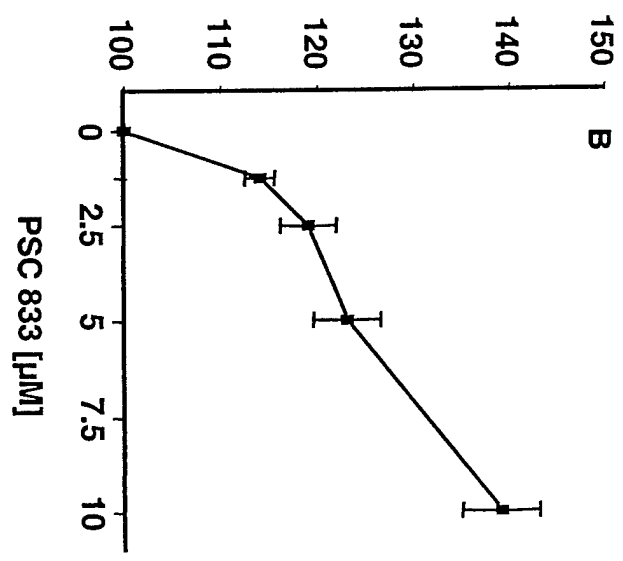
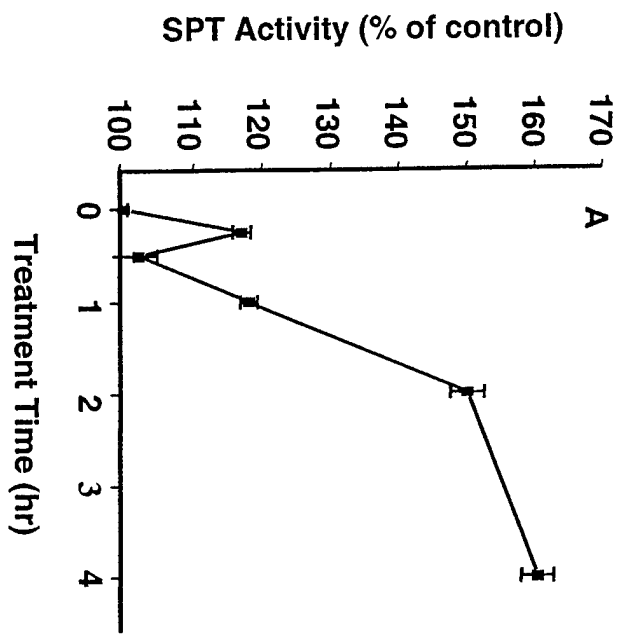


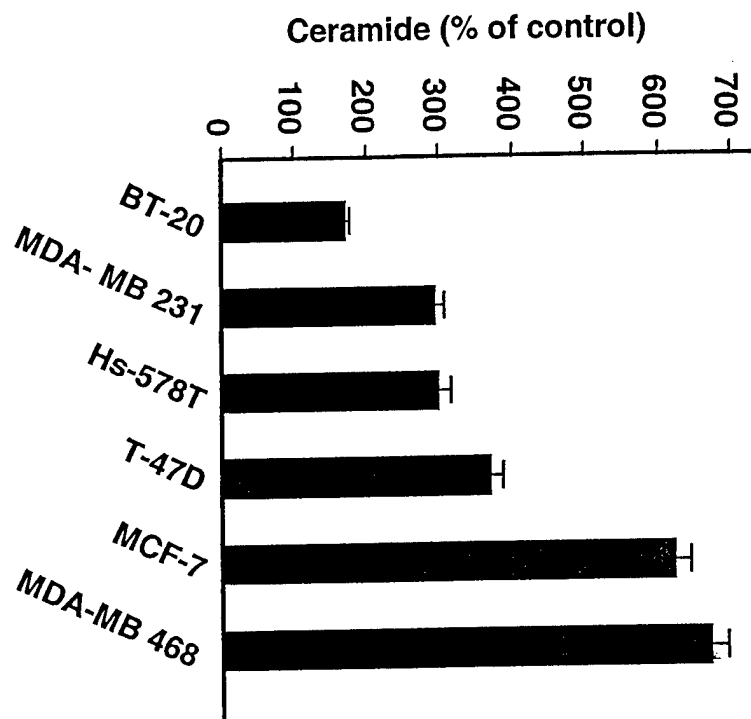












Myles Cabot

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