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13. ABSTRACT ***(Maximum 200 Words)***

Our previous studies showed that the drug resistance modulator, PSC 833, increased cellular ceramide levels, thus initiating caspase-apoptotic signaling. The mechanism of PSC 833 induced ceramide generation was, however, unknown. In order to use ceramide targeting as a therapeutic approach to chemotherapy sensitization in breast cancer, mechanism information is essential. During the past three years, our studies demonstrate that PSC 833 induces ceramide generation via the *de novo* biochemical pathway, as opposed to degradation of sphingomyelin. We have shown, for the first time, that serine palmitoyltransferase (SPT), and not ceramide synthase is activated by PSC 833. Furthermore, we also demonstrate a close structure-activity relationship for activation of SPT. 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. In combination with a downstream ceramide modulator, PSC 833 greatly elevates ceramide and synergizes chemotherapy-elicited cytotoxicity in breast cancer cells. 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, verapamil, cyclosporine A, PSC 833, reverse drug resistance (13,14). While studies have shown that some 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 previous studies have shown that PSC 833 markedly enhances cellular ceramide formation (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). We will characterize the biochemical mechanism of action of PSC 833 on cellular ceramide metabolism and use PSC 833 as a tool, together with conventional drugs like adriamycin and taxol, in an effort to enhance activity. This work will establish the contribution of ceramide to chemotherapy sensitization in breast cancer, and set the stage for alternative modes to treat drug resistant disease. 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 focused our studies (listed in the Statement of Work) in the second grant year and there after on characterizing SPT activity in breast cancer. We have assessed 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 such as, 4-HPR [N-(4-Hydroxyphenyl)retinamide], daunorubicin, etoposide, and taxol. These experiments have clarified the role of SPT activation in drug induced apoptosis in breast cancer, and provide a novel approach for anticancer drug design strategy, based upon targeting enzymes of sphingolipid metabolism.

BODY

1. **Development of research methods:**
   a. **Thin-layer chromatographic analysis of sphingolipids.** I have developed various techniques of cellular lipid radiolabeling and thin-layer chromatography (TLC) for analysis of 3-ketoshinganine, sphinganine, and sphingosine, and formulated systems that distinguish between ceramide generated de novo versus generation by sphingomyelin hydrolysis. Using these techniques, I have shown that PSC 833 induces ceramide generation via de novo synthesis, and that an enzyme upstream of ceramide synthase is activated, namely SPT.
   b. **Enzyme Assays.** Several enzymes, such as palmitoyl coenzyme A synthetase, serine palmitoyltransferase, and ceramide synthase, are involved in de novo synthesis of ceramide. Test tube assays are useful to clarify the enzymes responsible for PSC 833 induced ceramide generation. However, in vitro assays for palmitoyl coenzyme A synthetase, SPT, and ceramide synthase have not been developed for human breast cancer cell lines. I have successfully developed and standardized assays for these three important enzymes. Establishing these new enzyme assay techniques in breast cancer cells was crucial to the project goals, and the progress was key to our accomplishments.

2. **PSC 833 induces apoptosis and reverses multidrug resistance in breast cancer cells.** PSC 833 induces apoptosis in both human MDA-MB 468 and in human MCF-7 breast cancer cell lines (in a dose-dependent manner). PSC 833 also reverses resistance to adriamycin in MCF-7/AdrR cells, a breast cancer cell line resistant to adriamycin and other natural product anticancer drugs.

3. **PSC 833 increases ceramide generation in a time- and dose-dependent manner.** (see Appendix 1, p. 721, Figure 2).

4. **PSC 833 increases ceramide generation via the de novo pathway and not through sphingomyelin hydrolysis.** (see Appendix 1, p. 722, Figures 3, 4).

5. **PSC 833 activates cellular SPT.** (see Appenidx 1, p. 723, Figure 6).

6. **PSC 833 has no influence on either ceramide synthase or palmitoyl CoA synthetase activities.** (see Appendix 1, p. 723, Table 1).

7. **C_6-Ceramide is cytotoxic to human breast cancer cell lines.** C_6-Ceramide is cytotoxic to the human breast cancer cell line, MDA-MB 468 (EC_{50} = 1.9 μM). This supports the activity of ceramide as a second messenger in chemotherapy induced cytotoxicity in human breast cancer.

8. **Molecular 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 (prostate cancer) 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 clarity 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.** (see Appendix 1, p. 723, Figure 5).

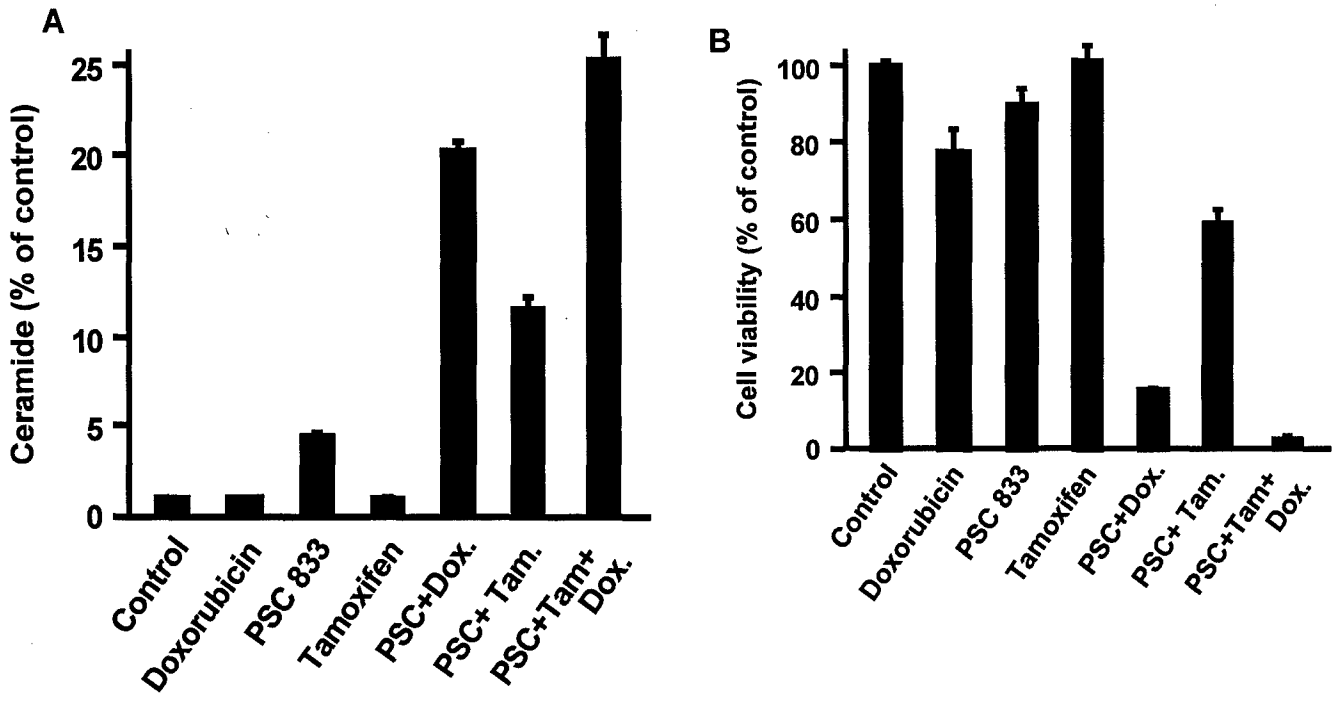
9. **PSC 833 does not stimulate SPT activity directly:** (see Appendix 1, p.724). Preincubation of microsomes (complete cell-free enzyme assay system minus substrate) with PSC 833 (10 μM) for 10 min at 37°C, followed by substrate addition and incubation for 10 min, failed to stimulate SPT activity compared to controls (minus PSC 833).

This shows that enzyme activation is not direct. However, it is important to note that stimulation of SPT can be achieved in intact cells upon exposure to PSC 833, followed by isolation of microsomes for cell-free assay. This suggests that the machinery of the intact cell is first required for the SPT activation sequence.

10. **Sphingolipid generation initiated by PSC 833 in different human breast cancer cell lines, and SPT activation initiated by different chemotherapeutic drugs:**
    a. **PSC 833 elicits ceramide generation in a number of human breast cancer cell lines.** (see Appendix 1, p. 724, Figure 7).
    b. **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.** (see Appendix 1, p. 724).

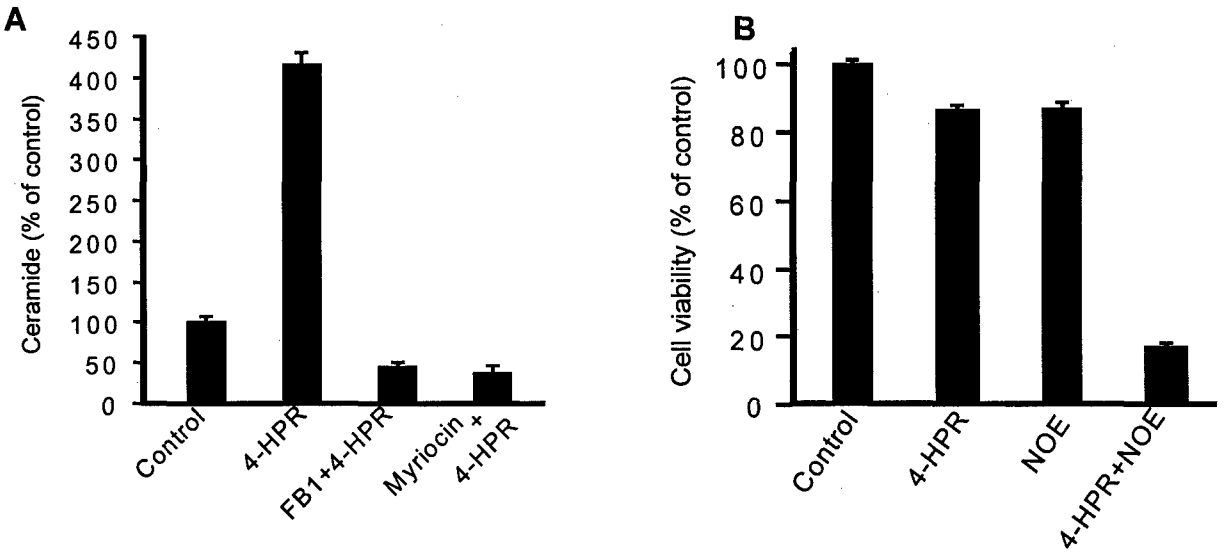
11. **Evidence that PSC 833 has a P-glycoprotein independent pathway and therapeutic effects:**
    a. **MDA-MB 468 is a P-gp negative human breast cancer cell line.** (see Appendix 1, p. 723).
    b. **PSC 833 elicits ceramide generation in a number of human breast cancer cell lines, independent of P-gp and estrogen receptor status.** (see Appendix 1, p. 724, Figure 7).
    c. **PSC 833 cytotoxicity is independent of P-gp expression in MCF-7 cells.** MCF-7 is a P-gp negative cell line, whereas MCF-7/MDR1 is a P-gp positive cell line generated by transfection of MCF-7 with the mdr1 gene. This cell line shows a stable MDR phenotype, in vitro. The observed EC_{50} for PSC 833 was 7.2 ± 0.6 μM in MCF-7 cells and 11.0 ± 1.0 μM in MCF-7/MDR1 cells. This shows that overexpression of MDR1 decreased but did not eliminate the cytotoxic effect of PSC 833.
    d. **P-gp expression does not influence PSC 833-induced ceramide elevation in MCF-7 cells.** Ceramide synthesis in response to 5.0 μM PSC 833 increased 4.5-fold in P-gp negative MCF-7 and in P-gp positive MCF-7/MDR1 cells. Thus, PSC 833 increases intracellular ceramide levels by a P-gp-independent pathway.

12. **Effect of combination drug treatment on ceramide metabolism and viability in MCF-7/AdrR adriamycin resistant cells:**
    a. **Influence of combination drug treatment with PSC 833 on (A) ceramide levels and (B) cell viability.**



Cells were treated with vehicle (control), doxorubicin (2.5 μM), PSC 833 (5 μM), tamoxifen (5 μM), or the combination. After 24 hr of exposure, ceramide was analyzed by TLC; after 3 days of exposure, cell viability was evaluated.

b. **Influence of combination treatment with 4-HPR, another de novo ceramide generator similar to PSC 833, on ceramide levels and cell survival:**



A. Ceramide generation in MCF-7/AdrR cells treated with vehicle (control), 4-HPR (5 μM), combination with FB_1 (50 μM), or myriocin (0.1 μM) for 24 hr. Ceramide was analyzed by TLC; **B.** Cells were treated with vehicle (control), 4-HPR (5 μM), ceramidase inhibitor N-oleoyl-ethanolamine (NOE) (5 μM), or the combination. After 3 days of exposure, cell viability was evaluated.

The above experiments show that use of a ceramide generator (PSC 833; 4-HPR) in conjunction with an agent that retards ceramide metabolism (tamoxifen; NOE) is synergistic for ceramide elevation and cell killing.

KEY RESEARCH ACCOMPLISHMENTS

- developed TLC methods for 3-keto-shinganine, sphinganine, and sphingosine analysis;
- developed a method for quantitative analysis of the ceramide generated from different biochemical pathways;
- developed and standardized enzyme assays for SPT, ceramide synthase, and palmitoyl coenzyme A synthetase;
- determined that PSC 833 induces apoptosis and reverses multidrug resistance in human breast cancer cells;
- demonstrated that PSC 833 increases ceramide generation in a time- and dose-dependent manner;
- determined that PSC 833 increases ceramide generation via the de novo pathway, and not by sphingomyelin hydrolysis;
- demonstrated that PSC 833 activates SPT in in vitro assays using microsomes obtained from PSC 833-pretreated cells;
- determined that PSC 833 has no influence on ceramide synthase and palmitoyl CoA synthetase activities;
- determined that the effect of PSC 833 on SPT activity is closely related with molecular structure, showing a stringent structure-activity relationship.
- determined that C_6-ceramide is cytotoxic to human breast cancer cells;
- determined that PSC 833 increases SPT activity independently of SPT gene transcription;
- development of cell lines: MDA-MB 468/PSC 833R (PSC 833 resistant breast cancer cell line) and MCF-7/PSC 833R (PSC 833 resistant breast cancer line);
- determined that activation of SPT by PSC 833 treatment is independent of PKC and MAP kinase signal transduction pathways;
- 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 P-gp and ER status;
- demonstrated that SPT, and not palmitoyl coenzyme A synthetase or ceramide synthase, is the target enzyme activated by several well known chemotherapeutic drugs;
- demonstrated that drug combinations consisting of PSC 833 and various modulators of ceramide metabolism are effective regimen for breast cancer cell killing.
- From this we suggest that elevation of de novo ceramide synthesis through SPT activation will provide a new approach for modulating multidrug resistance in breast cancer.

REPORTABLE OUTCOMES

A. **Original papers.**

1. Wang H, Giuliano AE, Cabot MC. Enhanced de novo ceramide generation through activation of serine palmitoyltransferase by the P-glycoprotein antagonist SDZ PSC 833 in breast cancer cells. Mol. Cancer Ther., 2002; 1: 719-726.
2. Gupta MS, Wang H, Cabot MC, Gennings C, Park M, Gewirtz DA. Influence of the vitamin D_3 analog, EB 1089 on senescence, apoptosis and sensitivity to fractionated radiation in MCF-7 breast tumor cells. (Submitted to: Int J Rad Oncol, 2002).
3. Han TY, Liu YY, Wang H, Bleicher R, Gouaze V, Gottesman MM, Bitterman A, Giuliano AE, Cabot MC. Enhanced ceramide glycosylation in cancer cells selected to resistance to vinblastine adriamycin. (in progress).

2. Abstracts:

1. Wang H, Giuliano AE, Cabot MC. Ceramide elevated by the P-glycoprotein antagonist, SDZ PSC 833, synergizes chemotherapy-elicited cytotoxicity in breast cancer cells. (Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Sept 25-28, 2002, Orlando, FL.)
2. Wang H, Giuliano AE, Cabot MC. Serine palmitoyl-transferase is the target enzyme in synthesis of ceramide elicited by anticancer drugs in breast cancer cells. Proceeding of American Association for Cancer Research, 2002; 43: 412.
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4. Liu YY, Wang H, Litvak DA, Giuliano AE, Cabot MC. Targeting ceramide-A therapeutic strategy for cancer treatment. Proceedings Molecular Biology and New Therapeutic Strategies: Cancer Research in the 21st Century. American Association for Cancer Research. P Feb. 12-16, 2001. Maui, Hawaii.
5. Liu YY, Wang H, Giuliano AE, Cabot MC. Targeting ceramide metabolism enhances chemotherapy cytotoxicity in drug resistant cancer cells. 18th UICC International Cancer Congress, June 30 - July 5, 2002, Oslo, Norway.

6. Bleicher R, Liu YY, Wang H, Gouaze V, Yu JY, Giuliano AE, Cabot MC. Glucosylceramide synthase upregulation - a new potential target for doxorubicin resistance in breast cancer. (Submitted to annual meeting of the Society of Surgical Oncology, Aug. 2002).

C. **Presentation:**

1. Serine palmitoyltransferase is the target enzyme in de novo synthase of ceramide elicited by anticancer drugs. (seminar, John Wayne Cancer Institute, Aug. 2002, Santa Monica, CA).

D. **Development of Cell lines:** MDA-MB 468/PSC 833R and MCF-7/PSC 833R PSC 833 resistant breast cancer lines.

E. **Employment or Research Opportunity:** Will assume position of Research Scientist, Breast Cancer Research Program, at the John Wayne Cancer Institute, Santa Monica, CA.

CONCLUSIONS

During the past 3 years I have developed various new lipid analysis techniques using cultured human breast cancer cells as models, and developed and standardized several key enzyme assays. Our studies demonstrate that PSC 833 induces ceramide generation in breast cancer cell lines via a de novo pathway. We have shown, for the first time, that serine palmitoyltransferase is activated by PSC 833. This is important, as etoposide has also been shown to elicit cytotoxicity by activation of serine palmitoyltransferase (23). PSC 833 activates SPT independently of SPT gene transcription and independently of MAP kinase, and PKC signal transduction pathways. PSC 833 also induces ceramide generation in a broad spectrum of human breast cancer cell lines, independently of either estrogen receptor or P-gp status. Furthermore, we have demonstrated a close structure-activity relationship (of PSC 833) for induction of ceramide generation and serine palmitoyltransferase activation. Furthermore, and most importantly we have demonstrated that SPT is the target enzyme of several well-known chemotherapeutic drugs. Elevation of de novo ceramide synthesis through SPT activation may provide a new approach for modulating multidrug resistance in breast cancer. 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

Appendix 1:

Wang H, Giuliano AE, Cabot MC. Enhanced de novo ceramide generation through activation of serine palmitoyltransferase by the P-glycoprotein antagonist SDZ PSC 833 in breast cancer cells. Mol Cancer Ther 1:719-726, 2002.

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Enhanced *de Novo* Ceramide Generation through Activation of Serine Palmitoyltransferase by the P-Glycoprotein Antagonist SDZ PSC 833 in Breast Cancer Cells[1]

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Abstract

SDZ PSC 833 (PSC 833), a P-glycoprotein-targeted multidrug resistance modulator, sensitizes cancer cells to chemotherapy. Here we show that PSC 833 also potentiates the formation of ceramide. Because ceramide is a second messenger in chemotherapy-induced apoptosis, knowledge of the lipid pathways influenced by PSC 833 is of relevance. In intact MDA-MB 468 breast cancer cells, ceramide generation increased 3-fold 1 h after PSC 833 addition (5.0 μM). Cyclosporine A, a structural analogue, failed to impact ceramide metabolism. Sphinganine, the upstream precursor of ceramide, also increased in response to PSC 833, and this could be blocked by adding L-cycloserine, a serine palmitoyltransferase (SPT) inhibitor. Exposure of cultured cells to PSC 833 (30 min to 4 h; 1–10 μM), followed by isolation of microsomes for *in vitro* assay, increased SPT activity 60%, whereas palmitoyl CoA synthetase and ceramide synthase activities were not altered. SPT activity was also heightened by pretreating cells with either paclitaxel, *N*-(4-hydroxyphenyl)retinamide, etoposide, or daunorubicin; however, activation was half that attained by PSC 833. PSC 833 stimulated ceramide generation in other breast cancer cell lines as well, including BT-20, MDA-MB 231, Hs 578T, T-47D, and MCF-7. In summary, several types of anticancer agents and the P-glycoprotein modulator PSC 833 share the ability to increase cellular ceramide levels by activation of SPT, the rate-limiting enzyme in the *de novo* pathway of ceramide synthesis. These data provide novel insight in the area of lipid-mediated cell death.

Introduction

Multidrug resistance is the major cause of cancer treatment failure (1, 2). Overexpression of P-gp,[3] a M_r 170,000 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). MDR modulators bind directly to P-gp and thereby interfere with cellular export of anticancer drugs. This approach appears to be a useful avenue for restoring cytotoxicity in drug-resistant cells; however, results from clinical trials are as yet inconclusive.

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 modulator, 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 part of the cytotoxic activity 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 tumor necrosis factor-α, 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, including palmitoyl CoA 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.

Because the ceramide *de novo* pathway inhibitor, FB_1, has been shown to block daunorubicin-induced ceramide formation and retard apoptosis in murine leukemia (21), it has been

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[3] The abbreviations used are: P-gp, P-glycoprotein; MDR, multidrug resistance; FB_1, fumonisin B_1; SPT, serine palmitoyltransferase; PSC 833, SDZ PSC 833 ([3′-keto-Bmt-1]-[Val-2]-cyclosporin); LSC, liquid scintillation counting; FBS, fetal bovine serum; 4-HPR, *N*-(4-hydroxyphenyl)retinamide, fenretinide.

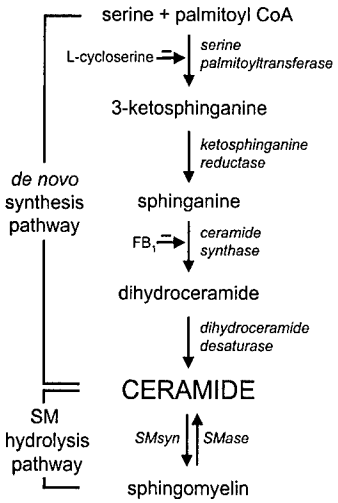

Fig. 1. Schematic of ceramide metabolism and the influence of FB_1 and L-cycloserine on the *de novo* pathway. *SM,* sphingomyelin; *SMase,* sphingomyelinase; *SMsyn,* sphingomyelin synthase.

widely accepted that the enzyme, ceramide synthase, is the sole target for various anticancer agents that use ceramide. More recent studies have, however, demonstrated that some anticancer drugs activate SPT (23), and we have shown in work with neuroblastoma that 4-HPR (or fenretinide) activates both SPT and ceramide synthase (15). Here, we have 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 (Fig. 1), in a cell line devoid of P-gp. We also show in breast cancer cells that other anticancer drugs, among them paclitaxel, 4-HPR, and etoposide, share this activity.

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 lines, MCF-7 and MCF-7 Adriamycin-resistant (MCF-7/AdrR), were obtained from Drs. Kenneth Cowan (University of Nebraska Medical Center Eppley Cancer Center, Omaha, NE) and 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). Paclitaxel, daunorubicin, and etoposide were from Sigma Chemical Co. (St. Louis, MO). 4-HPR was kindly provided by R. W. Johnson Pharmaceuticals (Spring House, PA). FB_1 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-^{3}H(N)]Palmitic acid (50 Ci/mmol) was from DuPont/NEN (Boston, MA). [5,6-^{3}H]Sphinganine (60 Ci/mmol, and L-[^{3}H(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). Monoclonal antibody, C219, to P-glycoprotein was from Signet Laboratories (Dedham, MA). Fluorescein-conjugated secondary antibody against mouse was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. MDA-MB 468, MDA-MB 231, MCF-7 and MCF-7/AdrR cells were cultured in RPMI 1640 containing 10% FBS and 584 mg/liter L-glutamine. T-47D cells were cultured in RPMI 1640 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 DMEM 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 nonessential 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% CO_2 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- or 96-well plates, or 6- 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 before use. Ethanol vehicle was present in controls.

Metabolic Labeling and Analysis of Cellular Lipids. After radiolabeling (1.0 μCi [^{3}H]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. [^{3}H]Ceramide was resolved from other radiolabeled lipids by TLC using a solvent system containing chloroform/acetic acid (90:10, v/v). [^{3}H]Sphinganine, sphingosine, and glucosylceramide were resolved by TLC in chloroform:methanol:ammonium hydroxide (70:20:4, v/v/v), and [^{3}H]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 interest 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 of homogenization buffer [20 mM HEPES (pH 7.4), 5 mM DTT, 5 mM EDTA, 2 μg/ml leupeptin, and 20 μg/ml aprotinin]. Cell suspensions were sonicated over ice for 60 s (20% output, alternating 15-s sonication and 20-s pause) using a Micro Ultrasonic Cell Disrupter from Konte (Vineland, NJ). Lysates were centrifuged at 10,000 × *g* for 10 min. The postnuclear supernatant was isolated and centrifuged at 100,000 × *g* for 60 min

at 4°C. The microsomal membrane pellet was resuspended in 100 μl of homogenization buffer by sonication for 5 s and frozen at −80°C (27).

SPT Assays. Enzymatic activity was determined by measuring the incorporation of [^{3}H]serine into 3-ketosphinganine. Each tube (final volume, 0.1 ml) 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 of 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 [^{3}H]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 of 0.5 N NH_4OH. Organic-soluble products were isolated by addition of 3 ml of chloroform:methanol (2:1), 25 μg of sphingosine carrier, and 2.0 ml of 0.5 N NH_4OH. 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), [^{3}H]sphinganine was used as radiolabeled precursor. The reaction mixtures contained 25 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 0.5 mM DTT, 10 μM sphinganine, and 100 μg of microsomal protein. Sphinganine was dried under nitrogen from a stock solution in chloroform:methanol (2:1) and dissolved with sonication in the reaction mixture before 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 [^{3}H]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 described previously (29). Microsomal protein (100 μg) was added to a buffer mixture containing 200 mM Tris-HCl (pH 7.5), 2.5 mM ATP, 8 mM $MgCl_2$, 2 mM EDTA, 20 mM NaF, 0.1% Triton X-100, and 10 μM palmitic acid. Reactions were initiated by simultaneous addition of acetyl CoA (0.1 mM) and 1.0 μCi [^{3}H]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 H_2SO_4 (40:10:1, v/v/v). After addition of 0.65 ml of H_2O and 1.5 ml of 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 of heptane containing 5 mg/ml palmitic acid, and 0.2 ml was analyzed by LSC. In control experiments, either the microsomes or acetyl CoA was omitted.

P-gp Expression. P-Glycoprotein expression was evaluated by immunofluorescence staining using C219 monoclonal antibody. Cells were grown on sterile coverslips in 6-well tissue culture plates. The coverslips were incubated in 4% paraformaldehyde for 5 min, fixed with cold acetone for 5 min, and preincubated with horse serum for 30 min. The coverslips were then incubated with C219 monoclonal antibody (diluted 1:100) in a humidified chamber at 4°C overnight. After washing with PBS, coverslips were incubated for 30 min with fluorescein-conjugated secondary antibody against mouse (diluted 1:200). Immunofluorescence staining was evaluated using an Olympus IX70 fluorescence microscope (Olympus, Inc., Tokyo, Japan). Coverslips were incubated with PBS instead of the primary antibody as a negative control. MCF-7/AdrR cells were used as positive controls.

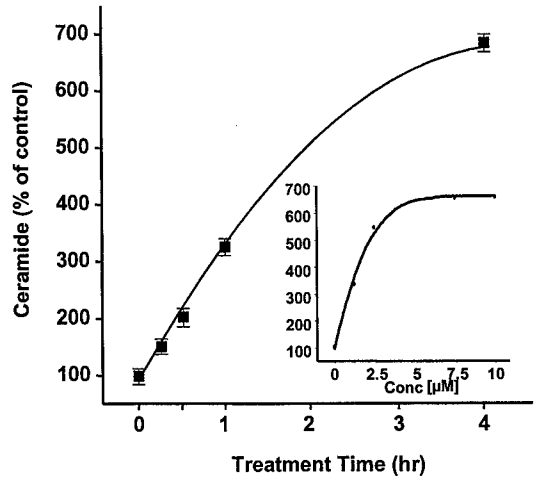

Fig. 2. Time course and dose-response effects of PSC 833 on ceramide generation in MDA-MB 468 cells. MDA-MB 468 cells were seeded in 6-well plates and experiments initiated at ~80% confluence. For the time study, PSC 833 (10 μM) and [^{3}H]palmitic acid (1.0 μCi/ml medium) were added simultaneously for the times indicated. For the dose-response study (*inset*), PSC 833, at the doses indicated and [^{3}H]palmitic acid (1.0 μCi/ml medium), were added simultaneously to the cells for 4 h. After exposure to PSC 833 under the conditions specified, total cellular lipids were extracted, and ceramide was quantitated by TLC and LSC. Data represent the means of triplicate samples; *bars*, SD. The data are representative of three independent experiments that gave similar results.

Results

The main pathways for cellular production of ceramide are shown in Fig. 1. L-Cycloserine and FB_1 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 ~5 μM (Fig. 2, *inset*).

Results using inhibitors of SPT and ceramide synthase indicated that PSC 833 targeted *de novo* ceramide synthesis upstream of ceramide synthase. As shown in Fig. 3*A*, PSC 833 alone activated sphinganine formation by 1.5-fold. Exposure of cells to FB_1, a ceramide synthase inhibitor, promoted sphinganine build-up that was 5-fold over control. When FB_1 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, sphinganine formation was halted in response to PSC 833. Under the same conditions, PSC 833 increased cellular ceramide levels 2.5-fold over control (Fig. 3*B*). FB_1

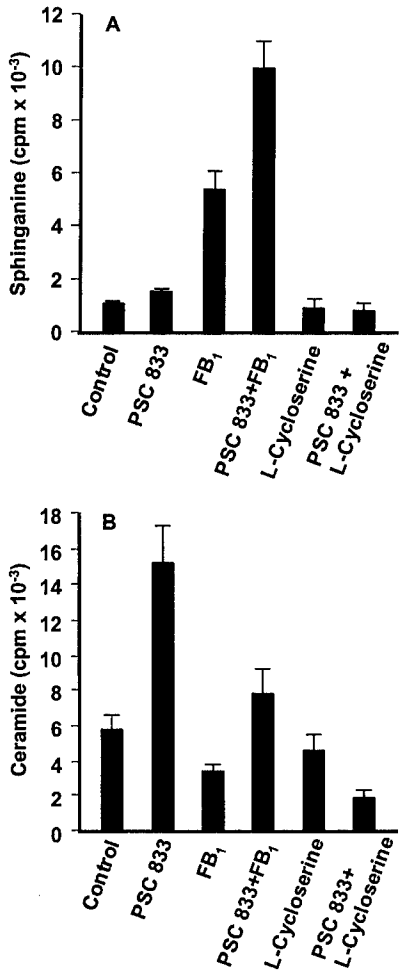

Fig. 3. Effect of *de novo* enzyme inhibitors FB_1 and L-cycloserine on PSC 833-induced sphinganine and ceramide formation. *A*, sphinganine formation. *B*, ceramide formation. At ~80% confluence, MDA-MB 468 cells were treated with either 50 μM FB_1 or 2 mM L-cycloserine for 30 min before the simultaneous addition of PSC 833 (10 μM) and [^{3}H]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 in specified lipid/500,000 cpm total lipid tritium and represent the means of triplicate samples; *bars*, SD. Experiments were conducted three times.

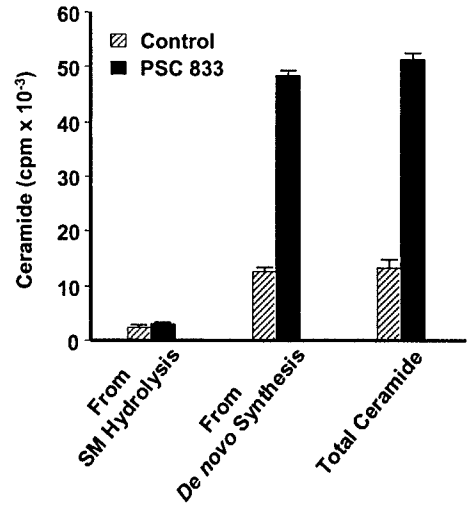

Fig. 4. Analysis of ceramide generated via *de novo* synthesis and by sphingomyelin hydrolysis. To measure ceramide generated by sphingomyelin hydrolysis, cells were prelabeled with [^{3}H]palmitic acid (1.0 μCi/ml medium) for 24 h. After washing and a 3-h chase in medium containing 5% FBS, cells were 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 [^{3}H]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 were treated with 10 μM PSC 833 and also supplemented with [^{3}H]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 means of triplicate samples; *bars*, SD. The experiments were conducted three times.

treatment decreased basal ceramide synthesis by ~40%, and FB_1 also decreased the formation of ceramide in response to PSC 833 treatment (PSC 833 + FB_1; Fig. 3*B*). Inhibition of SPT by L-cycloserine reduced the amount of baseline ceramide generated and severely retarded PSC 833-induced ceramide formation. Myriocin, a more specific SPT inhibitor, was also used to confirm the role of SPT in the PSC 833-governed lipid response. The addition of myriocin (0.25 μM) inhibited PSC 833-induced sphinganine formation by 80% and ceramide formation by 95%. Whereas this work demonstrates that PSC 833 accelerates ceramide synthesis through SPT/sphinganine, the experiments were conducted with cultured cells, and the data are not intended to relate kinetic or stoichiometric information.

The contributions of *de novo* synthesis and sphingomyelin hydrolysis to the production of ceramide by PSC 833 were compared by differential radiolabeling of cellular lipid pools. Twenty-four h prelabeling of cellular sphingomyelin with [^{3}H]palmitic acid followed by a wash to deplete cytoplasmic tritium and subsequent PSC 833 treatment yielded no increase over control in intracellular [^{3}H]ceramide (Fig. 4, *left*). However, when [^{3}H]palmitic acid and PSC 833 were added simultaneously, before palmitate was incorporated into sphingomyelin, intracellular [^{3}H]ceramide levels increased 4-fold over control (Fig. 4, *middle*). By the same token, combining both of the radiolabeling techniques, 24 h prelabeling of sphingomyelin pools followed by simultaneous addition of PSC 833 with a fresh bolus [^{3}H]palmitic acid, yielded the same 4-fold increase in [^{3}H]ceramide (Fig. 4, *right*). These experiments show that equilibrium radiolabeling of sphingomyelin does not enhance ceramide formed in response to PSC 833 exposure, demonstrating that sphingomyelin is not contributory to ceramide production when PSC 833 is present.

Because ceramide can be hydrolyzed by ceramidase and glycosylated to form glucosylceramide by glucosylceramide synthase, we also investigated the impact of PSC 833 on these metabolic pathways. Treatment of MDA-MB-468 cells with PSC 833 (10 μM) for 4 h in the presence of [^{3}H]palmitic acid increased the cellular glucosylceramide fraction. This would represent glycosylation of newly formed ceramide generated by PSC 833 exposure and as such demonstrates that the elevation of ceramide after PSC 833 exposure is not through glucosylceramide synthase inhibition. On the other

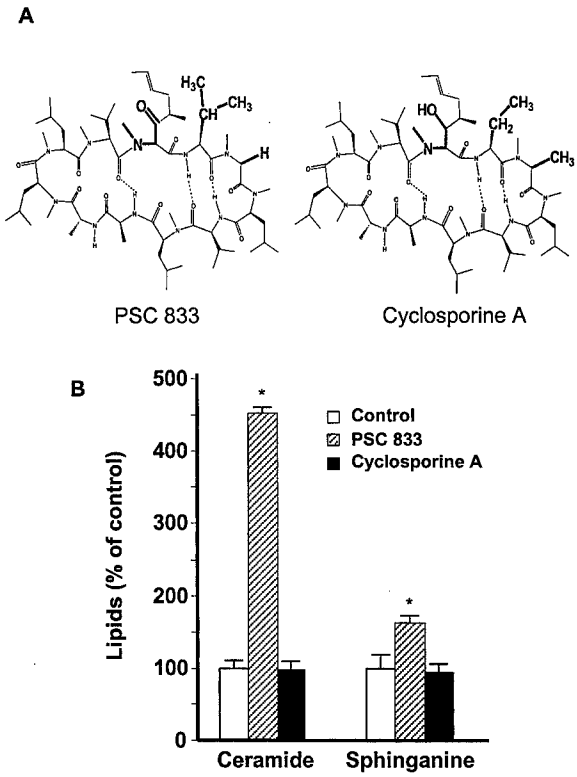

Fig. 5. Chemical structures and effects of cyclosporine A and PSC 833 on ceramide and sphinganine metabolism in MDA-MB 468 cells. *A,* chemical structures. *B,* lipid response. Cells were treated with either cyclosporine A (10 μM) or PSC 833 (10 μM) and simultaneously radiolabeled with [^{3}H]palmitic acid for 4 h. Cellular lipids were extracted, and ceramide and sphinganine were analyzed by TLC and LSC. Data represent the means of triplicate samples; *bars,* SD. The experiments were conducted three times.

hand, PSC 833 had no impact on cellular ceramidase activity, because no changes in levels of sphingosine, the product of ceramidase, were apparent with treatment.

Both PSC 833 and cyclosporine A are P-gp substrates and structurally nearly identical (Fig. 5*A*); however, a comparison of both agents shows that only PSC 833 increases cellular ceramide levels (Fig. 5*B*). After a 4-h exposure to PSC 833 (10 μM), cellular levels of [^{3}H]ceramide were >4-fold control values. PSC 833 also promoted formation of sphinganine, ~50% over control (Fig. 5*B*), whereas cyclosporine A was without influence. The results from immunofluorescent staining showed that MDA-MB 468 cells are P-gp negative; therefore, these experiments also illustrate that the influence of PSC 833 on ceramide production is independent of P-gp.

Cell-free experiments were carried out to assess whether exposure of intact cells to PSC 833 would modify enzyme activity *in vitro*. MDA-MB 468 cells were cultured with PSC 833 before harvesting and isolation of microsomes for *in vitro* assays. Of the major enzymes in the *de novo* synthesis pathway (see Fig. 1), only SPT activity was enhanced by pretreatment of cells with PSC 833 (Table 1). SPT activity, measured by sphinganine formation, was 57 ± 0.2 and 91 ± 7.1 pmol/mg protein/min in control and PSC 833-treated cells, respectively, accounting for a 60% increase in enzymatic activity. Palmitoyl-CoA synthetase and ceramide synthase activities were not influenced. Further studies revealed that stimulation of SPT by PSC 833 was both time and dose dependent (Fig. 6). SPT activation was biphasic with regard to time (Fig. 6*A*), with an early peak at 15 min (17% increase) followed by prolonged and greater activation thereafter (50%

Table 1 Effect of cellular exposure to PSC 833 on the enzymes of *de novo* ceramide synthesis measured in cell-free assays

After PSC 833 exposure (10 μM) 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 ± SD of triplicate samples. The data shown are representative of three independent experiments; all gave similar results.

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

[a] Pal-CoA syn, palmitoyl-CoA synthetase, palmitoyl-CoA formed; SPT, sphinganine formed; Cer syn, ceramide synthase, ceramide formed.
[b] Statistically significant from control, $P < 0.01$.

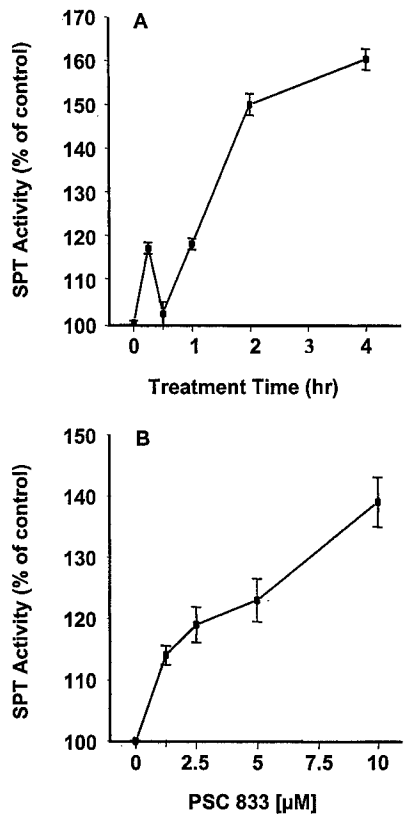

Fig. 6. Time course and dose response of PSC 833 on cellular SPT activity measured *in vitro*. *A,* time course study. MDA-MB 468 cultures were pretreated with 10 μM PSC 833 for the indicated times. *B,* dose-response study. Cultures were treated with PSC 833 at the concentrations indicated for 2 h. After treatment, cells were harvested, microsomes were isolated, and SPT activity was determined using cell-free assays. Data represent the means of duplicate samples, and calculations are based on pmol sphinganine/mg protein/min; *bars,* SD. These experiments were conducted two times. Both sets gave the same results.

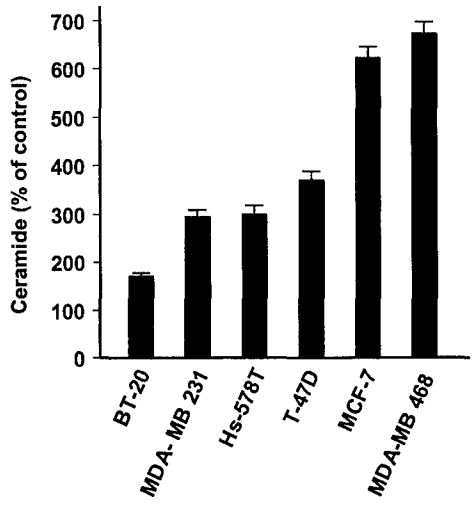

Fig. 7. Effects of PSC 833 on ceramide generation in various human breast cancer cell lines. Experiments were performed at ~80% confluence. The cells were treated with 5 μM PSC 833 for 1 h, before the addition of [^{3}H]palmitic acid (1.0 μCi/ml medium), for an additional 3 h. Total lipids were extracted, and ceramide was quantitated by TLC and LSC. Data represent the means of triplicate samples; *bars,* SD. These experiments were conducted two times, both yielding similar results.

increase by 2 h). SPT activity was enhanced over a concentration range of 1–10 μM PSC 833 (Fig. 6*B*). When added directly to the enzyme incubation, PSC 833, at levels up to 10 μM had no influence on SPT activity. Thus, there is no direct effect on the enzyme.

We evaluated other human breast cancer cell lines selected from a spectrum of estrogen receptor-positive and -negative cells to determine the cell type specificity of the ceramide response to PSC 833. As shown in Fig. 7, all cell lines tested responded to PSC 833. The range of activation of ceramide synthesis was from 3-fold to nearly 7-fold, with the exception of BT-20 cells which showed the weakest 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 μM (data not shown). Whether this is because of low ceramide burden remains to be shown.

We next compared PSC 833 to several known anticancer agents for influences on ceramide metabolism. When added to cultures of MDA-MB 468 cells, all of the drugs evaluated were shown to accelerate *de novo* [^{3}H]ceramide generation, with 4-HPR being the most potent (nearly 300% over control; Table 2). Paclitaxel and etoposide enhanced the production of [^{3}H]ceramide 189 and 161%, respectively. Daunorubicin was the least effective in enhancing *de novo* ceramide production. Cell-free enzyme assays conducted after exposure of cells (6 h) to the various chemotherapy drugs showed that SPT was targeted (data not shown). Paclitaxel (1.0 μM), 4-HPR (10 μM), and etoposide (1.0 μM) enhanced *in vitro* SPT activity similarly, by 24–29% over control, whereas daunorubicin had minimal impact (114 ± 4.7%). Neither palmitoyl CoA synthetase nor ceramide synthase activities were significantly altered by pretreating cells with the chemotherapy drugs. These experiments suggest that SPT may be a target in reactions involving *de novo* ceramide-mediated cell death.

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 paclitaxel (1.0 μM), 4-HPR (10 μM), etoposide (10 μM), daunorubicin (10 μM), or PSC 833 (10 μM) with simultaneous addition of [^{3}H]palmitic acid. Cellular lipids were extracted and analyzed as detailed in "Materials and Methods." The data are representative of two to four independent experiments; all gave similar results.

Drugs	Ceramide (% of control)
Paclitaxel	189 ± 2.4[a]
4-HPR	298 ± 6.7[a]
Etoposide	161 ± 3.9[a]
Daunorubicin	136 ± 0.8[a]
PSC 833	530 ± 8.9[a]

[a] Statistically significant compared with control, $P < 0.01$.

Discussion

PSC 833, a second generation P-gp antagonist developed to treat MDR, is being evaluated in patients with advanced cancers including acute myeloid leukemia and refractory ovarian carcinoma (30–32). Many studies demonstrate that PSC 833 retards drug efflux (5, 33); however, our group has determined that PSC 833 also activates ceramide formation in cancer cells (16, 17). Because ceramide has been linked with apoptosis pathways elicited by chemotherapy drugs (reviewed in Ref. 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, PSC 833 acts independently of P-gp to enhance apoptosis through sphingomyelin/ceramide-linked events (34), and in prostate cancer cells, 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 MDR modulators hold promise as co-drugs in cancer therapy, and therefore knowledge of mechanisms and targets is essential for furthering therapeutics in this area.

C_6-Ceramide causes MDA-MB-468 cell death with an EC_{50} of <2.0 μM (data not shown). With drugs that generate ceramide such as etoposide (23), daunorubicin (21), and paclitaxel (14), the inclusion of ceramide synthesis inhibitors has been shown to reverse drug cytotoxicity. Recently, it has been reported that *de novo* ceramide synthesis inhibitors also significantly reduce PSC 833-induced apoptosis in the human T leukemia cell lines, Molt-4 and Jurkat (36). In the current study, use of *de novo* ceramide synthesis inhibitors did not reduce PSC 833-induced apoptosis in MDA-MB-468 cells. These dissimilar findings may be reflective of cell type-specific responses to PSC 833.

Experiments using the ceramide synthase inhibitor, FB_1, and SPT inhibitors, L-cycloserine and myriocin, indicate that PSC 833 enhances *de novo* ceramide synthesis, targeting upstream of ceramide synthase. The FB_1 used in this study was 98% pure by TLC. We used a level that was not cytotoxic for MDA-MB 468 cells but that would also decrease the PSC 833-enhanced complement of ceramide production

through ceramide synthase (PSC 833 + FB_1; Fig. 3*B*). Depending on the cell line, some cells are extremely sensitive to FB_1. The 50 μM concentration was optimal for inhibition of ceramide synthesis while not being cytotoxic in MDA-MB 468 cells. The *in vitro* enzymology experiments demonstrate that PSC 833 promotes ceramide formation by enhancing SPT activity and not by stimulation of ceramide synthase or palmitoyl CoA synthetase activities. Therefore, both the use of inhibitors and *in vitro* assays support the idea that PSC 833 targets SPT.

Our preliminary studies show that no gross changes occur 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. The rapid activation time (Figs. 2 and 6) is further support for a nontranscriptional effect of PSC 833 on SPT. Although P-gp is not required for ceramide formation (18), influences on lipid transport and substrate localization caused by PSC 833 may play a role in enhancing ceramide formation in intact cells. MDR3 P-gp can function as a phosphatidylchcline translocase (37). Similarly, PSC 833 has been shown to influence sphingolipid translocation in CHO cells (38). Similar physical effects may be in operation at the ER/Golgi level with PSC 833; however, the enhanced SPT activity in cell-free incubations using exogenously added radiolabeled substrate (Fig. 6) would argue against physical changes contributing to SPT activation in intact cells.

Several known chemotherapy drugs also stimulated ceramide generation through SPT in MDA-MB 468 cells. The degree of enhancement (Table 2 and *in vitro* SPT results) may simply be reflective of drug lipophilicity enhancing cellular uptake. SPT may be a common target in the cytotoxic mechanism of some anticancer agents, because other studies show similar results. In Molt-4 human leukemia cells, etoposide enhances ceramide formation through activation of SPT (23), and in a human neuroblastoma cell line, 4-HPR enhances ceramide generation by activating both SPT and ceramide synthase (14). The anticancer agents studied in this paper are all of dissimilar structure, with the exception of PSC 833 and cyclosporine A. One could argue that PSC 833 and other agents that induce cell death share the ability to cause SPT activation and contribute to *de novo* ceramide synthesis. These observations provide novel insight in the field of lipid-mediated cell death. That cyclosporine A was devoid of activity compared with PSC 833 is noteworthy, because 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 (see Fig. 5*A*). Use of structural intermediates would be helpful in characterizing chemical and stereochemical specificity of the ceramide response.

Results from this work provide strong evidence that PSC 833 activates SPT. 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, because we demonstrated that all breast cancer cells tested exhibited a response, albeit the BT-20 response was minimal by comparison. Regarding the dose-response studies conducted with PSC 833, similar curves were achieved in Figs. 6 and 2; however, in the enzyme assay, the response did not plateau. These differences are likely attributable to the dissimilar experimental protocols. The whole cell experiment used *in vivo* radiolabeling and relied on coordinated responses of three enzymes in the ceramide synthesis pathway, whereas the *in vitro* assay was limited to one enzyme catalyzing sphinganine formation, the product of SPT. That the curve of Fig. 6*B* did not plateau may be attributable to incomplete reaction conditions and failure to test concentrations in excess 10 μM. Pharmacokinetically, the dose-response effect of PSC 833 on ceramide metabolism is likely more typical in the whole cell experiment of Fig. 2 *versus* the enzyme reconstitution study conducted for activation purposes.

Knowledge of sentinel cellular targets of agents such as PSC 833 and other anticancer drugs is necessary for the design of more effective treatments. In this specific instance, drugs that target ceramide metabolism have been shown to be effective in the elimination of cancer cells (11, 39). Strategies for targeting ceramide synthesis and degradation to enhance the cytotoxic effects of chemotherapy have been reviewed recently (11). This approach holds promise as a clinical design for the treatment of cancer.

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INFLUENCE OF THE VITAMIN D_3 ANALOG, EB 1089 ON SENESCENCE, APOPTOSIS AND SENSITIVITY TO FRACTIONATED RADIATION IN MCF-7 BREAST TUMOR CELLS.

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ABSTRACT

Purpose: Previous work from this laboratory has demonstrated that vitamin D_3 and vitamin D_3 analogs such as EB 1089 and ILX-23-7553 enhance the response of MCF-7 breast tumor cells to both the chemotherapeutic drug, adriamycin, and to a single (10 Gy) dose of ionizing radiation, conferring susceptibility to apoptotic cell death. The current work examines the capacity of EB 1089 to modulate the response of these breast tumor cells to more clinically relevant radiation doses of 2 Gy (administered daily over a period of 5 days) and also addresses the induction of senescence and generation of the signaling molecule ceramide. In addition, the influence of EB 1089 on sensitivity to fractionated radiation was evaluated in two normal cell lines, human breast epithelial cells and human fibroblasts.

Materials and Methods: MCF-7 human breast tumor cells, normal human breast epithelial cells and human fibroblasts were treated with EB 1089 (100 nM) for 72 h followed by 5 daily doses of 2 Gy radiation. Following the last dose of radiation, cell viability and/or colony-forming ability were determined. Apoptosis was detected using the TUNEL assay and quantitated by alkaline unwinding. Senescent cells were identified by staining with SA-β-galactosidase. Generation of ceramide from [^{3}H] palmitic acid was assessed by thin layer chromatography and liquid scintillation counting.

Results: Fractionated radiation and EB 1089 each alone reduced viable MCF-7 breast tumor cell number by 75 % and 84 %, respectively while the combination of EB 1089 followed by radiation reduced viable cell number by 98%. EB 1089 and radiation alone each reduced clonogenic survival by ~ 28% while the combination of EB 1089 with radiation resulted in an ~ 80% reduction in survival. EB 1089 conferred susceptibility to apoptosis in MCF-7 cells exposed to radiation based on complementary assays for the induction of DNA fragmentation. Radiation alone, but not EB 1089, and the combination of EB 1089 with radiation induced a senescence response. EB 1089, radiation as well as the combination promoted ceramide generation in MCF-7 cells. However, EB 1089 failed to enhance the response to radiation in the normal breast epithelial cells or BJ fibroblast cells.

Conclusion: These studies indicate that the combination of EB 1089 with fractionated radiation results in the promotion of apoptosis and the induction of senescence in the breast tumor cell, both of which may prove to be linked to the generation of ceramide. The failure of EB 1089 to increase sensitivity to fractionated radiation in either normal breast epithelial cells or human

fibroblasts suggests that vitamin D_3 analogs have the potential to enhance the effectiveness of fractionated radiation therapy in breast tumor cells without sensitizing normal tissues to radiation.

Breast Cancer, Radiation therapy, Vitamin D, Apoptosis, Ceramide

INTRODUCTION

Many common cancers occur in hormonally responsive tissues including breast, endometrium and ovary in women and prostate in men. The occurrence of such cancers has been causally linked to endogenous steroidal hormones or their metabolites and possibly to other environmental chemicals (1). The inability to effectively control advanced breast cancer with successive applications of surgery, radiotherapy, endocrine therapy and chemotherapy has made it imperative to develop new clinical strategies (2). One approach has been to utilize various chemotherapeutic agents as radiation sensitizers in order to enhance cell killing while exposing the host to non-overlapping toxicities, thereby enhancing the therapeutic ratio (3, 4).

The growth of a variety of cultured human cancer cells, including breast tumor cells, has been shown to be sensitive to the metabolically active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) (5-7). Furthermore, $1,25(OH)_2$ D_3 has been reported to inhibit growth of tumor xenografts of colon carcinomas and melanomas in immunodeficient mice (6). Several $1,25(OH)_2D_3$ analogs have been synthesized to separate the hypercalcemic activity from the antiproliferative and differentiation promoting actions displayed by $1,25(OH)_2D_3$. One such second generation analog, EB 1089 (seocalcitol) is 50 times more effective than $1,25(OH)_2D_3$ against breast tumor cells in vitro (7). The vitamin D_3 analogs exert their antiproliferative effects via multiple pathways which include cross-talk with the estrogen signaling pathway (8), modulation of growth factor responses (9), modulation of cell cycle regulators (10-18) and interaction with retinoids (19).

Studies in this laboratory have shown that breast tumor cells are refractory to apoptosis in response to DNA damage by adriamycin (20) or ionizing radiation (21). However, apoptosis does occur in response to adriamycin and radiation in the presence of vitamin D_3 analogs,

indicating that these compounds may selectively manipulate the signaling pathway that is permissive for apoptotic cell death in the breast tumor cells (22-24).

The previous work in this laboratory describing the interaction of vitamin D_3 and its analogs with ionizing radiation (22, 24) involved a high (10 Gy) supra-clinical dose of radiation. As breast cancer is routinely treated using multiple fractionated low doses of radiation, in the range of 2 Gy, to minimize normal tissue toxicity (25), the present studies extends the previous findings by assessing the interaction of the vitamin D_3 analog, EB 1089 with a cumulative dose of 10 Gy administered daily in 2 Gy fractions. We further evaluated the effects of this combination in two normal cell lines (human breast epithelial cells and human fibroblasts) in order to address therapeutic ratio. Finally we assessed the influence of EB 1089 and radiation on a marker of senescence and evaluated the generation of ceramide, as several groups have demonstrated the association of ceramide with radiation induced cell death (26-28) as well as regulation of telomerase (29, 30).

METHODS AND MATERIALS

Materials

The p53 wild-type human breast tumor cell line, MCF-7 was obtained from NCI, Frederick, MD. The normal human breast epithelial cells and the BJ fibroblast cells were a gift from Dr Shawn Holt, Medical College of Virginia, VA. EB 1089 was provided by Dr. Lise Binderup, Leo Pharmaceuticals, Denmark. RPMI 1640 and supplements were obtained from GIBCO Life Technologies, Gaithersburg, MD. Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Boehringer Manheim,

Indianapolis, IN. [9,10-^{3}H]Palmitic acid (51 Ci/mmol) was from DuPont New England Nuclear, Boston, MA. All other reagents used in the study were analytical grade.

Cell Culture

All cell lines were grown from frozen stocks in basal RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin at 37°C under a humidified, 5% CO_2 atmosphere. MCF-7 cells were routinely checked for functional p53 status by western blotting for induction of p53 and p21 in response to radiation. The normal human cell lines have wild-type p53 function (Shawn Holt and Lynne Elmore, personal communication).

Cell viability

The effects of fractionated radiation and EB 1089, alone and in combination on viability of MCF-7 breast tumor cells and on normal human fibroblasts and breast epithelial cells were determined by trypan blue exclusion. The cells were treated with EB 1089 (100 nM), fractionated radiation (5 x 2 Gy) or their combination. For the combination studies, cells were treated with EB 1089 (100 nM) for 72 hrs after which EB 1089 was removed and cells washed twice with phosphate buffered saline. This approach was utilized based on the studies by Wang and coworkers (31) which have demonstrated a requirement for prolonged incubation with vitamin D_3 or its analogs to promote sensitivity to adriamycin as well as our own previous work (22-24). Cells were then subjected to fractionated radiation (5 x 2 Gy) with each 2 Gy dose administered daily. Approximately 6 hrs after the last dose of radiation, cells were harvested using trypsin, stained with 0.4% trypan blue dye and trypan blue negative cells were counted using phase contrast microscopy.

Fractionated Radiation Protocol

MCF-7 cells were exposed to five fractions of 2 Gy gamma radiation alone or in combination with EB 1089 on five consecutive days using the cesium irradiator at a dose rate of 3 Gy/m. The cells were maintained at 37°C between radiation doses and were prepared for assays (cell viability, clonogenic survival, TUNEL, morphology and alkaline unwinding), 6 hours following the fifth dose of radiation.

Clonogenic survival

MCF-7 cells were treated with EB 1089 (100 nM) for 72 hrs followed by radiation (5 x 2 Gy). Cells were trypsinized under sterile conditions following radiation and plated in triplicate in 6 well tissue culture dishes at the appropriate density for each condition. After 14 days, the cells were fixed with 100% methanol, air-dried for 1-2 days and stained with 0.1% crystal violet. For computing the survival fraction, groups of 50 or more cells were counted as colonies.

Cell Morphology

Following treatments, the cells were washed and cytocentrifuged onto microscopic slides. The cells were air-dried, stained with Wright-Giemsa stain and photographed under a Nikon light microscope.

TUNEL assay for apoptosis

The method of Gavrielli and coworkers (32) was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the

In Situ Cell Death Detection Kit (Boehringer-Manheim). In this assay, the fragmented DNA in individual cells was end labeled using fluorescein dUTP at strand breaks by the enzyme terminal transferase. The cells were then washed, mounted in Vectashield and photographed using a Nikon fluorescent microscope.

Alkaline unwinding assay

The induction of DNA fragmentation was substantiated using the alkaline unwinding assay as described previously (22). Briefly, this involved determination of the ratio of double stranded and single stranded DNA after exposure to the various agents. F values (33) were converted to radiation equivalence based on standardization of the assay using graded doses of radiation.

Senescence

Following treatment with EB 1089, radiation or the combination, MCF-7 cells were trypsinized and plated in 6-well plates. The cells were assayed for senescence on day 5 after the last dose of fractionated radiation. Attached cells were fixed in 2% formaledehyde and stained for SA-β-gal activity using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) at pH 6.0, as described (34). The SA-β-gal + cells, indicated by blue staining, were determined by bright-field microscopy.

Ceramide analysis

Cell radiolabeling with [^{3}H]Palmitic acid, lipid extraction and analysis [^{3}H]ceramide by TLC were conducted described previously (35). Lipids were visualised in iodine vapor.

Commercial standards were cochromatographed and spots were scraped and analyzed for tritium by liquid scintillation counting.

Synergism vs Additivity

The predicted response for the cell viability assay were determined using the following model: $y=\gamma\exp(-\exp(-(\beta_0 + \beta_1 x_1 + \beta_2 x_2))) + \varepsilon$ where y is the predicted response (% reduction in cell viability), x_1 is concentration of EB 1089 (nM), x_2 is the fractionated radiation (Gy), γ is an unknown parameter associated with the maximum effect response, β_0 is an unknown parameter associated with the intercept, β_1 is an unknown parameter associated with the slope of x_1 and β_2 is an unknown parameter associated with the slope of x_2. ε is an unobserved random error term assumed to have mean 0 and constant variance. Parameter estimates were found using a generalized least squares criterion for nonlinear models. A constant variance was assumed across the concentration range of the drug/radiation. The Gauss-Newton iterative algorithm was used in PROC NLIN in SAS (version 8.01) to find parameter estimates. An overall test for additivity (36) was based on testing the hypothesis that the mean response under the hypothesis of additivity is the true mean response at the observed mixture points. The estimated responses for the true means were provided by the sample means at each mixture group.

RESULTS

Effects of EB 1089 and fractionated radiation on MCF-7 viable cell number

Previous studies in this laboratory have demonstrated that EB 1089 enhances sensitivity of MCF-7 breast tumor cells to adriamycin (23) or single doses of ionizing radiation (22). In the current studies we determined whether EB 1089 would enhance the response to radiation administered in 2 Gy fractions over the course of 5 days and whether such an effect could be shown to be selective for the tumor cell. Although this protocol does not precisely simulate the clinical treatment of breast cancer, where a patient may receive cumulative doses of fractionated radiation of up to 40-60 Gy, we believe that fractionating the dose over a period of 5 days represents a reasonable approximation of the clinical approach.

Figure 1 indicates that while fractionated radiation (5 daily doses of 2 Gy) and 100 nM EB 1089 each alone produced a 75% and 84% reduction in cell number respectively, treatment with EB 1089 for 72 hrs prior to fractionated radiation reduced cell number by 98%. The effect of the combination of EB 1089 with fractionated radiation was not significantly different from either individual treatment alone in the p53-mutated MDA-MB 231 cells (data not shown), similar to our previous reports utilizing a single dose of radiation (22).

Effects of EB 1089 and radiation on clonogenic survival of MCF-7 cells

Clonogenic survival assays are routinely used to measure long-term survival of tumor cells as defined by their ability to form a multicellular colony from a single cell. Figure 2 shows that EB 1089 and fractionated radiation alone each reduced clonogenic survival of MCF-7 cells by approximately equal amounts (29% and 28% reductions respectively) while the combination of EB 1089 with fractionated radiation reduced clonogenic survival by ~80%.

Indications of apoptotic cell death in MCF-7 cells after combined treatment with EB1089 and fractionated radiation

Previous studies in this laboratory have demonstrated that breast tumor cells exposed to radiation are refractory to apoptosis in response to DNA damage by radiation (21) as well as clinically relevant doses of adriamycin (37, 20). However apoptosis does occur in response to adriamycin and radiation in the presence of vitamin D_3 analogs (22-24), suggesting that these compounds may selectively manipulate the signaling pathway that is permissive for apoptotic cell death in the breast tumor cells. The capacity of EB 1089 to promote apoptosis in MCF-7 cells in response to fractionated radiation was determined based on DNA fragmentation by TUNEL analysis and by alkaline unwinding.

The TUNEL assay in Figure 3 indicates that there was minimal evidence for apoptosis in MCF-7 control cells or cells treated with EB 1089 (100 nM) or fractionated radiation alone. However, the number of fluorescent cells was significantly increased by the combination of EB 1089 and fractionated radiation, consistent with the induction of apoptosis. EB 1089 and fractionated radiation, alone or in combination, did not exhibit any evidence of apoptosis in MDA-MB 231 cells as evaluated by TUNEL assay (data not shown).

The induction of DNA fragmentation in the MCF-7 cells by the combination of EB 1089 and fractionated radiation was further substantiated using the alkaline unwinding assay. Table I indicates that no DNA fragmentation was detected by fractionated radiation alone while a relatively small degree of fragmentation (equivalent to ~ 1 Gy when corrected for baseline levels of fragmentation) was evident with EB 1089 alone. The combination of EB 1089 with fractionated radiation produced a significant amount of DNA fragmentation, consistent with the

data presented in Figure 3. It should be emphasized that this assay is being utilized to measure (delayed) DNA fragmentation rather than direct and immediate DNA strand breaks produced by irradiation.

Although changes in cell morphology are not a rigorous approach for assessment of apoptosis in epithelial cells such as the MCF-7 breast tumor cell line, we did monitor cell morphology under the different experimental conditions. Figure 4 indicates that neither EB 1089 (100 nM) nor fractionated radiation alone produced a significant degree of apoptosis based on alterations in cell morphology such as cell shrinkage, nuclear condensation and apoptotic body formation; in contrast, treatment of the cells with EB 1089 prior to fractionated radiation resulted in distinct changes in cell morphology indicative of apoptosis, although this occurred in a relatively small fraction of the cell population.

Beta galactosdiase expression, a marker of senescence

Senescence, a physiological process that limits the proliferative span of normal cells, is characterized by distinct phenotypic changes including enlarged and flattened shape, increased granularity and shortening of telomeres (38). These authors have also suggested that tumor cells have retained at least some of the components of the senescence like program of terminal proliferation arrest. In the present study, Figure 5 shows that treatment of MCF-7 breast tumor cells with fractionated radiation or the combination of EB 1089 with fractionated radiation results in induction of SA-β-gal, a commonly used surrogate marker for senescence. However, a senescence response was not seen with EB 1089 alone. Further, the cells exposed to radiation alone or in combination with EB 1089 appeared enlarged and flattened consistent (34, 38) with the senescence like phenotype exhibited by senescent cells.

Generation of ceramide by EB 1089 and ionizing radiation in the breast tumor cell

Ceramide generation has been associated with apoptosis (39) and recently, with regulation of telomerase (29). To evaluate whether ceramide was generated in association with EB 1089 and radiation-induced cell senescence and cell death, MCF-7 cells were treated with EB 1089, ionizing radiation or the combination of EB 1089 with radiation and analyzed for ceramide generation. Table II (A) indicates that immediately after the 72 hour exposure to EB 1089, ceramide levels were 200% of control values while 24 hours after the EB 1089 was removed, ceramide levels declined to 132% of control values (Table II B). A single dose of radiation (10 Gy) likewise elicited an increase (148% of control) in ceramide. Interestingly, other studies (unpublished) in our laboratory have demonstrated that a single 10 Gy dose of radiation also promotes senescence in MCF-7 breast tumor cells. The combination of EB 1089 with radiation resulted in almost exactly additive increase in ceramide levels (to 172% of control values). Treatment with taxol, which was utilized as a positive control, increased ceramide levels to 196% of control values.

Effects of the combination of EB 1089 and fractionated radiation on viable cell number in normal cells

The effects of EB 1089, fractionated radiation and the combination of EB 1089 with fractionated radiation were also determined in normal breast epithelial cells (Figure 6) as well as BJ fibroblast cells (Figure 7) to ascertain effects of these modalities on normal tissues. Since these cell lines fail to form distinct colonies under normal culturing conditions (unpublished data) we determined the effect of these agents on cell viability. Figures 6 and 7 show that, in both

cell lines, the decrease in cell viability in response to the combination of EB 1089 and fractionated radiation were not significantly different from either of the agents alone. These data suggest that EB 1089 may not enhance the toxic effects on ionizing radiation in normal tissues.

Quantitative analysis of the interaction between EB 1089 and fractionated radiation in MCF-7 cells

The studies described in this manuscript show that EB 1089 in combination with fractionated radiation results in apoptotic morphology, decreased cell viability and decreased clonogenic capacity in MCF-7 cells. Statistical analysis was performed to determine if the observed effects of the combination of EB 1089 and fractionated radiation on cell viability were occurring through a synergistic or additive interaction between the two modalities. The study design included dose-effect data of fractionated radiation alone (5x0.5, 5x1.0, 5x2.0 and 5x3.0 Gy), concentration-effect data of EB 1089 alone (5, 10, 50, 100, 200 nM), and one mixture point of the combination (EB 1089 at 100 nM in combination with radiation doses of 5x2 Gy). Two separate experiments were conducted. The endpoint of interest was percent reduction in cell viability. The results of these assays were compared to those predicted by the statistical model of additivity (Table 3). The observed percent reduction in cell viability was determined to not be significantly different from those predicted by the model of additivity ($p=0.917$). Therefore, it was concluded that EB 1089 and fractionated radiation interact additively in terms of their effects on viable cell number.

DISCUSSION

Several studies have demonstrated the utility of combining vitamin D_3 analogs with conventional chemotherapeutic drugs such as tamoxifen, platinum compounds and adriamycin (31, 40-44). Breast tumor cells, in general, tend to be refractory to apoptotic cell death in response to modalities that induce DNA damage (21, 37, 45). Our recent work (22-24) has focused on utilizing vitamin D_3 analogs such as EB 1089 and ILX-23-7553 to confer susceptibility to apoptosis in response to adriamycin or ionizing radiation in the breast tumor cells. These studies have demonstrated that pretreatment with EB 1089 or ILX-23-7553 in combination with an acute dose of adriamycin (1 μM) or a single (10 Gy) dose of radiation resulted in enhanced cell killing as well as increased DNA fragmentation indicative of apoptosis (22-24).

This paper extends our previous work by evaluating the capacity of EB1089 to promote apoptosis in response to radiation delivered in a manner that more closely reflects clinical protocols. The data in this report indicates that EB 1089 interacts effectively with fractionated radiation to interfere with MCF-7 (which exhibit a wt p53) breast tumor cell growth based on the reduction in viable cell number as well as clonogenic survival. These data also establish the utility of EB 1089 in the promotion of apoptosis (as shown by TUNEL and alkaline unwinding assays) in breast tumor cells in response to fractionated radiation. In contrast, MDA-MB 231 cells, a p53 mutated cell line, fail to demonstrate an enhanced response to the combination of EB 1089 and fractionated radiation (data not shown). This latter finding is consistent with our previous reports suggesting that p53 may be required for promotion of apoptosis and chemo- and radio-sensitization by vitamin D_3 analogs in breast tumor cells (22, 24).

A comparison of the data generated using the cell viability and clonogenic survival assays indicates that the combination of EB 1089 with fractionated radiation is only additive in the cell viability assay and yet clearly greater than additive in the clonogenic survival assay. We believe that the promotion of apoptosis by the combination of EB 1089 with radiation may have a more pronounced impact on clonogenic survival than cell viability, as cells in the early stages of apoptosis are known to maintain their membrane integrity and would therefore appear viable in this shorter term assay.

A related question is why the fraction of viable cells (after either EB 1089 or fractionated radiation alone) is less than the surviving fraction in the clonogenic assay. It should be noted that similar observations were evident in our previous publication (22). We believe that this apparent discordance is a consequence of the fact that these cells have the capacity to recover proliferative capacity within a 10-14 day interval after removal of the vitamin D_3 analogs or subsequent to radiation (unpublished data). Similarly, in a recent study, colony survival assays show that at high doses of gamma radiation, human diploid fibroblasts do not irreversibly arrest and that a fraction of cells are capable of re-entering the cell cycle after exposure to ionizing radiation (46). Preliminary work in our laboratory suggests that the combination of the vitamin D_3 analogs with radiation, which promotes apoptosis, ~~blunts this~~ interfere with recovery of proliferative capacity.

We were surprised that the surviving fraction after 5 doses of 2 Gy did not differ substantively from the surviving fraction after a single dose of 2 Gy (22). It is likely that the 24 hour interval between radiation doses allows the cells to repair sublethal damage (47). Furthermore, the recovery of proliferative capacity after radiation may blunt the expected additive effect of multiple 2 Gy doses on clonogenic survival.

Ceramide has been shown to play a role in anthracycline-induced cell death (48, 49); radiation-induced apoptosis (50) and in inhibiting telomerase activity (29). Previous studies have shown that $1,25(OH)_2D_3$ stimulates the hydrolysis of sphingomyelin (51, 52) and a more recent study suggests that ceramide acts as a downstream effector of TNF α-induced apoptosis in breast cancer cells (53). Here for the first time we demonstrate that a vitamin D_3 analog elicits cellular ceramide generation and that when combined with radiation, ceramide levels are further increased while the cells demonstrate both apoptosis and senescence. These studies suggest that enhanced cell killing by radiation in the presence of EB 1089 could result, at least in part, from ceramide governed cellular sensitization initiated by pre-exposure to EB 1089. This mechanism would be similar to $1,25(OH)_2D_3$-enhanced Taxol antitumor activity in prostate (44) and the enhancement of Taxol-induced apoptosis by ceramide in leukemia (54). Furthermore, the senescence-like phenotype may also prove to be linked to ceramide generation. Current studies in our laboratory are focused on evaluating the relationship between ceramide generation, apoptosis and senescence in breast tumor cells.

Perhaps the most significant observation in the current study is that EB 1089 fails to enhance the response to fractionated radiation in two normal human cell lines, the normal breast epithelial cells and fibroblasts, suggesting that this (and possibly other) vitamin D_3 analogs might have the potential to enhance the therapeutic ratio for radiation treatments. A recent study indicated that the in vitro sensitivity of skin fibroblasts is correlated with complication in different organs and normal tissues following radiotherapy in a variety of tumors (55). Although we were unable to perform clonogenic survival with these cells, our previous work indicates that the cell viability assay is generally predictive of clonogenic survival (22-24). Studies are in progress to examine the basis for the lack of interaction between EB 1089 and fractionated

radiation in normal breast epithelial cells and human fibroblasts. In the broadest sense, we expect that apoptosis in these cells may be regulated through different signaling pathways than in the breast tumor cell. That is, these cells may respond to EB 1089 or radiation by undergoing apoptosis and/or senescence, while the combination of EB 1089 with radiation may fail to enhance the apoptotic response or drive the cells out of a senescent state. A primary focus of our future studies will be to determine the basis for the apparent selectivity of EB 1089 in combination with fractionated radiation, at least at the level of cellular models systems.

The mechanistic basis for refractoriness of breast tumor cells to DNA damage induced apoptosis as well as for the permissive effects of EB 1089 on the promotion of apoptosis in response to radiation remain to be fully elucidated. One hypothesis is that MCF-7 cells are refractory to apoptosis in response to radiation as radiation activates mitogenic pathways including epidermal growth factor (EGF) and insulin-like growth factor (IGF) pathways which are cytoprotective (56,57). It is possible that EB 1089 may increase the susceptibility of breast tumor cells to radiation-induced cell death by abrogating the survival signals generated through the growth factor signaling pathways. Several reports have also indicated that vitamin D_3 analogs, including EB 1089, can induce apoptosis in breast tumor cells by modulating genes in the bax/bcl-2 family (58-60). Recent work in this laboratory suggests that the lack of an apoptotic response to adriamycin in MCF-7 cells may be related to its capacity to induce phosphorylation and subsequent inactivation of the pro-apoptotic Bad protein (unpublished data). Studies are in progress to determine whether EB 1089 increases susceptibility to adriamycin and/or radiation-induced apoptosis by abrogating Bad phosphorylation.

In summary, this report demonstrates that pretreatment of breast cancer cells, but not human normal breast epithelial cells or fibroblasts, with EB 1089 potentiates the response to

fractionated radiation. Our data also show that these breast tumor cells exhibit increased apoptotic sensitivity and a senescence like phenotype, effects that may be linked to increased ceramide generation. These studies also suggest that the effects of EB 1089 and fractionated radiation may be mediated via both apoptosis and senescence. Since a majority of breast cancer patients are treated with radiation therapy, EB 1089 may be a potentially useful drug that could have clinical utility in combination with fractionated radiation.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Influence of EB 1089 on the antiproliferative effects of fractionated radiation in MCF-7 cells. Cells were treated with EB 1089 (100 nM) for 72 h and replaced with fresh media prior to fractionated radiation (2 Gy x 5). Following the fifth dose of radiation, viable cell number was determined by trypan blue exclusion assay. Data presented are means ± SEM of three experiments. * Significantly different than control ($p<0.05$); ** Significantly different than fractionated radiation or EB 1089 alone ($p<0.05$).

Figure 2: Clonal growth assay of MCF-7 cells following fractionated radiation with or without pretreatment with EB 1089 (100 nM). Data represent means ± range from two independent experiments.

Figure 3: Effects of combining EB 1089 with fractionated radiation (2 Gy x 5) in inducing DNA fragmentation in MCF-7 cells was determined by fluorescent end-labeling. Cells were isolated following the fifth dose of radiation, cytospun onto glass slides and stained according to the TUNEL protocol as described in materials and methods. Magnification 20 X.

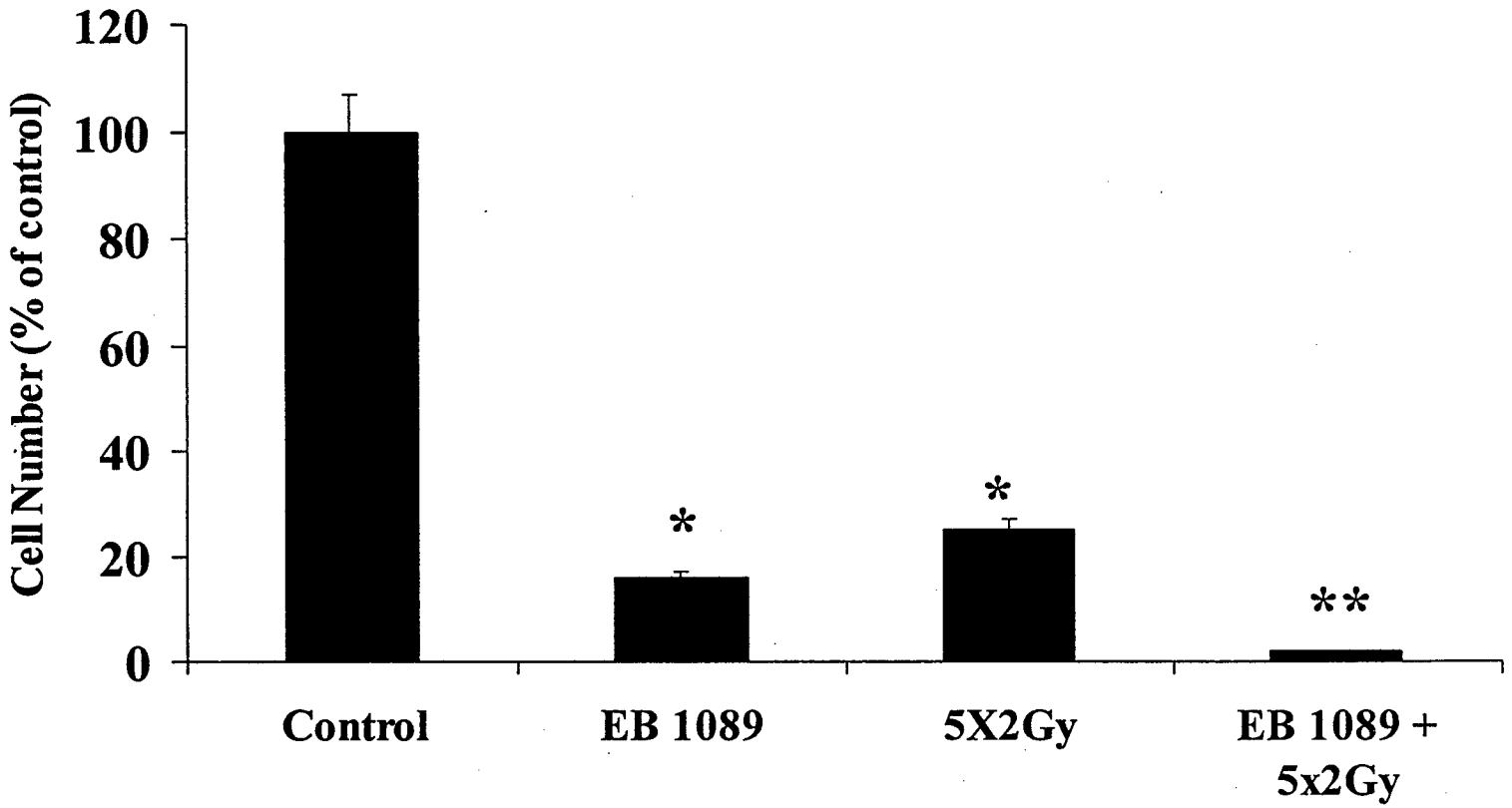
Figure 4: Effects of combining EB 1089 with fractionated radiation (2 Gy x 5) on MCF-7 cell morphology. Cells were isolated following the fifth dose of radiation, cytospun onto glass slides and stained with Wright-Giemsa stain. Arrows indicate cells displaying apoptotic morphology. Magnification 50 X.

Figure 5: Induction of SA-β-gal activity in MCF-7 cells following fractionated radiation with or without pretreatment with EB 1089 (100 nM). Attached cells were fixed with 2% formaldehyde and stained for SA-β-gal activity. Magnification 20X.

Figure 6: Influence of EB 1089 on the antiproliferative effects of fractionated radiation in normal human breast epithelial cells. Cells were treated with EB 1089 (100 nM) for 72 h and replaced with fresh media prior to fractionated radiation (2 Gy x 5). Following the fifth dose of radiation, viable cell number was determined by trypan blue exclusion assay as described in materials and methods. Data represent means ± range from two independent experiments.

Figure 7: Influence of EB 1089 on the antiproliferative effects of fractionated radiation in BJ fibroblast cells. Cells were treated with EB 1089 (100 nM) for 72 h and replaced with fresh media prior to fractionated radiation (2 Gy x 5). Following the fifth dose of radiation, viable cell number was determined by trypan blue exclusion assay as described in materials and methods. Data represent means ± range from two independent experiments.

MCF-7


Cell Number (% of control)
120
100
80
60
40
20
0
*
*
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Control
EB 1089
5X2Gy
EB 1089 +
5x2Gy

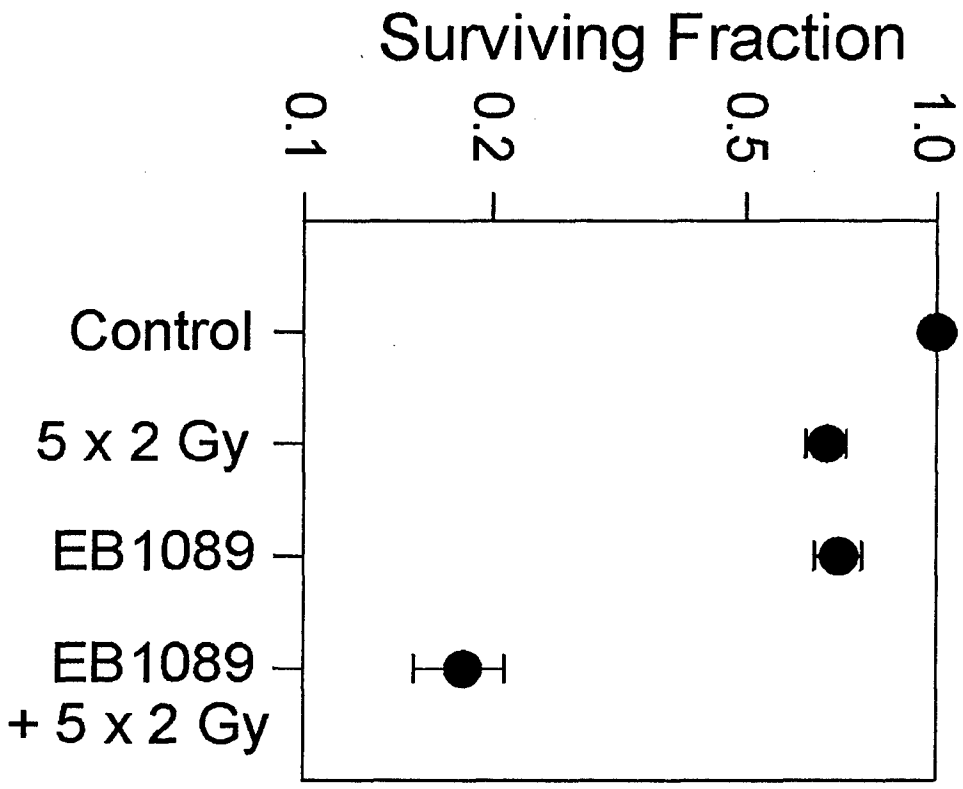
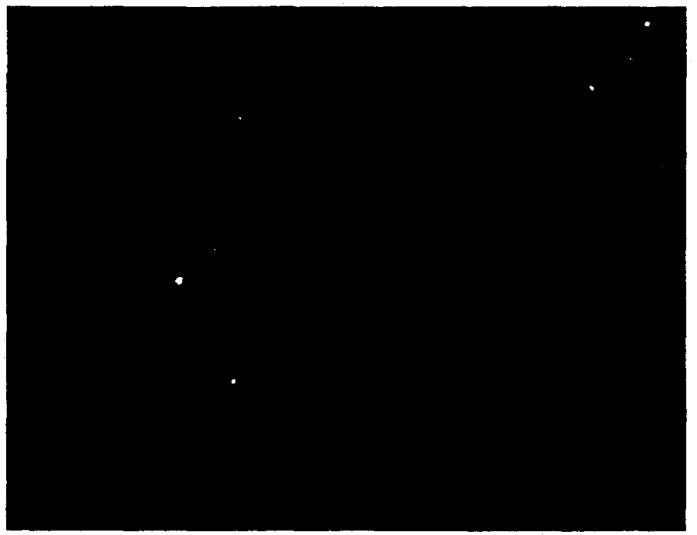

Surviving Fraction
0.1
0.2
0.5
1.0
Control
5 x 2 Gy
EB1089
EB1089
+ 5 x 2 Gy

Fig 3

control

EB 1089



5x2 Gy

5x2 Gy + EB 1089

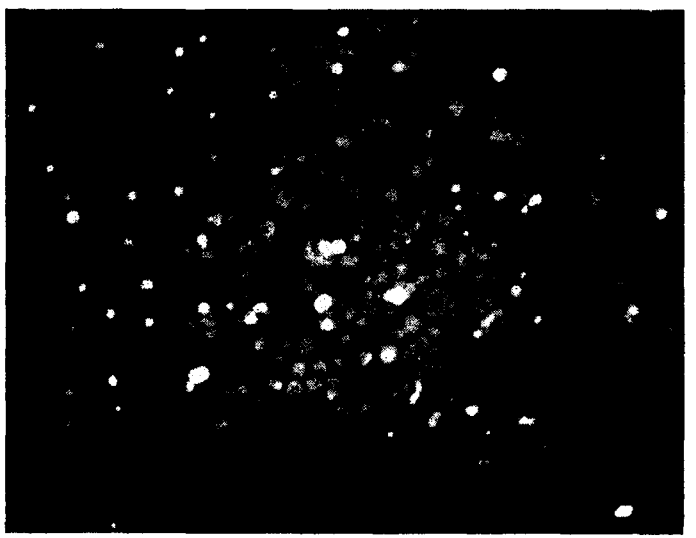


Fig 4

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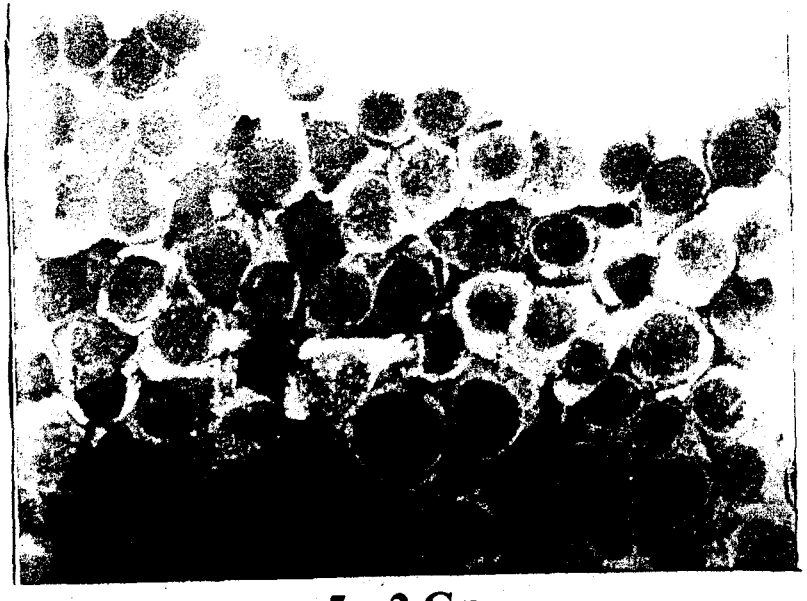
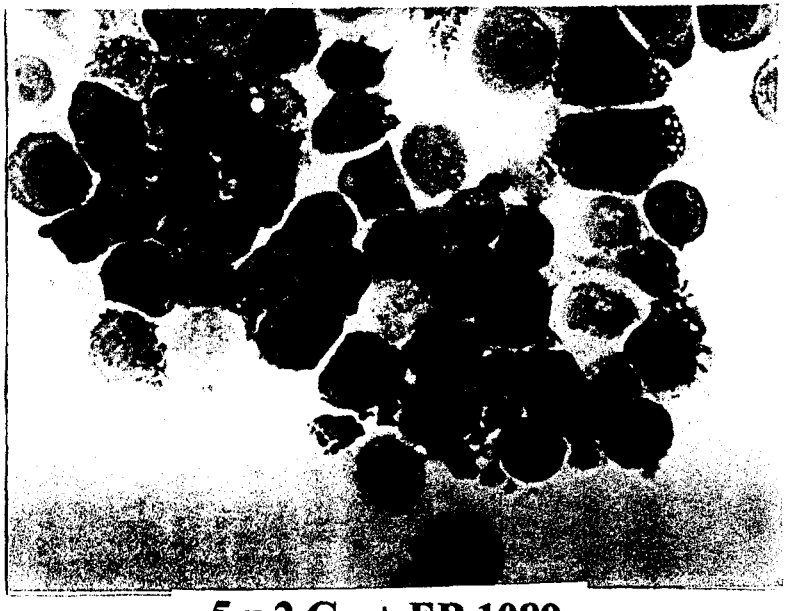
control

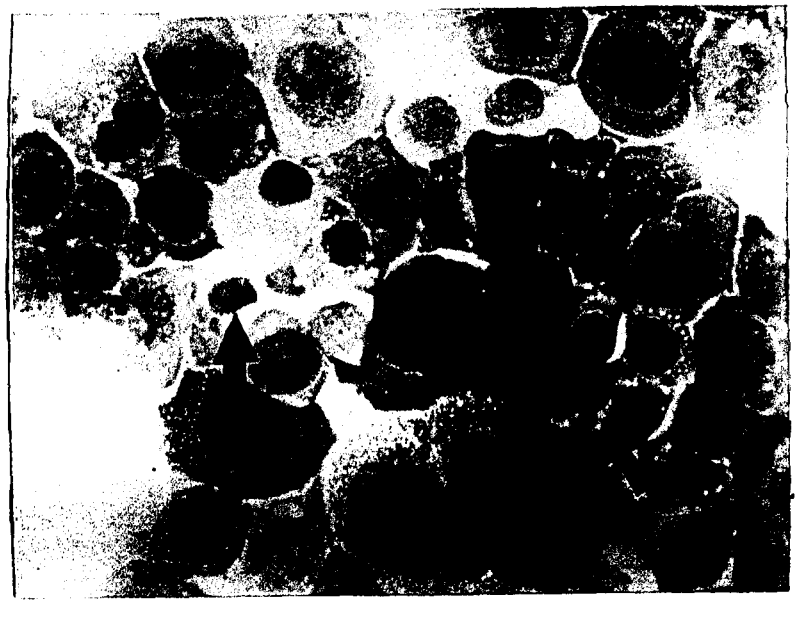


EB 1089

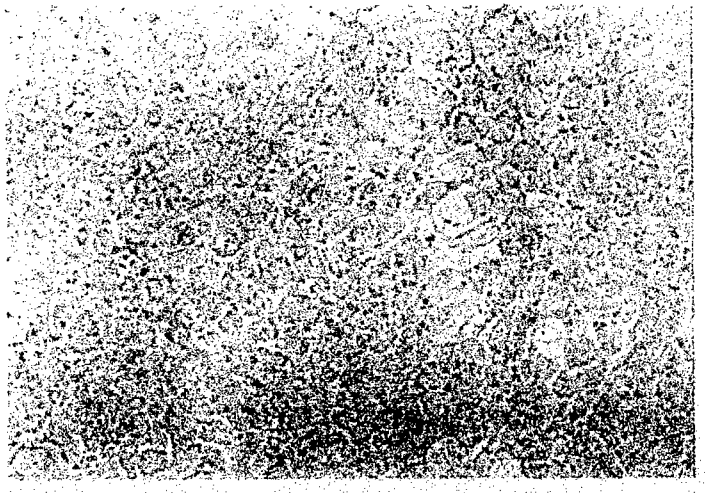


5 x 2 Gy

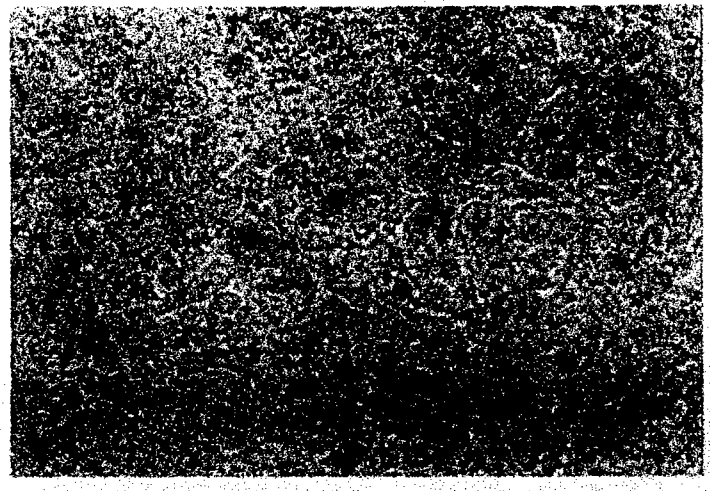
5 x 2 Gy + EB 1089



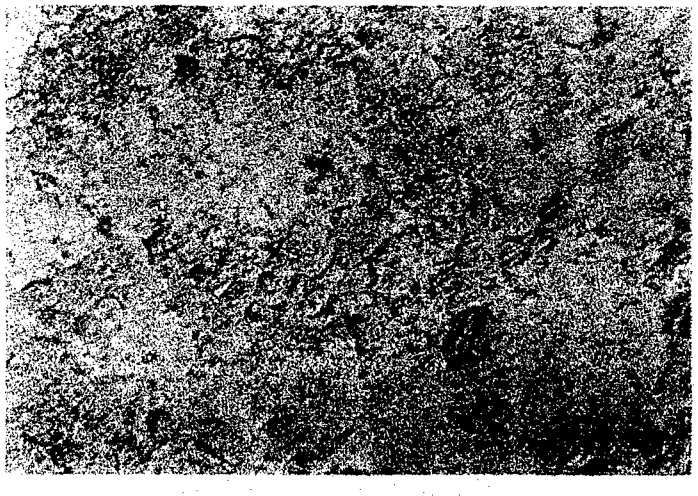
control



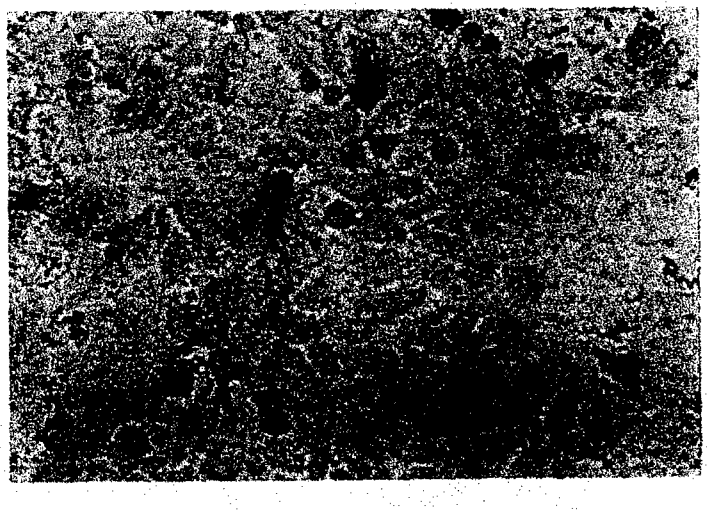
EB 1089

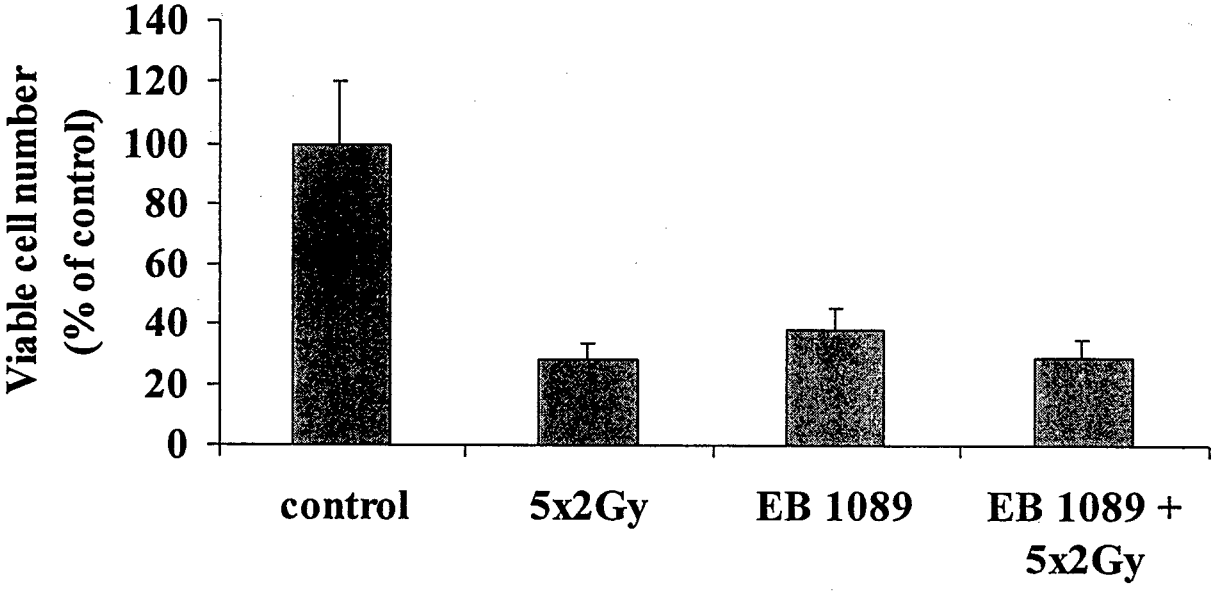


5 x 2 Gy



5 x 2 Gy + EB 1089




Normal human breast epithelial cells
Viable cell number
(% of control)
140
120
100
80
60
40
20
0
control
5x2Gy
EB 1089
EB 1089 +
5x2Gy

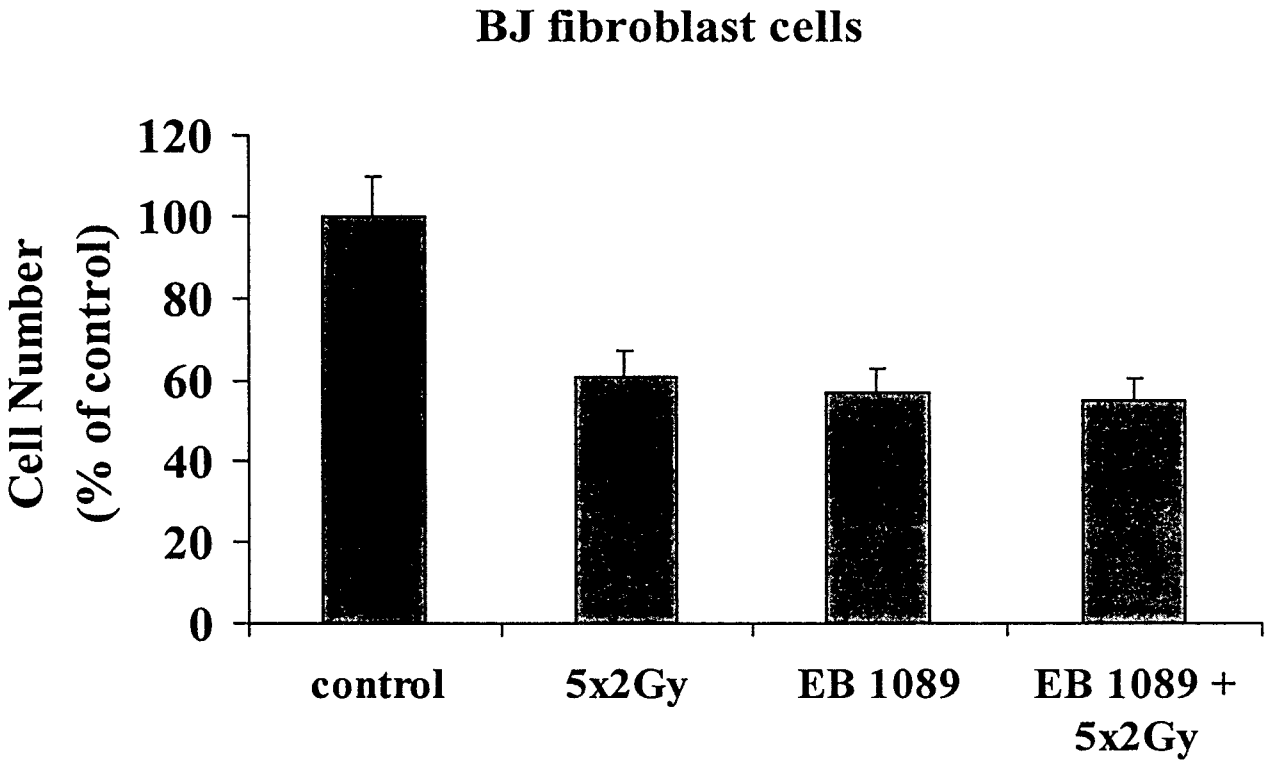

BJ fibroblast cells
Cell Number
(% of control)
120
100
80
60
40
20
0
control
5x2Gy
EB 1089
EB 1089 +
5x2Gy

Table 1

Alkaline Unwinding assay in MCF-7 cells

Treatment	**RAD Equivalents**
Control	96 ± 2
EB 1089	187 ± 4
IR (5x 2Gy)	100 ± 3
EB1089 + IR	1082 ± 8

MCF-7 cells were pretreated with 100 nM EB 1089 for 72 hr. After 72 hr, EB 1089 was removed and cells were irradiated (5 x 2 Gy). Following the last dose, cells were prepared for the alkaline unwinding assay as described in materials and methods.

* Data expressed as means ± range from two independent experiments.

Table 2

(A) Ceramide generation in MCF-7 cells

Treatment	Ceramide (% of control)
Control	100 ± 5.5
EB 1089	132.2 ± 4.3
Irradiation (10 Gy)	148.1 ± 6.7
EB1089 + IR (10 Gy)	171.8 ± 4.5
Taxol (T)	196.3 ± 7.0

(B) Ceramide generation after EB 1089 Exposure for 72 hr

Treatment	Ceramide (% of control)
Control	100 ± 5.8
EB1089	200.7 ± 6.4

(A) MCF-7 cells were treated with 100 nnM EB 1089 for 72 hr. After 72 hr, EB 1089 was removed; [^{3}H]palmitic acid was added and cells were irradiated (10 Gy) or treated with Adriamycin or Taxol for 24 hr. (B) MCF-7 cells were treated with EB 1089 and radio-labeled with [^{3}H]palmitic acid simultaneously. Lipid was extracted and analyzed for ceramide as described in materials and methods.

Table 3

Observed and predicted % reduction in cell viability under the hypothesis of additivity for MCF-7 cells pretreated with EB 1089 and followed by FIR

EB (nM)	Radiation (5 x 2 Gy)	Observed % Reduction	Predicted % Reduction Under Additivity	95% Prediction Interval Under Additivity
100	2.0	0.82	0.85	[0.41, 1.19

Observed results from cell viability assay of EB 1089 in combination with FIR were compared to values predicted using a statistical model of additivity. Observed and predicted values were not significanly different (p=0.917); the assumption of additivity is not rejected. EB 1089 and FIR interact additively.

Enhanced Ceramide Glycosylation in Cancer Cells Selected for Resistance to Vinblastine and Adriamycin[1]

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Running Title:

Keywords: ceramide, glucosylceramide, multidrug resistance, Adriamycin, vinblasti

ABSTRACT

Resistance to natural product chemotherapy agents continues to deal major setbacks in the successful treatment of cancer patients. In some instances, drug resistance can be acquired in response to drug exposure; however, the mechanisms for this remain elusive. Here we sought to determine the influence of vinblastine (VBL) and Adriamycin (doxorubicin) selection pressure on ceramide metabolism by glycosylation via the enzyme glucosylceramide synthase (GCS) in cultured human epidermoid carcinoma (KB-3-1 series) and in MCF-7 human breast cancer cells.

As free ceramide has been shown to be contributory to the cytotoxic response of natural product chemotherapy, knowledge of ceramide metabolism becomes important. Experiments revealed that levels o the glycosylated form of ceramide, glucosylceramide (GC), increased in parallel with increasing resistance to both VBL and adriamycin in the KB-3-1 series of cell lines (listed in order of increasing drug resistance), KB-V.0l, KB-V.l, KB-Vl, and KB-A.05, KB-Al, respectively. GC metabolism, measured using [^{3}H3palmitic acid in intact cells and by cell-free enzyme assay, likewise showed highest synthesis rates in highly resistant KB-Vl cells, with KB-V.0l and KB-V.l, intermediate, and highest GCS activity in KB-Vl compared to KB-3-1, 10.2 ± 0.6 versus 21.8 ± 1.5 nmol GC/mg protein/hr, respectively. Reverse transcription – polymerase chain reaction using RNA isolated from cell lystates demonstrated significantly higher levels of GCS expression in both the VBL – and Adriamycin-resistant cell lines compared to drug naïve parent cell lines. This work demonstrates that selection pressure for resistance to natural product chemotherapy results in enhanced ceramide metabolism through GCS gene overexpression.

INTRODUCTION

Inherent or acquired resistance, which can include development of simultaneous resistance to multiple drugs, is a frequent characteristic of cancer cells. It is difficult to predict and to manage. Approximately 40% of cancer patients with resectable disease and 80% of cancer patients with unresectable disease have poor response to chemo- and radiotherapy. Several mechanisms of drug resistance have been examined. Overexpression of a membrane efflux transporter, P-gp[3], is one of the most consistent alterations in drug resistance (1,2). P-gp has come to be an important clinical target and the object of many studies (3,4). Anticancer drug resistance can also be caused by overexpression of multidrug resistance – associated protein (5,6), changes in topoisomerase II activity (7,8) modifications in glutathione –S- transferase (9,10), altered expression of important apoptosis – associated proteins, Bcl-2 (11) and tumor suppressor protein p53 (12), the synthesis of vaults (13), and the overexpression of caveolne (14).

Apoptosis is an essential element in the cytotoxic response to many anticancer agents (15), and the neutral lipid, ceramide, has been shown to play a major role in this response (16). Studies have demonstrated that chemotherapy resistance in cancer cells is in some instances allied with an enhanced capacity for ceramide glycosylation (17-19). This would suggest that for agents employing ceramide as an apoptosis signal, the cytotoxic response to therapy would be blunted in tumors that harbor upregulated ceramide metabolism via glycosylation. Therefore, the importance of GCS, the enzyme that regulates synthesis of glucosylceramide, becomes paramount, especially when considering the large number of front-line drugs that elicit ceramide formation (reviewed in 18).

The present work was undertaken to test the hypothesis that selection of cancer cells for resistance to chemotherapy drugs, in this case natural product drugs, adriamycin and VBL, alters the expression of GCS. This is a new and intriguing idea regarding the mechanisms of acquired resistance to chemotherapy. To this end we have demonstrated using a series of epidermoid carcinoma and breast cancer cell lines selected for resistance to chemotherapy, heightened GCS levels in terms of elevated enzyme activity (cell-free assays), increased rates of glucosylceramide synthesis in intact cells, higher GC mass, and increased GCS mRNA all expression, in parallel with increases in the level drug resistance.

MATERIALS AND METHODS

Materials. KB-3-1, the parent human epidermoid carcinoma cell line, and the vinblastine- and adriamycin-resistant sublines were grown as monolager cultures in high glucose (4.5 gm/liter) Dulbecco's modified Eagle's medium with 10% FBS, HyClone (Logan, UT) and additives as described (17,20). The human breast cancer cell lines, MCF-7 and MCF-7-AdrR (adriamycin-resistant) were provided by Drs. Kenneth Cowan (University of Nebraska Medical Center, Eppley Cancer Center, Omaha, NE) and Merrill E. Goldsmith (National Cancer Institute, Bethesda, MD). A description of the cell lines used in this study is given in Table 1.

RPMI-1640 and DMEM culture media were from Gibco BRL (Grand Island, NY). Vinblastine sulfate, Adriamycin (doxorubicin hydrochloride), and other chemicals were purchased from Sigma. [9,10-^{3}H(N)]Palmitic acid (50 Ci/mmol) was from DuPont/NEN (Boston, MA), and [^{3}H]UDP-glucose (40 Ci/mmol) was a product of American Radiolabeled Chemicals (St. Louis, MO). C219, monoclonal antibody against human P-gp was from Signet Laboratories (Dedham, MA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), and EcoLume (liquid scintillation mixture) was from ICN (Costa Mesa, CA). Silica Gel G prescored TLC plates were purchased from Analtech (Newark, DE)

RNA was isolated using RNAgents Total isolation System, Promega (Madison, WI) following the instructions from the manufacturer. RT-PCR was carried out using ProStar HF Single Tube PCR System, from Stratatgene (Cedar Creek, TX). Housekeeping primer, Classic 18S, was from Ambion (Austin, Tx). The thermocycler, Mastercycler Gradient, was product of Eppendorf Scientific (Westburg, NY).

Cell Culture. KB-3-1 and the drug resistant sublines and MCF-7 and MCF-7-AdrR cells were maintained and subcultured as previously described (17,20). All culture media contained 10% FBS (v/v), 50 units/ml penicillin, 50 µg/ml streptomycin, and 584 mg/ liter L-glutamine. Cells were subcultured using 0.05% trypsin/0.53 mM EDTA solution.

Cell radiolabeling for GC analysis. Cells in 6-well plates at approximately 60% confluence were given fresh 5% FBS medium containing [^{3}H]palmitic acid (1.0µCi/ml) for various times (2-24 hr). Total cellular lipids were extracted from washed monolayers according to previous methods (17,19) and tritium labeled GC was resolved from the total lipid extract by TLC using a solvent system containing chloroform/methanol/ammonium hydroxide (80:20:2, v/v/v). GC, co-chromatographed with commercial standard, was visualized in iodine vapor and scraped into water and Ecolume (17,19) for quantitation of tritium by liquid scintillation counting.

Autoradiographic analysis of GC in the KB series of cell lines was conducted by growing cells in 10-cm dishes to 60% confluence, and replacing the medium with fresh 5% FBS medium containing [^{3}H]palmitic acid (1.0µCi/ml) for 24 hr. Monolayers were then rinsed twice with cold PBS, and total cell lipids were extracted (17,19). Equal aliquots based radioactivity (500,000 dpm/lane) were applied to the origin of TLC plates, and GC was resolved using the above cited

solvent system. Radiochromatograms were sprayed with EN^{3}HANCE and exposed for 40 hr at -80°C for autoradiography.

Glucosylceramide Synthase Assay. Cells, harvested from log phase growth, were homogenized by sonication in lysis buffer (50 mM Tris-HCl, pH 7.4, 1.0 µg/ml leupeptin, 10 µg/ml aprotinin, 25 µM phenylmethylsufonyl fluroide). Microsomes were isolated by centrifugation (129,000 x *g* for 60 min). The enzyme assay, modified from a previous method (21), contained 50 µg of microsomal protein, in a final volume of 0.2 ml, and was performed in a shaking water bath at 37 °C for 60 min. The reaction contained liposomal substrate composed of C_6-ceramide (1.0 mM), phosphatidylcholine (3.6 mM; molecular weight, 786), and brain sulfatides (0.9 mM; molecular weight, 563). The liposomal substrate was prepared by mixing the components, evaporating the solvents under a stream of nitrogen, and sonicating in water over ice for 1 min using a microtip at 50% output, Micro Ultrasonic Cell Disrupter, Kimble Knotes (Vineland, NJ). Other reaction components included sodium phosphate buffer (0.1 M), pH 7.8, EDTA (2.0 mM), $MgCl_2$ (10 mM), dithiothreitol (1.0 mM), β-nicotinamide adenine dinucleotide (2.0 mM), and [^{3}H]UDP-glucose (0.5 mM). Radiolabeled and unlabeled UDP-glucose were diluted to achieve the desired radiospecific activity (5,000 dpm/nmol). To terminate the reaction, tubes were placed on ice, and 0.5 ml isopropanol and 0.4 ml Na_2SO_4 were added. After brief vortex mising, 3 ml *t*-butyl methyl ether was added, and the tubes were mixed for 30 sc. After centrifugation, 0.5 ml of the upper phase, which contained GC, was withdrawn and mixed with 4.5 ml of EcoLume for analysis of radioactivity by liquid scintillation spectroscopy.

Western Blot. Confluent cells, KB-3-1 and KB VBL series, were washed, harvested in PBS, and lysed in a PBS buffer containing 10% glycerol, 1% Triton X-100, I mM $NaVO_3$, 10 mM β glycerophosphate, 50 mM NaF, 0.1 mM phenylmethylsulfonyl fluroide, 2 µg/ml leupeptin, and 10 µg/ml aprotinin for 30 min

on ice. The mixture was centrifuged at 11,000 x *g* for 15 min at 4°C. Equal aliquots of protein (25 µg) were resolved using 4-20% gradient SDS-polyacrylamide gel electrophoresis. The transferred nitrocellulose blot was blocked with 5% fat-free milk powder in PBS containing 0.1% Tween-20 at room temperature for 1 hr. The membrane was the immunoblotted with murine monoclonal antibody C219 against human P-gp (5 µg/ml) in 5% fat-free milk in PBS-Tween-20, 0.1%. Detection was performed using ECL (Amersham Pharmacia Biotech).

RNA Analysis. Equal amounts of mRNA (5.0 ng) were used for RT-PCR. Under upstream primer (5' –CCTTTCCTCTCCCCACCTTCCTCT-3') and downstream primer conditions (5' –GGTTTCAGAAGAGAGACACCTGGG-3'), a 302-base pair fragment in the 5' –terminal region of the GCS gene was produced using the ProSTAR HF single-tube RT-PCR system in a thermocycler. mRNAs were reverse transcribed using Moloney murine leukemia virus reverse transcriptase at 42 °C for 15 min. DNA was amplified with *TaqPlus* Precision DNA polymerase in a 40-cycle PCR reaction, using the following conditions: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 120 sec. RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. Classic 18*S* primer was used for even loading.

RESULTS

The drug-resistant KB-3-1 sublines were first evaluated for GC content. For this, cells were radiolabeled to steady state using [^{3}H]palmitic acid, and GC was, characterized by TLC autoradiography. As demonstrated in Fig.1, GC levels increased with increased VBL selection pressure, and the KB-V1 cells, subcloned to grow with 1.0 μg/ml VBL, had the highest GC content. The parent KB-3-1 drug sensitive cell line was by comparison, nearly devoid of GC, and KB-V.01 and KB-V.1 cells showed incrementally increased GC mass, respectively. All KB sublines selected for growth in VBL showed the characteristic GC doublet, with the lower band corresponding to predominantly N-palmitoyl ceramides and the upper band, a mixture of longer amide chains (lignoceroyl, nervonoyl) (17,19). Analysis of KB cells selected for growth in adriamycin showed a similar pattern of GC increase which corresponded to increasing anthracycline resistance (Fig. 2 and Table 1).

Using the VBL series of cell lines we next investigated the rates of synthesis of GC. [^{3}H]Palmitic acid was utilized as glycolipid precursor to tag the aliphatic moieties of GC, and synthesis was followed in intact cells over a 2- 24 hr period. Figure 3 shows that amoung the cell line series, the lowest rate of GC synthesis was found in KB-3-1 cells, with the highest rate associated with KB-VI cells. After 8 hr in the presence of [^{3}H]palmitic acid the difference in the rate of synthesis of GC between KB-3-1 and KB-V1 cells was approximately 2-fold. KB-V.01 and KB-V.1 cell lines showed an intermediate rate of GC synthesis. Cell–free assays using microsomes as enzyme source were conducted to measure actual GCS activity under defined *in vitro* conditions. The data of Table 1 show that cell-free GCS activity was higher in cells selected to grow in the presence of the natural product

anticancer agents compared to the chemotherapy naïve cell line. A comparison of the parental cell line, KB-3-1, with highly drug-resistant KB-V-1 cells shows GCS activity of 10.2 ± 0.6 and 21.8 ± 1.5 nmol GC/mg protein/hr, respectively, which amounts to a 2-fold increase in GC synthesis. Likewise, although not following the increased selection pressure pattern, GCS activity was also higher in adriamycin resistant cells (Table 1).

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[3]The abbreviations used are: P-gp, P-glycoprotein; GCS, glucosylceramide synthase; GC, glucosylceramide; FBS, fetal bovine serum; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; VBL, vinblastine; RT-PCR, reverse transcription polymerase chain reaction.

Table 1 Cell lines employed in this study

Cell Line	Media additions [a]	MDR status [b, c]	
		VBL	Adria
KB-3-1	none	negative	
KB-V.01	Vinblastine, 10 ng/ml	2.8	1.8
KB-V.1	Vinblastine, 100 ng/ml	23	5.1
KB-V1	Vinblastine, 1 µg/ml	213	422
KB-A.05	Adriamycin, 50 ng/ml	38	31
KB-A1	Adriamycin, 1 µg/ml	43	97
MCF-7	none	negative	
MCF-7-AdrR	none	N.D.	33

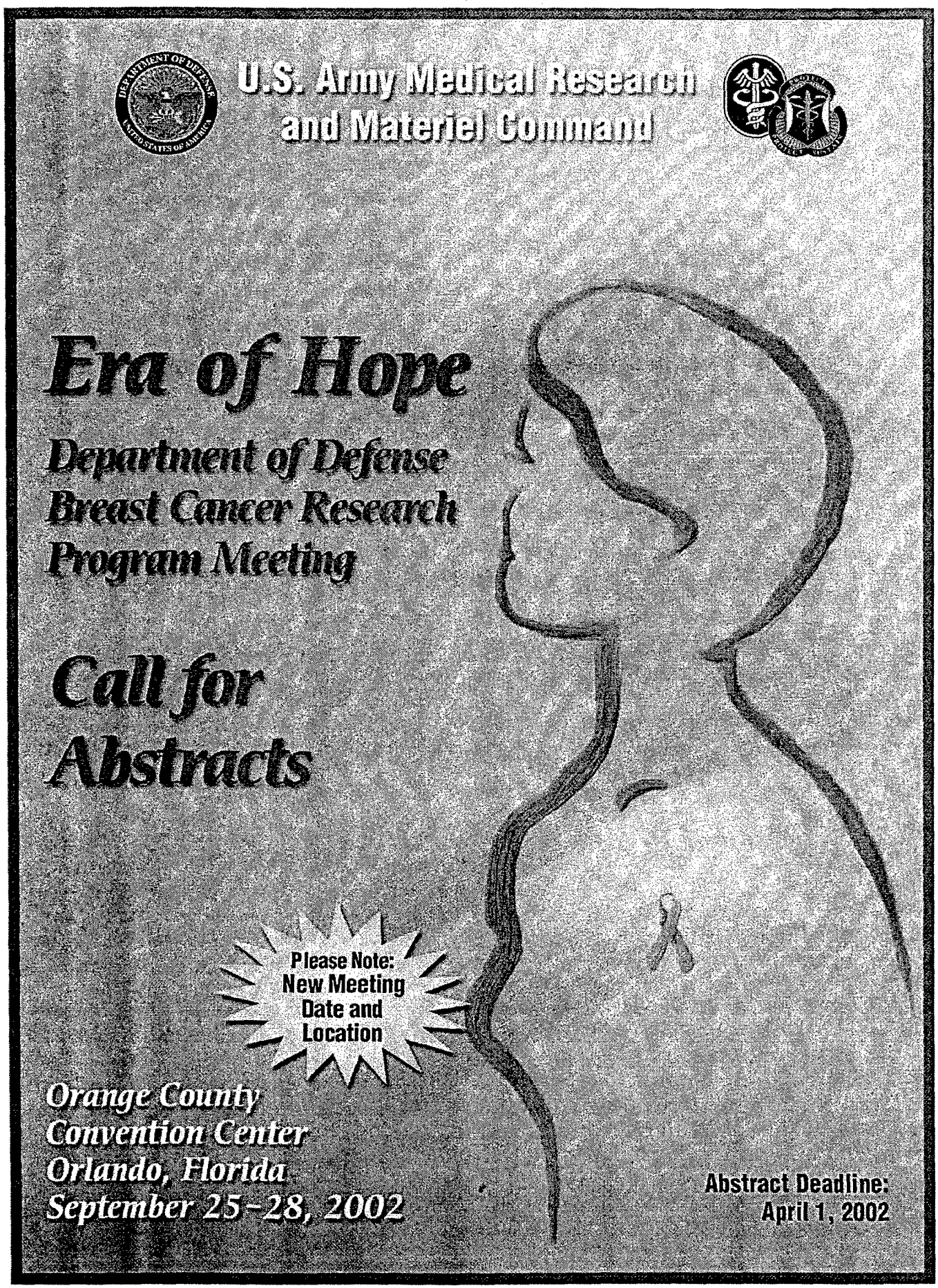
[a] Cells continually maintained in culture medium containing the drugs at concentrations indicated.

[b] MDR status shown as relative resistance and calculated from D_{10} values (concentration of drug which reduced the cloning efficiency of the sublines to 10% of the control without drugs, ref.20) by dividing D_{10} of the resistant line by the D_{10} of the KB-3-1 parental drug-sensitive cell line.

[c] MDR status of MCF-7-AdrR derived by dividing Adriamycin EC_{50} in MCF-7-AdrR by Adriamycin EC_{50} in MCF-7 cells.

Table 2 Glucosylceramide synthase activity in cells selected for resistance to VBL and adriamycin

Cell line	GCS activity (nmol/mg/hr)
KB-3-1	10.2 ± 0.6
KB-V.1	14.6 ± 0.6
KB-V1	21.8 ± 1.5
KBA 0.5	14.7 ± 0.3
KBA1	12.4 ± 0.7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CERAMIDE ELEVATED BY THE P-GLYCOPROTEIN ANTAGONIST, SDZ PSC 833, SYNERGIZES CHEMOTHERAPY-ELICITED CYTOTOXICITY IN BREAST CANCER CELLS

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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. Because ceramide is a critical component of the apoptosis signaling cascade, this study examined a side reaction of the P-glycoprotein-targeted multidrug resistance modulator, SDZ PSC 833 (PSC 833), **that of ceramide elevation.** Using cultured human breast cancer cells under various treatment protocols and [^{3}H]palmitic acid as tracer, ceramide and sphingolipid metabolism was analyzed from cell extracts using thin-layer chromatography and liquid scintillation counting. Palmitoyl CoA synthetase, serine palmitoyltransferase (SPT), and ceramide synthase activities, all enzymes involved in ceramide synthesis, were measured by *in vitro* assay using microsomes isolated from cell lysates. In cells being treated, viability was quantitated spectrophotometrically using a cell proliferation assay kit. In MDA-MB 468, a P-glycoprotein-poor human breast cancer cell line, PSC 833 elicited ceramide generation through activation of the *de novo* synthesis pathway and not via sphingomyelin hydrolysis by sphingomyelinase. Exposure of MDA-MB 468 cells to PSC 833 (15 min – 4 h, 2.5 – 10 μM) elicited ceramide generation as high as 7-fold over control. Cyclosporine A, a close chemical cousin of PSC 833, was not active. Ceramide elevation could be blocked by adding myriocin, a chemical inhibitor of SPT, to the culture medium. Exposure of intact cells to PSC 833 (30 min – 4 h; 1 – 10 μM) followed by isolation of microsomes for cell-free *in vitro* assay, increased SPT activity as much as 60%, whereas palmitoyl CoA synthetase and ceramide synthase activities were not altered. SPT activity and ceramide levels were also enhanced, as measured *in vitro*, by pretreatment of cells with either paclitaxel, N-(4-hydroxyphenyl)retinamide (4-HPR), or etoposide. PSC 833 also activated ceramide formation in other breast cancer cell lines, including BT-20, MDA-MB 231, Hs 578T, T-47D, MCF-7, and drug resistant MCF-7/AdrR. Both C_6-ceramide and PSC 833 were cytotoxic to MDA-MB 468 cells, with EC_{50}'s of 2.0 and 2.1 μM, respectively. In MCF-7/AdrR cells, when used in combination with tamoxifen and doxorubicin, PSC 833 increased ceramide levels 26-fold and brought cell viability to zero. In conclusion, PSC 833 increases ceramide synthesis and induces cytotoxicity in breast cancer cells by activation of SPT, the rate-limiting enzyme in the *de novo* synthesis pathway. As such, in addition to it's intended use as a P-glycoprotein antagonist, PSC 833 may be of utility therapeutically to enhance apoptosis when used in conjunction with modulators of ceramide metabolism. Elevation of *de novo* ceramide synthesis through SPT activation may provide a new approach for modulating multidrug resistance in breast cancer.

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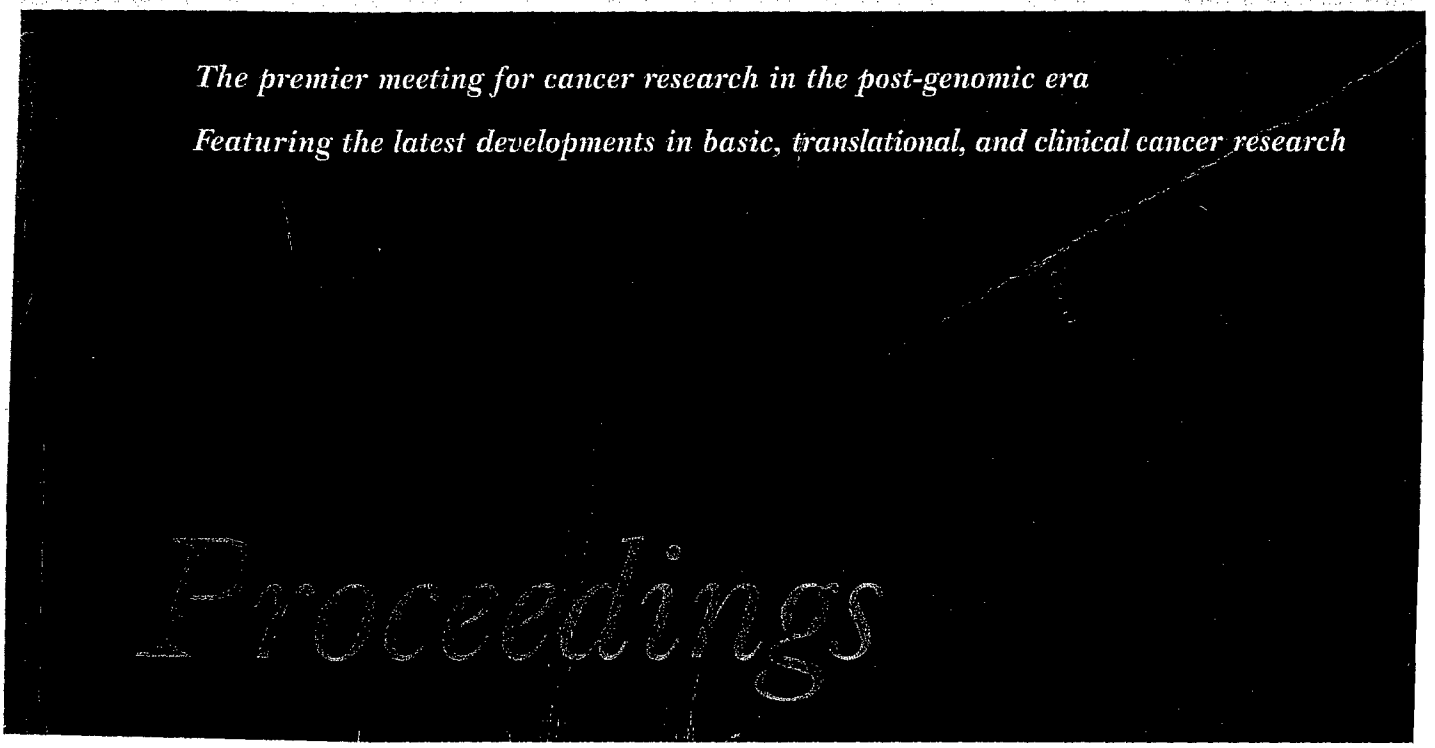

compounds for a various lengths of time. Cell cycle distributions and apoptotic fractions were analyzed with standard flow cytometry. Surprisingly, B201 but not B317 or B429 produced a dose and time dependent new population of cells with an apparent hyper-diploid DNA content. To determine whether the new population had new genetic changes or merely changes in DNA binding, we performed FISH analysis before and after exposure to the compounds. There were no genetic changes indicating that B201 had merely changed the DNA binding properties. Cells with this altered DNA binding subsequently became apoptotic. Despite of different effects on the DNA binding in the treated cells, B201, B317 and B429 all activated pro-caspase 3. Anti-angiogenic effects of B201, B317 and B429 were evaluated in an *in vitro* cord formation assay, using spontaneously transformed human umbilical vascular endothelial cells ECV grew on a layer of preformed Matrigel. B201, B317 and B429 all effectively disrupted the vascular endothelial cord formation, suggesting that bradykinin may play a role in the tumor angiogenesis. These new classes of anti-angiogenic agents are further developed for the treatment of human lung cancers through the RAID (B201) and SBIR mechanisms (B317 and B429

#2049 Serine palmitoyltransferase is the target enzyme in synthesis of ceramide elicited by anticancer drugs in breast cancer cells. Hongtao Wang, Armando E. Giuliano, and Myles C. Cabot. *John Wayne Cancer Institute, Santa Monica, CA.*

Multidrug resistance (MDR) is the major cause of cancer treatment failure. SDZ PSC 833 (PSC 833) is a P-glycoprotein-targeted MDR modulator, but additionally it 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. Using cultured breast cancer cells and [^{3}H]palmitic acid tracer, ceramide and sphinganine metabolism were analyzed using thin-layer chromatography and liquid scintillation counting. Serine palmitoyltransferase (SPT), ceramide synthase, and palmitoyl CoA synthetase activities were measured by *in vitro* assay. In MDA-MB 468 human breast cancer cells, PSC 833 elicited ceramide generation through activation of the *de novo* synthesis pathway and not via sphingomyelin hydrolysis. Exposure of MDA-MB 468 cells to PSC 833 (15 min – 4 h, 2.5 – 10 μM) elicited ceramide generation as high as 7-fold over control. Cyclosporine A, a close chemical cousin of PSC 833, was not active. Sphinganine generation also increased in response to PSC 833 treatment, and this could be blocked by adding L-cycloserine, a SPT inhibitor, to the culture medium. Moreover, when Fumonisin B_1, a ceramide synthase inhibitor, was added to block conversion of sphinganine to ceramide, sphinganine increased nearly 10-fold in response to PSC 833 addition, verifying that SPT was activated. Exposure of intact cells to PSC 833 (30 min – 4 h; 1 – 10 μM) followed by isolation of microsomes for *in vitro* assay, increased SPT activity 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, N-(4-hydroxyphenyl)retinamide (4-HPR), or etoposide; however, activation was half that attained by PSC 833. PSC 833 also activated ceramide formation in other breast cancer cell lines, including BT-20, MDA-MB 231, Hs 578T, T-47D, and MCF-7. In conclusion, PSC 833 and perhaps other anticancer drugs increase ceramide synthesis 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. (Supported by Department of the Army, Grant No. DAMD17-99-1-9228).

#2050 Lipid rafts as gateway to ALP-induced apoptosis. Arnold H. Van der Luit, Marcel Verheij, Marianne Budde, Caan Wendy, Paula Ruurs, Harry Bartelink, and Wim J. Van Blitterswijk. *The Netherlands Cancer Institute, Amsterdam, Netherlands.*

Synthetic alkyl-lysophospholipids (ALPs), such as 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine, are antitumor agents known to accumulate in cell membranes. The aim of this study was to understand the mechanism by which ALP enters the cell and induces apoptosis. We demonstrate that in murine lymphoma S49 cells, ALP inhibits de novo biosynthesis of phosphatidylcholine (PC) at the CTP:phosphocholine cytidylyltransferase (CT) step. Exogenous lysoPC, providing an alternative route to generate PC (via acylation), rescued the cells from ALP-induced apoptosis. This indicates that a continuous rapid PC turnover is essential for cell survival. To reach CT, ALP needs to be internalized. This internalization did not involve receptor/clathrin-coated pit-mediated endocytosis, nor fluid phase endocytosis. Instead, intact lipid rafts in the plasma membrane were found essential, since artificial disruption of these microdomains resulted in reduced ALP endocytosis and inhibition of apoptosis. Interestingly, an ALP-resistant cell variant, S49AR, showed impaired ALP internalization and reduced levels of sphingomyelin, an essential component of lipid rafts. For the first time, lipid rafts are recognized as potential targets for anticancer therapy.

#2051 The regulation of p53 by protein kinase C in anticancer drug-induced apoptosis. Cassie L. Johnson and Alakananda Basu. *UNT Health Science Center, Ft. Worth, TX.*

The tumor suppressor protein p53 has been implicated in DNA damage-induced apoptosis. Previous studies demonstrated that the protein kinase C (PKC) signal transduction pathway regulates apoptosis induced by the DNA damaging agent cisplatin and is deregulated in cisplatin-resistant cells. The present study examined whether PKC influences p53 and, hence, cellular sensitivity to cisplatin. The basal p53 levels were low in HeLa cells due to E6 papillomavirus-mediated degradation and cisplatin alone had little effect on p53 content. PKC activators, such as PDBu and bryostatin 1 that sensitized cells to cisplatin, further decreased p53 levels in HeLa cells whereas PKC inhibitors alone had little effect on p53 content. Rottlerin, a PKC inhibitor that prevents cisplatin-induced apoptosis, however, caused p53 accumulation in HeLa cells when treated in conjunction with cisplatin. In contrast, a combined treatment with cisplatin and Gö 6976, an inhibitor of conventional PKCs, failed to increase p53 lev els in HeLa cells. Basal p53 levels were elevated in cisplatin-resistant HeLa (HeLa/CP) cells as compared to parental HeLa cells and cisplatin further increased p53 levels in HeLa/CP cells. The half-life of p53 was approximately 5 min in both parental and cisplatin-resistant HeLa cells. While cisplatin had no effect on the half-life of p53 in HeLa cells, it increased the half-life of p53 in HeLa/CP cells to greater than 60 min. Rottlerin increased the half-life of p53 in response to cisplatin in HeLa cells to 35 min but it had no additional effect on cisplatin-induced stabilization of p53 in HeLa/CP cells. These results suggest that PKC-mediated phosphorylation of p53 leads to its degradation and both inhibition of PKC as well as DNA damage were required to stabilize p53 in HeLa cells. Furthermore, since PKC is deregulated in HeLa/CP cells, DNA damage may be sufficient to stabilize p53 in cisplatin-resistant HeLa cells. Supported by grants CA71727 and CA85682 from NCI.

#2052 Synergism of apoptosis and mitotic catastrophe in chemotherapy against colorectal cancer; clinical validation for biphasic cytotoxic effects of five-fluorouracil (5-FU). Reigetsu Yoshikawa, Tomoko Hashimoto-Tamaoki, Hidenori Yanagi, Masafumi Noda, Masato Kusunoki, and Takehira Yamamura. *Hyogo College of Medicine, Nishinomiya, Hyogo, Japan, and Mie University, School of Medicine, Tsu, Mie, Japan.*

Efficacy of chemotherapy is believed to depend on the balance between cell proliferation, arrest, and death. The "Pharmacokinetic Modulating Chemotherapy (PMC)" regimen we have advocated, of weekly 24-hr intravenous infusion of high dose 5-FU combined with low dose of oral UFT, a 5-FU derivative, is proving to be highly effective in treating colorectal cancer. We investigated the effects on cell kinetics of clinically relevant concentrations of 5-FU during PMC regimen in two human colorectal adenocarcinoma cell lines with mutant p53 or wild-type p53, and its clinical implication on the prognosis of colorectal cancers. Findings: In the cell lines, cytotoxic mechanism of 5-FU was via two different pathways, depending on the integrity of their schedule-oriented cell cycle checkpoints; G2/M arrest and mitotic catastrophe, non-apoptotic cell death, at a continuous lower dose possibly through 14-3-3σ induction and nuclear cyclin B1 accumulation, and G1/S arrest and apoptosis at a shorter higher dose partly due to $p21^{WAF1/CIP1}$. Clinically, Cell kinetic profile of these surgical specimens showed dual forms of cell death consistent with the *in vitro* data. PMC reduced the tumor size (the proportion of tumor nest to background stroma: 72% versus 85% without PMC), and markedly improved the prognosis of unresectable colorectal cancer (median survival of 30 months). Conclusions: This study unravelled that our efficacy of PMC was attributed to target different artificial checkpoints by single agent, creating dual forms of cell death. These findings suggest a potential avenue for new effective anti-cancer strategy by exploiting synergism of apoptosis and mitotic catastrophe at different cell cycle checkpoints to treat colorectal cancer.

#2053 Bryostatin 1-mediated regulation of tumor necrosis factor results in caspase-8 activation during paclitaxel-induced apoptosis in human leukemia U937 cells. Shujie Wang, Zhiliang Wang, and Steven Grant. *Medical College of Virginia, Richmond, VA.*

Previous studies from this laboratory have shown that bryostatin 1, a PKC activator and down-regulator, potentiates paclitaxel (Taxol; Ptx)-induced cell death in human leukemia U937 cells. However, the molecular mechanisms by which bryostatin 1 enhances Ptx-induced apoptosis are not well characterized. Previously, we and others have demonstrated that Ptx-induced apoptosis is substantially reduced in U937 cells overexpressing Bcl-2 protein. Ectopic expression of a Bcl-2 N-terminal phosphorylation loop-deleted protein (Bcl-2Δ32-80) was more potent than its full length counterpart in blocking Ptx-induced loss of mitochondrial membrane potential, cytochrome c release, activation of multiple caspases, and apoptosis. Resistance conferred by Bcl-2 and Bcl-2Δ32-80 overexpression were substantially reversed by cotreatment with bryostatin 1 (10 nM). Addition of bryostatin 1 resulted in equivalent activation of caspase-8, an initiator caspase in death receptor-mediated cell death pathway, in these three cell lines, accompanied by equivalent degrees of Bid cleavage and cytochrome c release. To test the hypothesis that activation of the receptor-mediated apoptotic cascade might be involved in Ptx/bryostatin 1-induced lethality, the effects of ectopic expression of CrmA, which blocks activation of caspase-8, and overexpression of a dominant-negative caspase-8 mutant (C8DN) were monitored. CrmA and C8DN substantially protected cells from treatment with TNFA, but did not protect cells from Ptx-induced apoptosis. However, potentiation of Ptx-induced apoptosis by bryostatin 1 was abrogated in cells ectopically expressing CrmA protein or C8DN. Moreover, cotreatment of cells with TNF soluble receptor (TNF RI/Fc) completely prevented bryostatin 1-mediated potentiation of Ptx-induced apoptosis and activation of caspase-8. Bryostatin 1-mediated enhanced cell death and caspase-8 activation was completely blocked by Bisndolylmaleimide I (GF109203X), a highly selective inhibitor of PKC. Together, our data indicate that bryostatin 1-related potentiation of Ptx-induced apoptosis in U937 cells requires

resulted in apoptosis. By colony forming assay, transfection of both IκB α mutants resulted in enhanced radiosensitivity in all cell lines tested. F-IκB α was more effective in increasing radiosensitivity than DP-IκB α. The sensitization enhancement ratio (SER) for F-IκB α ranged from 1.21 to 1.71: 1.71 in HL-60 (myeloid leukemia), 1.57 in Jurkat (T cell leukemia), 1.55 in SK-N-DZ (neuroblastoma), 1.37 in MCF7 (breast cancer), 1.36 in CURCII (renal carcinoma), 1.33 in Caki-1 (renal carcinoma), and 1.21 in SK-MEL-2 (melanoma). For DP-IκB α, the SER ranged from 1.12 to 1.69. Also, pyrrolidine dithiocarbamate (PDTC), a chemical inhibitor of NF-κB enhanced radiosensitivity with SER ranging from 1.08 to 1.34. These results show that the inhibition of NF-κB activation using degradation-resistant IκB mutants increases radiation-induced apoptosis and enhances radiosensitivity in various human cancer cell lines.

#3221 UCN-01-mediated enhancement of radiation cytotoxicity is schedule-dependent in non-small cell lung carcinoma cells. Philip C. Mack, Matthew H. Gustafsson, Paul H. Gumerlock, and Zelanna Goldberg. *UC Davis Cancer Center, Sacramento, CA.*

DNA damage produced by ionizing radiation (IR) results in cell cycle arrest, most notably at the G2 checkpoint. This arrest checkpoint provides an opportunity for cells to repair DNA damage prior to mitosis. Thus, attenuation of IR-induced arrest may be an effective strategy to enhance the efficacy of radiation by curtailing the time available for DNA repair, potentially increasing tumor cell death. The Cdk and Chk1 kinase inhibitor UCN-01 has been shown to enhance the activity of a number of cytotoxic agents, including IR, via abrogation of DNA-damage checkpoints in S and/or G2 phase. Since our previous work demonstrated schedule-dependent potentiation of cisplatin by UCN-01, we hypothesized that enhancement of radiation efficacy by UCN-01 would also be schedule dependent. Using the non-small cell lung carcinoma cell line Calu1 (*p53*-null, wild-type *RB*), the cell cycle response to the clinically-relevant dose of 2Gy was assessed by flow cytometric analysis of DNA content. Following IR, an increased proportion of cells was detected in S and G2 as early as 3h and was maximal at 6 hours. By 18 hours, cell cycle profiles returned to near untreated control values. Enhancement of IR cytotoxicity by UCN-01 was measured by clonogenic survival and assessment of apoptosis. Cells were exposed to UCN-01 for 24 hours at 100-200nM beginning 0, 3, 6 or 9 hours after IR. When UCN-01 was administered immediately after IR exposure (0 hour), the effects of the two agents on colony formation were additive. When UCN-01 administration was delayed, the effects of the two agents were synergistic, achieving maximal interaction when UCN-01 was introduced 6 hours after IR. Extent of apoptosis was measured by DNA fragmentation (TUNEL assay) and by nuclear morphological changes (chromatin staining by DAPI), with all cells harvested at the same timepoint post-IR. Minimal evidence of apoptosis was observed using either technique, although some (less than 7%) was detected when UCN-01 was administered 6 or 9 hours after IR, suggesting that apoptosis does not correlate with colony forming ability. These data demonstrate that there is a schedule-dependency to the IR-plus-UCN-01 interaction that favors delayed administration of UCN-01 after IR exposure. The greatest benefit was observed when UCN-01 was introduced 6 hours after IR, coinciding with the timepoint where the proportion of cells in G1 was lowest. These observations are consistent with the paradigm that UCN-01 enhances IR efficacy by abrogation of DNA damage checkpoint(s). These studies show that cell cycle modulators, such as UCN-01, may be able to greatly potentiate radiation efficacy in the clinic if applied with appropriate regard to timing and sequencing. (Supported by: RSNA Scholars Grant, CA 62505 and ACS IRG-95-125-04)

#3222 Overexpression of DPC-4 induces Bax expression and enhanced radiation sensitivity in DPC-4-null pancreatic cancer cell line BxPC-3. Rachael A. Alcock, Damodaran Chendil, Swatee Dey, Mohammed Mohiuddin, Lee K. Chatfield, Vincent S. Gallicchio, and Mansoor M. Ahmed. *University of Kentucky, Lexington, KY, and University of Central Lancashire, Preston, UK.*

DPC-4 (Deleted in Pancreatic Carcinoma, locus 4 or Smad 4) is a tumor-suppressor gene mutated in approximately 50% of human pancreatic adenocarcinomas. DPC-4 plays an important role as an effector gene in the transforming growth factor-β (TGF-β) signaling pathway. TGF-β signaling plays a pivotal role in the regulation of radiation-induced apoptosis (Ahmed et al, JBC, 2001 In Press). Since, DPC-4 is an effector gene in TGF-β signaling pathway, we hypothesized that loss of this gene will contribute towards enhanced radiation resistance. To test the hypothesis, we selected a DPC-4-null pancreatic cancer cell line BxPC-3 to study the functional role of DPC-4 in radiation-inducible clonogenic inhibition and apoptosis. BxPC-3 cells were infected with Ad/Vector or Ad/Smad4/DPC-4 and irradiated to assess the response to radiation. BxPC-3 cells over-expressing DPC-4 were significantly sensitive ($p<0.00296$) to radiation-induced clonogenic inhibition ($SF_2= 0.17$; $D_0= 63$ cGy) when compared to cells over-expressing the adenoviral vector alone ($SF_2= 0.37$; $D_0= 190$ cGy). Bax protein was significantly elevated in the untreated and irradiated BxPC-3 cells over-expressing DPC-4. This elevation was found to be MOI (multiplicity of infection)-dose dependent. Gel-shift analysis indicated presence of a potential SBE (Smad binding element) site in promoter region of the Bax gene. Thus, the radiosensitization effect of DPC-4 is mediated through restoration of TGF-β signaling and elevation of Bax protein.

#3223 Selective enhancement of radiation responsiveness and apoptosis in MCF-7 breast tumor cells by the vitamin D3 analog, EB 1089. Mona S. Gupta, Hongtao Wang, Myles Cabot, Chris Gennings, Misook Park, and David A. Gewirtz. *Virginia Commonwealth University/Medical College Virginia, Richmond, VA, and John Wayne Cancer Institute, Santa Monica, CA.*

Vitamin D3 analogs such as EB 1089 and ILX 23-7553 enhance the sensitivity of breast tumor cells to ionizing radiation and promote apoptotic cell death. The current studies examined the combination of EB 1089 with fractionated ionizing radiation as fractionated radiation is routinely utilized in the treatment of breast cancer. p53 wild-type MCF-7 cells were exposed to fractionated radiation alone (5 x 2Gy over 5 days), EB 1089 alone (100nM) or EB 1089 followed by radiation. Fractionated radiation and EB 1089 each alone reduced viable cell number by 75% and 84% respectively. Pretreatment with EB 1089 followed by fractionated radiation reduced viable cell number by 98%. EB 1089 and fractionated radiation each alone reduced clonogenic survival by approximately 28% while the combination resulted in an 80% reduction in survival. EB 1089 conferred susceptibility to apoptosis in MCF-7 cells exposed to radiation based on complementary assays for the induction of DNA fragmentation (TUNEL assay and alkaline unwinding). Radiation alone, but not EB 1089 as well as the combination of EB 1089 with radiation induced the expression of beta galactosidase, a marker of replicative senescence. To assess selectivity, we examined the effects of EB 1089 on sensitivity to fractionated irradiation in wild-type BJ skin fibroblasts.and normal breast epithelial cells. EB 1089 failed to enhance radiation sensitivity in the fibroblasts or the breast epithelial cells. Finally, EB 1089, ionizing radiation, as well as the combination of EB 1089 with radiation promoted ceramide generation in the MCF-7 cells. In conclusion, the combination of EB 1089 with fractionated radiation results in the promotion of apoptosis and the induction of senescence in the breast tumor cell, both of which may prove to be linked to the generation of ceramide. The failure of EB 1089 to increase sensitivity to fractionated radiation in either the normal breast epithelial cells or human fibroblasts suggests that vitamin D3 analogs have the potential to enhance the effectiveness of fractionated radiation therapy in breast tumor cells without concurrent sensitization of normal tissues. Supported by the American Institute for Cancer Research and Leo Pharmaceuticals.

#3224 Selective enhancement of radiation responsiveness and apoptosis in MCF-7 breast tumor cells by the vitamin D3 analog ILX 23-7553. Mark Polar and David A. Gewirtz. *Virginia Commonwealth University/Medical College Virginia, Richmond, VA.*

Previous work from this laboratory has demonstrated that the vitamin D3 analogs EB 1089 and ILX 23-7553 enhance the sensitivity of breast tumor cells to ionizing radiation and promote apoptotic cell death. The current studies were designed to more closely simulate clinical radiotherapy in the treatment of breast cancer by examining the utility of ILX 23-7553 as an adjunct to fractionated ionizing radiation. p53 wild-type MCF-7 cells were treated with radiation alone (5 x 2Gy over 3 days), ILX 23-7553 alone (50nM) or ILX 23-7553 followed by radiation. Fractionated radiation and ILX 23-7553 each alone reduced viable cell number by 74.5% and 60.3% respectively. Pretreatment with ILX 23-7553 followed by radiation (5 x 2 Gy) reduced viable cell number by 90%. Statistical analysis supports the existence of an additive interaction between ILX 23-7553 and fractionated radiation in reducing viable cell number. The combination of ILX-23-7553 with fractionated radiation also promoted apoptotic cell death. Repopulation studies performed over a period of 15 days indicated that ILX 23-7553 exposure prior to irradiation significantly slowed the recovery of proliferative capacity whereas ILX-23-7553 after irradiation had a relatively modest effect on recovery. To assess selectivity, we examined the effects of ILX 23-7553 on sensitivity to fractionated irradiation in human BJ skin fibroblasts. ILX-23-7553 failed to enhance radiation sensitivity of the fibroblasts or to promote apoptosis. Preliminary mechanistic studies failed to implicate MAP kinase induction in sensitization to apoptosis in the breast tumor cell. In conclusion, ILX 23-7553 appears to selectively enhance the antiproliferative and apoptotic effects of fractionated ionizing radiation in p53 wild-type MCF-7 breast cancer cells. Current studies are focused on elucidating the mechanism(s) for promotion of apoptosis by fractionated radiation in the presence of vitamin D3 analogs, the basis for our previous findings of a requirement for functional p53 in the enhanced responsiveness to irradiation as well as understanding the basis for the apparent selectivity of ILX-23-7553 in radiosensitization of the breast tumor cell Supported by ILEX Oncology, Inc. and the American Institute for Cancer Research.

#3225 Radiosensitization of human tumor cell lines by flavopiridol. Jeffery S. Russell, Kelli A. Manspeaker, Lisa A. Ridnour, and Philip J. Tofilon. *National Cancer Institute, Bethesda, MD.*

Flavopiridol, a cyclin-dependent-kinase (CDK) inhibitor, structurally related to staurosporine, is currently undergoing Phase I/II clinical trials. Although flavopiridol has been shown to have anti-tumor activity in a number of experimental systems, its ability to influence cell cycle progression suggests that it may affect the response of tumor cells to ionizing radiation. To investigate the radiosensitizing potential of flavopiridol, U251 glioma cells were exposed to a dose of flavopiridol that allowed for greater than 50% survival. This dose (25 nM) was approximately 4-fold less than the reported IC50 values related to the inhibition of CDK activity. Growth curve analyses revealed that at this dose, flavopiridol had no effect on U251 cell proliferation rate. To determine whether pretreatment of

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 DT_{388} –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), DT_{388} – GM-CSF, and CPT-11, to activate ceramide formation, combined with drugs to augment or retard ceramide metabolism such as C_6-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.

REPORTABLE OUTCOME
ABSTRACT
#5

OSLO 2002
18th UICC International Cancer Congress

18th UICC International Cancer Congress

Final programme

Oslo - Norway

30 June – 5 July 2002

Research symposium – New drug development

Hall: Hall B, Oslo Kongressenter
Time: 14.00–16.00 (break 15.00–15.10)
Chairman: Ø. Fodstad, Norway
Co-chairman: E. Mihich, United States

14.00 I 188 Taxol, epothilone and discodermolide – Mechanisms of action and resistance
S. B. Horwitz
Albert Einstein College of Medicine, Department of Molecular Pharmacology, Bronx, New York, United States

14.20 I 189 Molecular targets drug discovery program, CCR, NCI
M. R. Boyd, R. H. Wiltrout, J. C. Barrett
National Cancer Institute (NCI), Center for Cancer Research, Frederick, Massachusetts, United States

14.40 O 174 Gene therapy for colon cancer using survivin antisense expressing replication – incompetent adenoviral vectors
T. Y. Yamamoto, J. O. Okuda, M. T. Toyoda, N. T. Tanigawa
Osaka Medical College, General and Gastroenterological Surgery, Takatsuki, Japan

14.50 O 175 Targeting ceramide metabolism enhances chemotherapy cytotoxicity in drug resistant cancer cells
Y. Y. Liu, H. Wang, A. E. Giuliano, M. C. Cabot
John Wayne Cancer Institute, Breast Cancer Research Program, Santa Monica, United States

15.10 I Expression genomics in drug development
E. Liu
Singapore EDB, Singapore, Singapore

15.30 I 190 Postgenomic approaches to new drug discovery
P. Workman
CRC Centre for Cancer Therapeutics at the Institute of Cancer Research, Belmont, Sutton, United Kingdom

16.00–16.30 Coffee break, served at Oslo Spektrum

Research symposium – Screening and early detection of lung cancer – New technology

Hall: Sentrum Scene
Time: 14.00–15.00
Chairman: F. R. Hirsch, United States
Co-chairman: P. A. Bunn Jr, United States

14.00 I The New York early lung cancer action program (ELCAP)
C. Henscke
N.Y. Presbyterian Hospital, Department of Radiology, Weill Cornell Medical Center, New York, New York, United States

14.20 I 191 The Colorado high-risk cohort study
F. R. Hirsch, T. Byers, S. A. Prindiville, Y. E. Miller, W. A. Franklin, P. A. Bunn, T. C. Kennedy
University of Colorado Cancer Center, Denver, Colorado, United States

14.40 I 192 Which population should be targeted for screening and chemo-prevention programs?
P. A. Bunn Jr
University of Colorado Cancer Center, Denver, Colorado, United States

16.00–16.30 Coffee break, served at Oslo Spektrum

Research symposium – Tobacco control – Do we care? What can the cancer control community do? Proffered papers

Hall: Sentrum Scene
Time: 15.10–16.00
Chairman: M. Daube, Australia

15.10 O 176 Case studies in international tobacco surveillance: the rise in cigarette smoking by women in Spain, 1948–1997
M. A. Corrao, O. Shafey, A. Schiaffino, E. Fernandez, V. E. Cokkinides, M. J. Thun
American Cancer Society, Epidemiology and Surveillance Research, Atlanta, United States

Country	United States
Presenting author	Yes

Abstract Preview

Targeting ceramide metabolism enhances chemotherapy cytotoxicity in drug resistant cancer cells

Y Y Liu, H Wang, A E Giuliano, M C Cabot
John Wayne Cancer Institute, Breast Cancer Research Program, Santa Monica, United States
Contact e-mail: cabot@jwci.org

Multidrug resistance (MDR) is a frequent characteristic of cancer cells and is difficult to predict and manage. MDR is caused by multiple mechanisms including a newly described dysfunction in the metabolism of ceramide, a lipid that promotes the cytotoxic response of various anticancer agents. We have shown that upregulated metabolism of ceramide through glycosylation catalyzed by glucosylceramide synthase (GCS) is responsible for Adriamycin resistance. Here we describe how targeting ceramide metabolism by either pharmacologic or genetic intervention can be effective in enhancing chemotherapy sensitivity. Combining agents that increase ceramide (Adriamycin, fenretinide) with agents that retard ceramide glycosylation (tamoxifen, mifepristone), causes synergistic increases in ceramide and enhanced cell kill. In PC-3 prostate cancer cells for example, 5 µM fenretinide exposure activated de novo ceramide synthesis (2-fold) and was only slightly cytotoxic (80% viability); however, when combined with nontoxic levels of tamoxifen, ceramide levels increased 9-fold over control and viability fell to 10%. For genetic intervention, we have introduced GCS antisense cDNA into Adriamycin-resistant breast cancer cells (MCF-7-AdrR), cells that demonstrate enhanced ceramide glycosylation. The antisense-transfected cell line exhibited decreases in GCS mRNA, GCS protein, and GCS enzymatic activity, and was 40-, 100-, and 200-times more sensitive to Adriamycin, vinblastine, and Taxol, respectively, compared to MCF-7-AdrR cells. Heightened drug sensitivity was accompanied by resumption of ceramide generation, caspase activation, and apoptosis. We have recently constructed antisense oligonucleotides to GCS and shown that a 4 hr exposure to 100 - 200 nM enhanced Adriamycin sensitivity by 30- and 10-fold in MCF-7-AdrR cells and in Adriamycin-resistant human ovarian cancer cells, respectively. These studies show that GCS is a viable target in cancer therapy.

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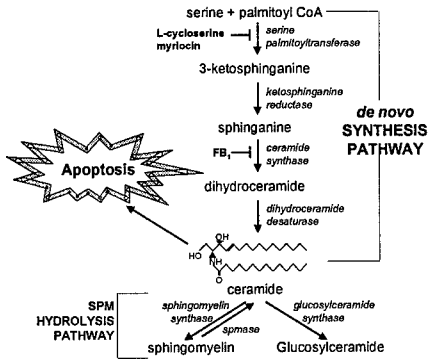
Targeting Ceramide Metabolism Enhances Chemotherapy Cytotoxicity in Drug Resistant Cancer Cells

YY Liu, H Wang, AE Giuliano, MC Cabot

Breast Cancer Research Program
John Wayne Cancer Institute
at Saint John's Health Center
Santa Monica, CA, USA

1



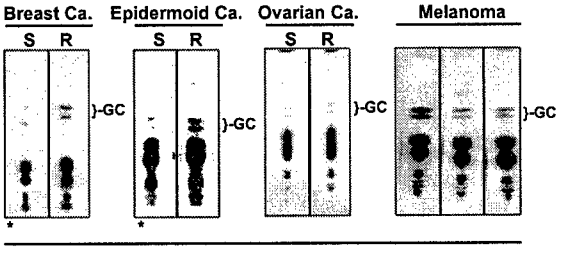
2

Anticancer Agents that Increase Cellular Ceramide Levels

- ⇒ Anthracyclines (dox, daun)
- ⇒ Vinblastine
- ⇒ Etoposide
- ⇒ Taxol
- ⇒ 4-HPR (fenretinide)
- ⇒ SDZ PSC 833 (P-gp drug)
- ⇒ DT_{388}-GM-CSF
- ⇒ CPT-11 (Irinotecan)

3

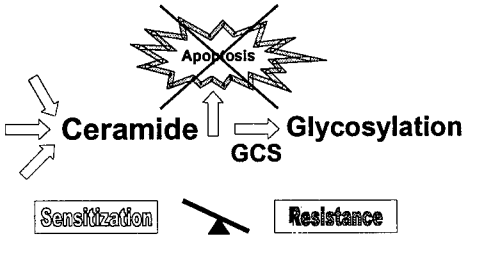
Elevated Glucosylceramide in MDR Cancer Cells



**Lavie Y, et al, J. Biol. Chem. (1996)*

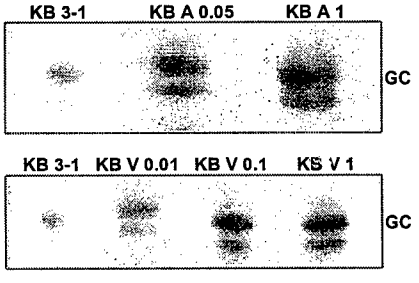
4

Developing Chemoresistance



5

GC is Increased in Cancer Cells Selected for Resistance



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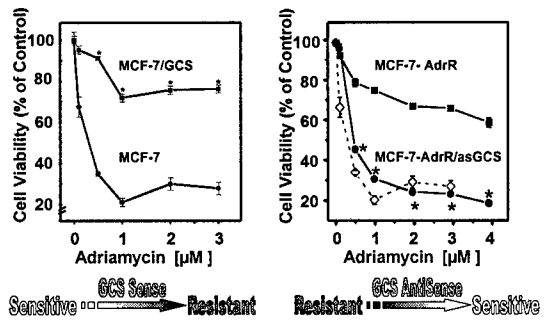

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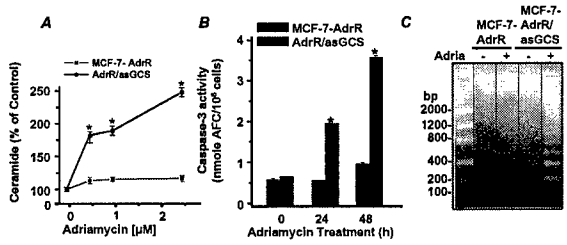
asGCS Restores Ceramide/Caspase Apoptosis Signal



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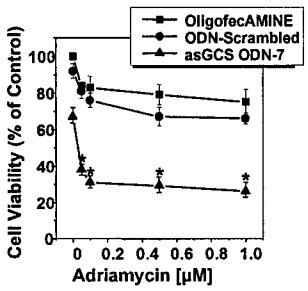

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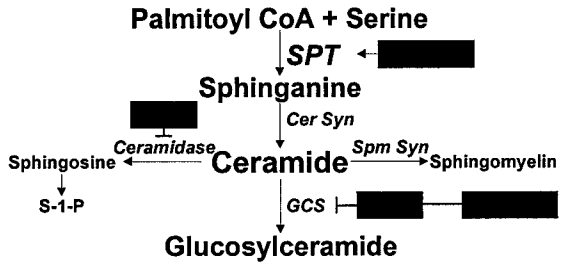
Prostate and Breast Cancer



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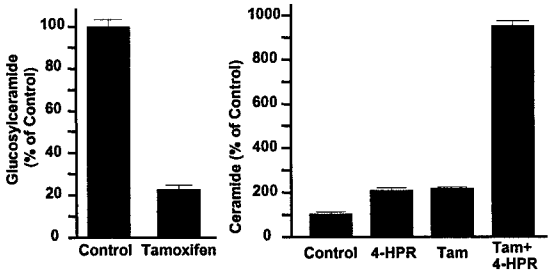
Drugs that Synergize for Ceramide Production in Prostate Cancer Cells



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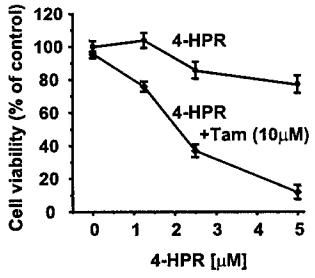
Tamoxifen Enhances 4-HPR Cytotoxicity in Prostate Cancer Cells



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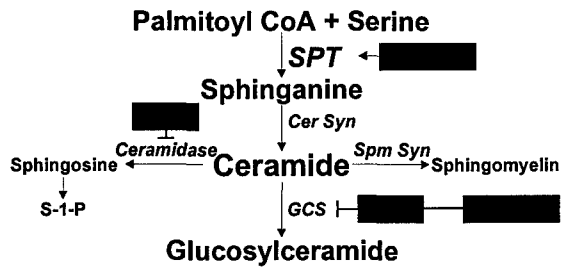
Prostate and Breast Cancer



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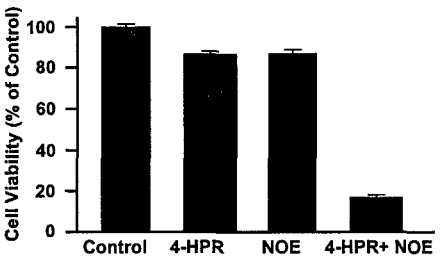
Ceramidase Inhibition Enhances 4-HPR Cytotoxicity in MDR Breast Cancer Cells



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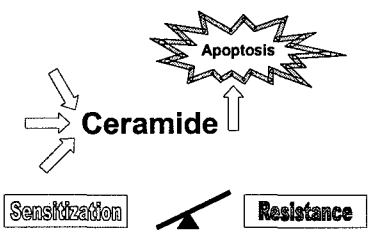
Harnessing Ceramide Metabolism to Restore Chemosensitivity



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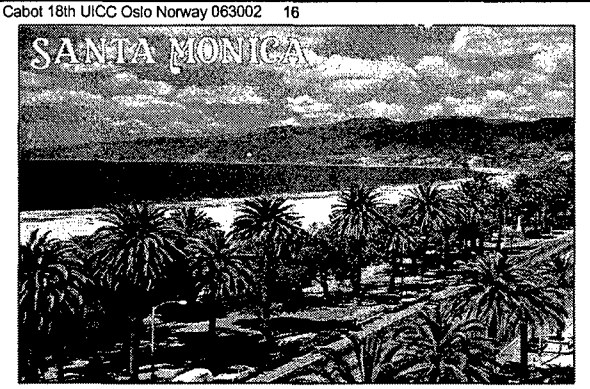

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Abstract

TITLE:

Glucosylceramide Synthase Upregulation - A New Potential Target For Doxorubicin Resistance In Breast Cancer

AUTHORS (ALL): Bleicher, Richard J[1]; Liu, Y Y[1]; Wang, H [1]; Gouaze, V [1]; Yu, J Y[1]; Giuliano, A E[1]; Cabot, M C[1]

INSTITUTIONS (ALL): 1. Surgical Oncology, The John Wayne Cancer Institute, Santa Monica, CA, USA;

ABSTRACT BODY:

Introduction: Multidrug resistance is a significant phenomenon after treatment with doxorubicin (dox)-based chemotherapy. We have demonstrated that this is due, in part, to glycosylation of ceramide (a messenger of apoptosis) via the action of glucosylceramide synthase (GCS). Because anthracyclines elevate ceramide in breast cancer, we hypothesized that upregulation of the GCS gene would be associated with breast cancer resistance to dox.

Methods: Human MCF-7 breast cancer cells and dox-resistant MCF-7-AdrR cells were used in all experiments. Cell sensitivities to dox were determined using an MTS assay. Glucosylceramide (GC) levels in cells were measured using [^{3}H]palmitic acid tracer and thin-layer chromatography, and GCS activity was evaluated by enzyme assay. Western blots were performed in standard fashion. Expression of the GCS gene was analyzed using RNA isolated from cell lysates with subsequent RT-PCR and 1% agarose gel electrophoresis.

Results: The EC_{50} of dox was 12.4 μM $\pm$ 0.7 and 0.37 $\pm$ 0.01 μM in MCF-7-AdrR and MCF-7, respectively. Microsomal GCS activity was higher in MCF-7-AdrR as compared with wild-type cells (27.4 $\pm$ 2.3 *vs* 17.2 $\pm$ 0.1 pmol/h/ μg protein). GC content was also found to be higher in the dox-resistant cells (2.5 $\pm$ 0.17% *vs* 1.5 $\pm$ 0.12% of total lipids). RT-PCR demonstrated a significantly higher level of GCS expression in the dox-resistant cell line compared with MCF-7 cells. Western blots also demonstrated a 3.5-fold higher level of GCS protein in MCF-7-AdrR cells.

Conclusion: Dox resistance may be partially mediated by increased GCS gene expression, thus circumventing the pathway to ceramide-mediated apoptosis. This is the first demonstration of GCS gene overexpression in dox-resistant breast cancer cells, raising the possibility that acquired resistance to anthracyclines is mediated this way. Further investigation is needed to determine whether GCS gene regulation can be used to reverse drug resistance to breast cancer.

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