INCREASING INTRACELLULAR CERAMIDE: AN APPROACH THAT ENHANCES THE CYTOTOXIC RESPONSE IN PROSTATE CANCER CELLS

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ABSTRACT

Objectives. To investigate the feasibility of targeting ceramide metabolism to enhance chemotherapy cytotoxicity in prostate cancer. Discovering new targets for cancer treatment is an important endeavor, especially in prostate malignancies, which often revert to hormone- and chemotherapy-refractory disease states.

Methods. Ceramide metabolism was measured in human prostate cancer cell lines using [3H]palmitic acid as the tracer. Cellular lipids were analyzed by thin-layer chromatography and liquid scintillation counting. Cell viability in response to drug exposure was measured spectrophotometrically using commercial cell proliferation reagents.

Results. LNCaP cells were five times more sensitive to N-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, compared with PC-3 cells. Ceramide levels increased only twofold in PC-3 cells versus 10-fold in LNCaP cells in response to 10 μM 4-HPR. PC-3 resistance to 4-HPR could be reversed by the addition of tamoxifen or other agents that block the metabolism of ceramide to glucosylceramide, and with tamoxifen this was marked by a ninefold increase in cellular ceramide levels. The influence of 4-HPR on ceramide metabolism was shown to be through activation of serine palmitoyltransferase, the rate-limiting enzyme in the ceramide synthesis pathway. Blocking the ceramide generated by 4-HPR reduced the extent of apoptosis.

Conclusions. Increasing intracellular concentrations of ceramide may be an avenue to enhance the cytotoxic response to chemotherapy in human prostate cancer.


Prostate cancer is the most common noncutaneous malignancy in men in the United States. Of the 190,000 cases estimated for 2002, nearly 30,000 will succumb to this disease.1 Metastatic prostate cancer is one of the most therapy-resistant neoplasms known; standard treatment consists of androgen ablation by surgical orchietomy or the use of luteinizing hormone-releasing hormone analogs such as Lupron or Zoladex, with or without antiandrogens to inhibit hormone uptake. With this, however, disease progression to androgen independence is eventually observed in most patients. Patients with a relapse after treatment with androgen ablation develop disease that is both hormone refractory and resistant to chemotherapy.2

Prostate cancer is the second-most leading cause of death in men in the United States. It is, therefore, crucial to develop therapies that outperform the currently available hormonal treatments. It is, however, a widely held notion that chemotherapy has little role in the treatment of hormone-refractory disease. In the past few years, estramustine-based regimens, camptothecin analogs, and doxorubicin-based combinations have shown encouraging responses, but, for the most part, these therapies have been palliative in nature with little significant impact on survival.3,4
Recent developments in cell and molecular biology are transforming our approaches to anticancer drug research. New cellular targets for testing anticancer agents based on molecular mechanisms of neoplastic transformation, cancer growth, and response to chemotherapy are being studied. Vitamin A analogs (retinoids) comprise an interesting group of agents being evaluated in clinical trials for therapy and the prevention of a variety of malignancies. The synthetic retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR or fenretinide) has been shown to be effective against prostate cancer in an in vivo animal model, and in vitro, it inhibits prostate cancer cell proliferation and induces apoptosis. 4-HPR also elicits apoptosis in androgen-dependent and independent human prostate carcinoma cells by mechanisms involving retinoic acid receptors and apoptosis-related genes, p21, c-myc, and c-jun.

Many front-line anticancer agents have been shown to induce ceramide formation in cancer cells. We have recently shown that 4-HPR enhances ceramide levels in neuroblastoma cells by coordinate activation of serine palmitoyltransferase (SPT) and ceramide synthase enzymes of the de novo pathway of ceramide synthesis and induces cell death by mixed apoptosis/necrosis.

In addition, several groups have shown that chemotherapy cytotoxicity can be heightened by including agents that block ceramide glycosylation; however, little work has been done in prostate models. We show that 4-HPR promotes ceramide formation in human prostate cancer cells and that the cytotoxic potential of 4-HPR can be enhanced by adding agents that sustain ceramide levels.

**MATERIAL AND METHODS**

**CELL CULTURE**

The human prostate cancer cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Rockville, Md). LNCaP cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 584 mg/L L-glutamine. PC-3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 584 mg/L L-glutamine. Cells were grown and passaged as described previously. For experiments, cells were subcultured into 6-well or 96-well plates or 5-cm dishes, and the FBS content of the medium was lowered to 5%. Culture medium containing 4-HPR was prepared just before use. Ethanol vehicle was present in controls.

**REAGENTS**

4-HPR was provided by R. W. Johnson Pharmaceuticals (Spring House, Pa). Culture media were products of Life Technologies (Grand Island, NY), and FBS was from HyClone (Logan, Utah). Tamoxifen was purchased from Sigma (St. Louis, Mo). Fumonisin B1 (FB1) and l-cycloserine were purchased from Biomol (Plymouth Meeting, Pa). Ceramide and sphingomyelin (brain derived) were from Avanti Polar Lipids (Alabaster, Ala). The glucosylceramide synthase inhibitor, dl-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), and sphinganine were from Matreya (Pleasant Gap, Pa). [9,10-3H(N)]Palmitic acid (50 Ci/mmol) was from DuPont/NEN (Boston, Mass). [5,6-3H]Sphinganine (60 Ci/mmol), and l-[3H]serine (20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, Mo). Silica Gel G thin-layer chromatography plates were purchased from Analtech (Newark, Del). Other chemicals were from Sigma (St. Louis, Mo).

**CELL RADIOLABELING AND LIPID ANALYSIS**

Cell radiolabeling with [3H]palmitic acid, lipid extraction, and thin-layer chromatography resolution of [3H]ceramide, glucosylceramide, sphinganine, and sphingomyelin were conducted as described previously.

**ENZYME ASSAYS**

SPT was assayed with [3H]serine as described, using microsomes (100 µg protein) as the enzyme source. Ceramide synthase was assayed using [3H]sphinganine, 100 µg microsomal protein, and 0.1 mM palmitoyl-coenzyme A as previously described.

**CYTOTOXICITY ASSAYS**

LNCaP and PC-3 cells (3000 cells/well) were seeded in 96-well plates in 0.1 mL of medium containing 5% FBS. Cells were cultured for 24 hours before the addition of the drugs. Drugs were diluted into 5% FBS-containing medium and added to each well to give a final volume of 0.2 mL. Cells were incubated at 37°C for 72 hours, and cell viability was determined using the Promega Cell Titer 96 Aqueous Cell Proliferation Kit, using an enzyme-linked immunosorbent assay plate reader (Biotek, Winooski, Vt).

**APOPTOSIS DETECTION**

Apoptosis was quantitatively measured using the Cell Death Detection ELISA-PLUS (Roche Diagnostics, Indianapolis, Ind). This is a photometric enzyme immunoassay using mouse monoclonal antibody directed against DNA and histone. The assays were conducted as detailed in the company instructions.

**RESULTS**

4-HPR, CERAMIDE FORMATION, AND CYTOTOXICITY

4-HPR exposure (2.5 to 10 µM) elicited a dose-dependent increase in ceramide in LNCaP cells (10-fold with 10 µM, Fig. 1A). By comparison, ceramide formation in PC-3 cells was minimal, at two-fold with 10 µM. Cells also displayed dissimilar sensitivity to 4-HPR, with LNCaP being sensitive (median effective concentration 2.2 µM), and PC-3 cells relatively refractory (Fig. 1B).

**SOURCE OF CELLULAR CERAMIDE AND INDUCTION OF APOPTOSIS**

Preliminary studies assessing sphingomyelin metabolism in LNCaP cells in response to 4-HPR suggested that ceramide was not formed by hydro-
lysis of sphingomyelin. On the other hand, exposure of LNCaP cells to FB1, a de novo inhibitor of ceramide synthesis, completely blocked 4-HPR-induced ceramide formation (Fig. 2A). This indicates that ceramide is generated in prostate cancer cells through targeting the de novo pathway enzymes, SPT and ceramide synthase. To explore this, cultured cells were preincubated with 4-HPR and lysed to obtain microsomes for cell-free enzyme assays. As shown in Fig. 2B, this procedure enhanced SPT activity in a dose-dependent fashion (70% more than controls at 5.0 μM). Ceramide synthase activity, assayed similarly, increased only slightly (10% at 5.0 μM 4-HPR, data not shown). Measurement of enzyme-linked immunosorbent assay-detected nucleosomes showed that 4-HPR elicited apoptosis (Fig. 2C). To determine whether ceramide was contributory to cell death, FB1 was added to the treatment regimen to block ceramide formation. As shown in Fig. 2D, FB1 reduced the extent of 4-HPR-elicited apoptosis by 50%, indicating that ceramide is causal in the 4-HPR cytotoxic response.

Enhancing the Ceramide Effect by Enzyme Targeting

Elevated levels of glucosylceramide (GC) are a characteristic of chemotherapy-resistant breast cancer cells and multidrug-resistant epidermoid carcinoma.9 PC-3 cells also have high levels of GC compared with drug-responsive LNCaP cells, as demonstrated by steady-state radiolabeling of cells with [3H]galactose (Fig. 3A). Because PC-3 cells were refractory to 4-HPR (Fig. 1B), we sought to determine whether sensitivity could be enhanced by increasing the formation of intracellular ceramide. For this, agents known to retard the conversion of ceramide to GC were evaluated. Exposure of PC-3 cells to either tamoxifen or PPMP inhibited GC synthesis by approximately 80% (Fig. 3B). Both agents enhanced ceramide levels approximately twofold (Fig. 3C) by disruption of ceramide glycosylation, and 4-HPR alone increased ceramide levels twofold. However, when given concurrently with 4-HPR, both tamoxifen and PPMP increased ceramide levels 9.5-fold and fivefold, respectively (Fig. 3C). The influence of enhanced ceramide generation on cell viability was evaluated. Although PC-3 cells were relatively resistant to 4-HPR, the inclusion of tamoxifen increased 4-HPR sensitivity, as demonstrated by the precipitous drop in cell viability (Fig. 3D).

Comment

The results of our study demonstrated that 4-HPR activates ceramide formation and elicits apoptosis in human prostate cancer cells. The enzymatic events underlying ceramide formation have been characterized. In that 4-HPR has been shown to exert myriad effects in prostate cancer cells, this work represents insight that may guide strategies for its use in the treatment of prostate cancer. A similar study supporting this idea was conducted in DU-145 cells, in which ceramide was shown to accumulate, in response to suramin treatment, before apoptosis.19

Several works have demonstrated the apoptosis-inducing effects of 4-HPR on prostate cancer
and other synthetic retinoids have shown similar properties with androgen-resistant cells. In a mouse model of prostate cancer progression, dietary 4-HPR suppressed development of bone metastasis. In another study, 4-HPR diminished neoplastic characteristics of prostate cancer cells, prompting the investigators to suggest that the retinoid be used as an agent for prevention. Although various lines of investigation implicate reactive oxygen species, retinoic acid receptors, and apoptosis-related genes in the mechanism of action of 4-HPR, this is the first study to show that 4-HPR elicits ceramide production in prostate cancer cells. In drug-resistant PC-3 cells, ceramide production in response to 4-HPR was many fold less than that observed in LNCaP; however, this could be reversed and cytotoxicity restored to the level inherent in LNCaP cells, when ceramide glycosylation blockers were added (Figs. 1B and 3D). These findings complement work by Wang and colleagues who showed, using camptothecin, that PC-3 resistance was associated with a defect in ceramide metabolism. Although the defect may be related to either deficient activation of SPT and/or ceramide synthase by camptothecin, or ceramidase overexpression, as recently demonstrated in prostate cancer, our results with agents that block ceramide glycosylation point to GC synthase as the determining factor.
That the cytotoxic principle of 4-HPR is in part related to ceramide has been shown by experiments with the ceramide synthase inhibitor, FB₁ (Fig. 2). FB₁ blocked 4-HPR elicited ceramide generation and depressed the apoptosis-inducing capacity of 4-HPR by 50%. Apoptosis was not completely blocked, because FB₁, although inhibiting conversion of sphinganine to ceramide, does not inhibit SPT, which catalyzes formation of sphinganine, another of the cytotoxic sphingoid bases.²⁹,³⁰

CONCLUSIONS

The finding that 4-HPR activates SPT, and that cell-killing synergy is created by adding tamoxifen to inhibit ceramide glycosylation, suggests that a unique-targeted approach to treating prostate cancer may be within reach (Fig. 4). We caution, however, that this was a study using a limited number of cell lines; we are currently exploring other androgen-independent models. New developments in cellular and molecular biology are transforming the avenues to anticancer drug research, and the discovery of new molecular targets is among the most important of endeavors. In prostate cancer, which can recur as therapy-resistant disease, the need for a targeted approach to control metastatic growth is well overdue. We encourage implementation of clinical studies using agents that target ceramide metabolism.
Palmitoyl CoA + Serine

\[ \text{SPT} \rightarrow 4-	ext{HPR} \]

Sphinganine

\[ \text{Cer Syn} \rightarrow \text{Ceramide} \]

GCS → Tamoxifen

\[ \text{Glucosylceramide} \]

**FIGURE 4. Ceramide metabolism and sites of drug interaction. Cer Syn = ceramide synthase; GCS = glucosylceramide synthase.**

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**REFERENCES**


