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Modification of sphingolipid metabolism by tamoxifen and *N*-desmethyltamoxifen in acute myelogenous leukemia—Impact on enzyme activity and response to cytotoxics

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ABSTRACT

The triphenylethylene antiestrogen, tamoxifen, can be an effective inhibitor of sphingolipid metabolism. This off-target activity makes tamoxifen an interesting ancillary for boosting the apoptosis-inducing properties of ceramide, a sphingolipid with valuable tumor censoring activity. Here we show for the first time that tamoxifen and metabolite, *N*-desmethyltamoxifen (DMT), block ceramide glycosylation and inhibit ceramide hydrolysis (by acid ceramidase, AC) in human acute myelogenous leukemia (AML) cell lines and in AML cells derived from patients. Tamoxifen (1–10 μM) inhibition of AC in AML cells was accompanied by decreases in AC protein expression. Tamoxifen also depressed expression and activity of sphingosine kinase 1 (SphK1), the enzyme-catalyzing production of mitogenic sphingosine 1-phosphate (S1-P). Results from mass spectroscopy showed that tamoxifen and DMT (i) increased the levels of endogenous C16:0 and C24:1 ceramide molecular species, (ii) nearly totally halted production of respective glucosylceramide (GC) molecular species, (iii) drastically reduced levels of sphingosine (to 9% of control), and (iv) reduced levels of S1-P by 85%, in vincristine-resistant HL-60/VCR cells. The co-administration of tamoxifen with either *N*-(4-hydroxyphenyl)retinamide (4-HPR), a ceramide-generating retinoid, or a cell-deliverable form of ceramide, C6-ceramide, resulted in marked decreases in HL-60/VCR cell viability that far exceeded single agent potency. Combination treatments resulted in synergistic apoptotic cell death as gauged by increased Annexin V binding and DNA fragmentation and activation of caspase-3. These results show the versatility of adjuvant triphenylethylene with ceramide-centric therapies for magnifying therapeutic potential in AML. Such drug regimens could serve as effective strategies, even in the multidrug-resistant setting.

1. Introduction

Sphingolipid metabolism is an area of cancer biology that has risen to prominence over the past decade and a half [1–4].This is because ceramide, the aliphatic backbone of sphingolipids, acts as a powerful tumor censor, whereas its glycosylated product, GC, formed by the action of glucosylceramide synthase (GCS), is anti-apoptotic and a biomarker of multidrug resistance [5]. Ceramidase, in particular acid ceramidase (AC), another sentinel enzyme regulator of cancer cell growth, has been identified as candidate gene for development of new cancer

Abbreviations: DMT, *N*-desmethyltamoxifen; AC, acid ceramidase; AML, acute myelogenous leukemia; SphK1, sphingosine kinase 1; S1-P, sphingosine 1-phosphate; GC, glucosylceramide; 4-HPR, fenretinide; GCS, glucosylceramide synthase; P-gp, P-glycoprotein; ATCC, American Type Culture Collection; C6-ceramide, *N*-hexanoyl-D-*erythro*-sphingosine; DMSO, dimethyl sulfoxide; LCL204, (1R,2R) 2-(*N*-tetradecylamino)-1-(4-NO2)-phenyl- 1,3-dihydroxy-propane HCl; TLC, thin-layer chromatography; ESI-MS/MS, electrospray ionization–tandem mass spectrometry; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PI, propidium iodide; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride; GADPH, glyceraldehyde 3-phosphate dehydrogenase; ANOVA, analysis of variance; CI, combination index; PPMP, D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-proapnol; SM, sphingomyelin

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diagnostics and touted as a therapeutic target in metastatic cancer [6,7]. Much like GCS, AC dampens the tumor suppressor properties of ceramide via ceramide hydrolysis, leading to the formation of sphingosine 1-phosphate (S 1-P), a problematic mitogen in cancer [8]. Thus, sphingolipid metabolism is a dynamic process with complex orchestration, impact, and clinical applications, presenting druggable targets for exploitation.

GCS, AC, and sphingosine kinase 1 (SphK1), which catalyzes formation of S 1-P, are all important regulators of the tumor suppressor actions of ceramide [2,9]. Numerous studies have demonstrated that the inhibition of these enzymes drives apoptotic responses and/or subdues mitogenicity [7,10]. Although many inhibitors of these enzymes have been studied, rarely does one find a single agent that demonstrates multihit capacity. The present study shows, in human acute myelogenous leukemia (AML) cell lines and in AML cells derived from patients, that tamoxifen inhibits ceramide glycosylation, AC activity, and Sphk1 activity, and downregulates AC and SphK-1 expression, and sensitizes AML cells to ceramide-centric therapeutics. These unique actions place tamoxifen and associated chemical relatives, *N*-desmethyltamoxifen (DMT) and raloxifene, in an interesting place for study, specifically as regards "off-target" activities of triphenylethylenes in cancer therapy.

Our earlier studies with tamoxifen showed that this gold standard therapy for the treatment of estrogen receptor-positive breast cancer, acted as a potent inhibitor of ceramide glycosylation [11], an action in common with other P-glycoprotein (P-gp) antagonists such as verapamil and cyclosporin A [12]. Whereas tamoxifen is not a specific GCS inhibitor, addition to cells in culture results in reduced glucosylceramide (GC) levels [12] and in some instances increases in intracellular ceramide levels [13]. Another commonality of tamoxifen with sphingolipid metabolism is the inhibition of AC, a property associated with elicitation of lysosomal membrane permeability and subsequent AC proteolysis by cathepsin B [14].

The focus of the present study is AML, the most common type of leukemia in adults. AML is aggressive, and only about 25% of patients that experience remission with cytotoxic chemotherapy remain disease free. Drug resistance is a major problem in the treatment of AML [15], and new therapeutic approaches are needed. The use of P-gp antagonists to overcome drug resistance has had limited clinical success in the treatment of leukemia [16].

An allied function of drug transporters such as P-gp is glycolipid trafficking [17], an area that has not been explored from a therapeutic standpoint. Building upon what has been learned from past work on the effects of tamoxifen on ceramide glycosylation [11,12], it appears appropriate that triphenylethylene anti-estrogens could have therapeutic application in AML, application divorced from their use as modulators of multidrug resistance and divorced from anti-estrogenic activities. Herein we report for the first time that tamoxifen targets three important junctures in sphingolipid metabolism in AML, ceramide glycosylation, ceramide hydrolysis, and sphingosine phosphorylation. This work is an introduction to future research on the use of cell-deliverable ceramides and/or ceramide-generating agents in combination with triphenylethylenes in the treatment of cancer and supportive of combination therapy with standard of care approaches to treating cancer.

2. Materials and methods

2.1. Cell lines, patient samples, and reagents

Human AML cell lines HL-60 and KG-1 were obtained from the American Type Culture Collection (ATCC), Manassas, VA. The cells were expanded and cryo-preserved in liquid nitrogen in the investigator's laboratory. Cell lines were authenticated by documentation provided by the ATCC, which include antigen expression, DNA profile, and cytogenic analysis. HL-60/VCR cells were provided by A.R. Safa (Indiana University School of Medicine, Indianapolis, IN); cells were grown in medium containing 1.0 μg vincristine sulfate/mL culture medium. Cell lines were maintained for approximately 30 passages in RPMI-1640 GlutaMAX medium (Life Technologies, Carlsbad, CA), supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA) and 100 U/mL of penicillin and 100 μg/mL streptomycin. For experiments with HL-60/VCR cells, vincristine was removed from the medium. Cells were cultured in a humidified atmosphere, 95% air, 5% CO_2 at 37 °C.

AML patient samples with 20% or greater blast count were obtained with informed consents signed for sample collection according to a protocol approved by the Institutional Review Board of the Milton S. Hershey Medical Center. Cells were cryo-preserved before use, and after thawing, cells were seeded for experiments based upon viable cell counts.

C6-ceramide (*N*-hexanoyl-D-*erythro*-sphingosine) was obtained from Avanti Polar Lipids, Alabaster, AL, and dissolved in 100% ethanol (10 mM stock) and stored at −20 °C. *N*-(4-hydroxyphenyl)retinamide (4-HPR), a product of Calbiochem (San Diego, CA), was dissolved in DMSO (10 mM stock) in amber glass vials and stored at −20 °C. Tamoxifen-HCl and DMT were purchased from Sigma Chemical Company, St. Louis, MO; all were dissolved 100% ethanol (10 mM stock) and stored at −20 °C. LY117018, a raloxifene analog, obtained from Eli Lilly, Indianapolis, IN, was dissolved and stored, as were the other triphenylethylenes. LCL204 ((1R,2R) 2-(N-tetradecylamino)-1-(4-NO2)-phenyl- 1,3-dihydroxy-propane HCl) was generously provided by Dhimant Desai and Shantu Amin, Penn State College of Medicine, Organic Synthesis Core Facility, Hershey, PA. Radiolabeled [9,10-^{3}H] palmitic acid, 40–60 Ci/mmol, was purchased from American Radiolabeled Chemicals, St. Louis, MO, and stored at −20 °C. Solvents, HPLC-grade, for thin-layer chromatography (TLC) were purchased from ThermoFisher Scientific (Waltham, MA), and Silica Gel G plates were purchased from Analtech, Newark, DE. Ecolume liquid scintillation fluid was a product of MP Biomedicals, Solon, OH.

2.2. Acid ceramidase activity assays

AC activity was measured in intact cells by fluorogenic assay as described [18], with slight modification. Briefly, cells (20,000/well) were seeded into 96-well plates, in 50 μl 10% FBS-containing culture medium. After 24 h, tamoxifen or DMT was added to the cells (in medium containing 1% FBS) to a final volume of 100 μL (controls contained ethanol vehicle), and cells were placed in a tissue culture incubator at 37 °C, 5% CO_2, for 24 h. Cell viability assays were conducted in parallel (see methods below). Fluorogenic substrate (ethanol vehicle) was then added to the wells to a final concentration of 16 μM (125 μL final well volume), and the cells were incubated for 3 h. At this point, the plates with cells were placed at −20 °C, and the chemistry was conducted the following day. To complete the chemistry, 50 μL methanol and 100 μL $NaIO_4$ (2.5 mg/mL) in 0.1 M glycine buffer, pH 10.6, were added, and the plates were incubated in the dark for 2 h at 37 °C. Fluorescence was measured in the UV range (365 nm excitation/410–460 nm emission) using a GloMax multidetection system (Promega, Madison, WI).

2.3. Sphk1 activity assays

Sphk1 activity was quantified by using a commercial Sphingosine Kinase Activity Assay Kit (Echelon Biosciences, Salt Lake City, UT) as the manufacturer instructed. In brief, 1×10^6 cells were incubated with tamoxifen for the indicated times, at 37 °C in culture medium containing 5% FBS, collected by centrifugation, and washed in ice-cold PBS. Cells were then lysed by freeze-thaw cycles in the reaction buffer provided, and lysates were then incubated in reaction buffer containing 100 μM sphingosine and 10 μM ATP for 1 **h** at 37 °C. Luminescence-attached ATP detector was then added to stop the reaction. Luminescence was determined by using a Perkin Elmer Victor-3 1420 microplate reader.