Lactoferricin-induced apoptosis in estrogen-nonresponsive MDA-MB-435 breast cancer cells is enhanced by C_6 ceramide or tamoxifen

SUZANNE J. FURLONG[1], JAMIE S. MADER[2] and DAVID W. HOSKIN[1,2]

[1]Department of Microbiology and Immunology, [2]Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada

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Abstract. Bovine lactoferricin (LfcinB) is a cationic peptide that selectively induces caspase-dependent apoptosis in human leukemia and carcinoma cell lines. Ceramide is a second messenger in apoptosis signaling that has been shown to increase the cytotoxicity of various anti-cancer drugs. In this study, we determined whether manipulation of intracellular ceramide levels enhanced LfcinB-induced apoptosis of estrogen-nonresponsive MDA-MB-435 breast carcinoma cells. LfcinB caused DNA fragmentation and morphological changes consistent with apoptosis in MDA-MB-435 breast cancer cell cultures, but did not affect the viability of untransformed mammary epithelial cells. MDA-MB-435 breast cancer cells also exhibited DNA fragmentation and morphological changes consistent with apoptosis following exposure to the cell-permeable ceramide analog C_6. An additive increase in DNA fragmentation was observed when both LfcinB and C_6 ceramide were added to MDA-MB-435 breast cancer cell cultures. A greater than additive increase in DNA fragmentation was seen when LfcinB was used in combination with tamoxifen, which prevents the metabolism of endogenous ceramide to glucosylceramide by glucosyl-ceramide synthase, as well as blocking estrogen receptor signaling. However, a selective inhibitor of glucosyl-ceramide synthase,1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, failed to further increase DNA fragmentation by LfcinB, suggesting that tamoxifen enhanced LfcinB-induced apoptosis in breast cancer cells via a mechanism that did not involve glucosylceramide synthase inhibition. We conclude that combination therapy with LfcinB and tamoxifen warrants further investigation for possible use in the treatment of breast cancer.

Correspondence to: Dr David Hoskin, Department of Microbiology and Immunology, Dalhousie University, Room 7E1, Sir Charles Tupper Medical Building, 5850 College Street, Halifax, Nova Scotia B3H 1X5, Canada
E-mail: d.w.hoskin@dal.ca

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Introduction

Bovine lactoferricin (LfcinB) is a cationic anti-microbial peptide generated by acid-pepsin hydrolysis of lactoferrin obtained from cow's milk (1). We have shown that LfcinB is a potent inducer of apoptosis in human cancer cell lines of hematopoietic and epithelial origin, including leukemia and carcinoma of the colon and ovaries (2). LfcinB-induced apoptosis results from reactive oxygen species- and caspase-2-dependent disruption of mitochondrial membrane integrity and the subsequent sequential activation of caspase-9 and caspase-3. Activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) may also play an important role in LfcinB-induced apoptosis of human cancer cells since exposure to pepsin-digested bovine lactoferrin causes JNK/SAPK activation in the human oral squamous cell carcinoma cell line SAS (3). Moreover, the apoptotic response is diminished when SAS cells are pretreated with a JNK/SAPK inhibitor. LfcinB has also been reported to exert potent *in vivo* anti-tumor activity in mouse models of cancer. Direct injection of LfcinB into solid Meth A tumors causes tumor cell lysis and a significant reduction in tumor size (4). In addition, subcutaneous administration of LfcinB inhibits tumor metastasis by highly metastatic murine L5178Y-ML25 lymphoma cells and B16-BL6 melanoma cells (5). However, in all cases, relatively high concentrations of LfcinB are required for the anti-cancer effect, which may pose a barrier to the use of LfcinB in cancer treatment.

Ceramide is a membrane sphingolipid that functions as an important second messenger during apoptosis induction in response to death receptor signaling and cellular stress (6,7). Cellular ceramide is generated by sphingomyelinase-mediated hydrolysis of sphingomyelin or *de novo* synthesis catalyzed by ceramide synthase or serine palmitoyl transferase. Although the mechanism by which intracellular ceramide accumulation promotes apoptosis remains controversial, evidence suggests that ceramide-induced apoptosis may involve mitochondrial ceramide generation and the subsequent formation of ceramide channels in the mitochondrial outer membrane (7). Administration of exogenous cell-permeable ceramide analogs also triggers apoptosis in various cell lines, including mouse fibrosarcoma cells and human leukemia and breast carcinoma cells (8,9). In addition, exposure to exogenous ceramide

augments paclitaxel-mediated cytotoxicity against human head and neck squamous carcinoma cells (10). Apoptosis can also be induced in human melanoma and leukemia cells by treatment with inhibitors of ceramide metabolism, which results in elevated levels of endogenous ceramide (11,12). Interestingly, adriamycin-resistant MCF-7 breast carcinoma cells are rendered sensitive to adriamycin by treatment with agents such as tamoxifen, which block ceramide glycosylation (13). Taken together, these findings suggest that manipulation of the ceramide metabolic pathway is a promising strategy to enhance the effectiveness of anti-cancer drugs.

In this study, we employed the cell-permeable ceramide analog C_6, as well as inhibitors of ceramide conversion to glucosylceramide, to determine the effect of intracellular ceramide accumulation on LfcinB-induced apoptosis in human estrogen receptor-nonresponsive MDA-MB-435 breast carcinoma cells. Our findings indicate that both exogenous C_6 ceramide and tamoxifen potentiated the cytotoxic effect of LfcinB on human breast cancer cells, although the enhancing effect of tamoxifen was independent of glucosylceramide synthase inhibition.

Materials and methods

Cell lines and reagents. Estrogen-nonresponsive MDA-MB-435 human breast carcinoma cells (14) were generously provided by Dr J. Mackey (University of Alberta, Edmonton, Alberta). MDA-MB-435 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Canada, Oakville, Ontario), supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mmol/l L-glutamine, 5 mmol/l N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer (pH 7.4) and 5% heat-inactivated fetal calf serum (FCS) (all from Invitrogen, Burlington, Ontario, Canada) at 37˚C in a 10% CO_2 humidified atmosphere. Untransformed human mammary epithelial cells were purchased from Cambrex Bio Science (Walkersville, MD) and maintained in fully supplemented Mammary Epithelial Basal Medium (Cambrex). Cultures were passaged every second day or as required by treatment with trypsin containing 0.25% EDTA for 2 min. LfcinB (amino acid sequence, FKCRRWQWRMKKLGAPSITCVRRAF) was synthesized in linear form with a purity of >95% by Sigma Genosys (Woodlands, TX). LfcinB was dissolved in serum-free medium and stored at -80˚C. All experiments with LfcinB were performed in DMEM containing 0.5% FCS since LfcinB has maximum cytoxic activity at low serum concentrations (15). Hoescht 33342 stain was from Sigma-Aldrich. C_6 ceramide, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), and tamoxifen were from EMD Biosciences Inc. (San Diego, CA). Stock solutions of C_6 ceramide and tamoxifen were prepared in dimethyl sulfoxide and stored at -20˚C. A stock solution of PPMP was prepared in water and stored at -20˚C.

DNA fragmentation (JAM) assay. DNA fragmentation was measured using the JAM assay, as described by Matzinger (16). Briefly, MDA-MB-435 breast cancer cells were labeled with 5 μCi/ml tritiated thymidine (MP Biomedicals, Irvine, CA) for 4 h at 37˚C in a 10% CO_2 humidified atmosphere.

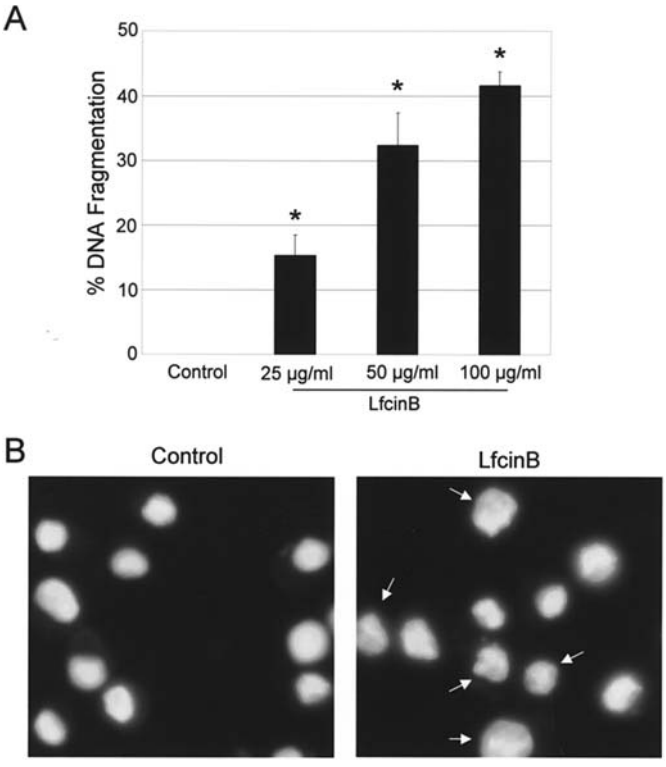

Figure 1. Dose-dependent induction of DNA fragmentation in breast cancer cells by LfcinB. (A) MDA-MB-435 breast carcinoma cells were cultured in the absence or presence of the indicated concentrations of LfcinB for 24 h. DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *Indicates a statistically significant difference ($p<0.001$) in comparison to the control by the Tukey-Kramer multiple comparisons test. (B) MDA-MB-435 breast carcinoma cells were cultured in the absence or presence of LfcinB (100 μg/ml) for 24 h. Cells were then fixed, stained with DNA-specific Hoescht 33342 dye, and visualized by fluorescence microscopy. Intense staining indicates chromatin condensation. Arrows indicate cells showing nuclear fragmentation.

Following 3 washes, radiolabeled cells were resuspended in DMEM containing 0.5% FCS, and $5x10^4$ cells were added in quadruplicate to 96-well flat-bottom tissue culture plates (Sarstedt Inc., St. Laurent, Quebec, Canada). MDA-MB-435 cells were incubated overnight at 37˚C in a 10% CO_2 humidified atmosphere to allow adherent monolayers to form prior to use in experiments. MDA-MB-435 cells were then cultured in the absence or presence of the indicated drugs for 24 h at 37˚C in a 10% CO_2 humidified atmosphere. DNA was harvested onto glass fiber filters using a multiple sample harvester (Skatron Instruments, Sterling, VA), and radioactivity in counts per minute (cpm) was measured using liquid scintillation counting. Percentage of DNA fragmentation was calculated using the formula $(C_{cpm}-E_{cpm})/C_{cpm}$ x100, where E is intact DNA from treated cells and C is intact DNA from control cells exposed only to the drug vehicle.

Hoechst staining. Cells with apoptotic nuclear morphology were distinguished from normal cells by staining with DNA-specific Hoescht 33342 dye (17). MDA-MB-435 cells were resuspended in fresh DMEM containing 0.5% FCS, and $5x10^5$ cells were added in duplicate to 24-well flat-bottom plates (Sarstedt Inc). Following an overnight incubation at 37˚C in a 10% CO_2 humidified atmosphere to allow adherent

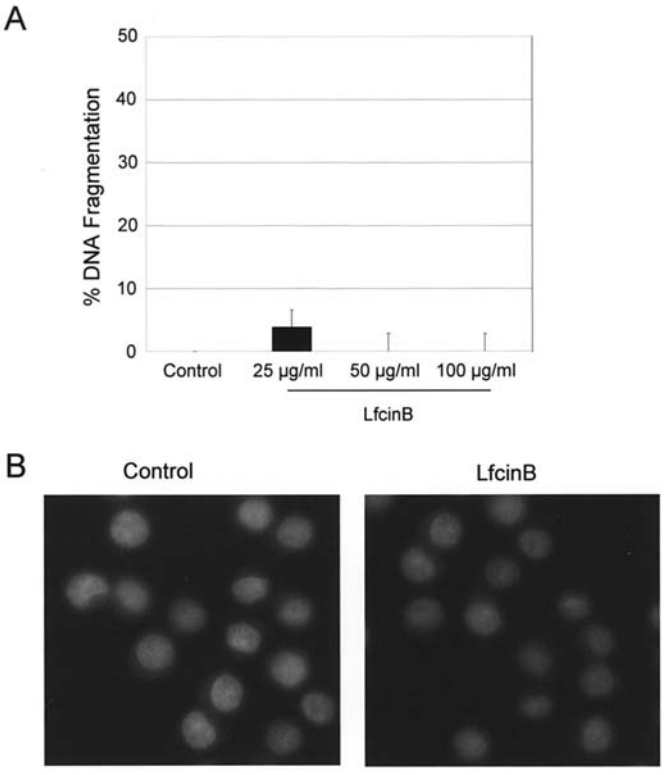

Figure 2. Human mammary epithelial cells are refractory to the cytotoxic effect of LfcinB. (A) Normal mammary epithelial cells were cultured in the absence or presence of the indicated concentrations of LfcinB for 24 h. DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. (B) Normal mammary epithelial cells were cultured in the absence or presence of LfcinB (100 μg/ml) for 24 h. Cells were then fixed, stained with DNA-specific Hoescht 33342 dye, and visualized by fluorescence microscopy.

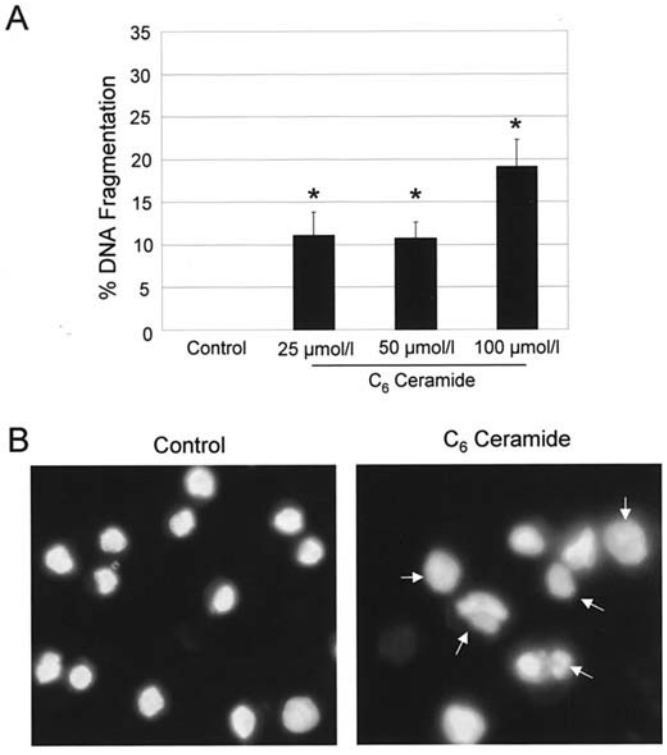

Figure 3. Dose-dependent induction of DNA fragmentation in breast cancer cells by C_6 ceramide. (A) MDA-MB-435 breast carcinoma cells were cultured in the absence or presence of the indicated concentrations of C_6 ceramide for 24 h. DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *Indicates a statistically significant difference ($p<0.001$) in comparison to the control by the Tukey-Kramer multiple comparisons test. (B) MDA-MB-435 breast carcinoma cells were cultured in the absence or presence of C_6 ceramide (100 μmol/l) for 24 h. Cells were then fixed, stained with DNA-specific Hoescht 33342 dye, and visualized by fluorescence microscopy. Intense staining indicates chromatin condensation. Arrows indicate cells showing nuclear fragmentation.

monolayers to form, MDA-MB-435 cells were then cultured in the absence or presence of the indicated drugs for 24 h at 37˚C in a 10% CO_2 humidified atmosphere. MDA-MB-435 cells were detached from plates by treatment with trypsin-EDTA, washed extensively, and resuspended in a 4% paraformaldehyde fixative solution. Aliquots of MDA-MB-435 cells were then placed onto silinated slides, air-dried, and stained with 10 μg/ml Hoecsht 33342 dye for 10 min. Slides were then washed with phosphate-buffered solution, and coverslips were mounted with 10% glycerol/phosphate-buffered saline solution. Chromatin condensation and nuclear fragmentation were visualized by fluorescence microscopy.

Statistical analysis. Data were analyzed using the Instat statistics program (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test; $p<0.05$ was considered to be statistically significant.

Results

LfcinB induces apoptosis in MDA-MB-435 breast carcinoma cells, but spares normal breast epithelial cells. Fig. 1A shows that 24 h incubation of MDA-MB-435 breast cancer cells in the presence of LfcinB (25, 50, or 100 μg/ml) caused DNA fragmentation to occur in a dose-dependent fashion. DNA fragmentation in LfcinB-treated breast cancer cells was accompanied by chromatin condensation and nuclear fragmentation (Fig. 1B), indicating that cell death was by apoptosis. In contrast, neither DNA fragmentation nor morphologic features of apoptosis were observed in untransformed human mammary epithelial cells that were exposed to LfcinB for 24 h (Fig. 2).

C_6 ceramide induces apoptosis in MDA-MB-435 breast carcinoma cells. MDA-MB-435 breast cancer cells were cultured for 24 h in the presence of the cell-permeable short-chain ceramide analog C_6 (25, 50, or 100 μmol/l) to determine the effect of increased levels of intracellular ceramide on cell viability. Fig. 3A shows that treatment with exogenous C_6 ceramide caused DNA fragmentation to occur in a dose-dependent fashion. C_6 ceramide-treated breast cancer cells also exhibited chromatin condensation and nuclear fragmentation (Fig. 3B), indicating that cell death was by apoptosis.

C_6 ceramide and LfcinB have an additive cytotoxic effect on MDA-MB-435 breast carcinoma cells. Since exogenous C_6 ceramide had a cytotoxic effect on MDA-MB-435 breast cancer cells, and intracellular ceramide is an important

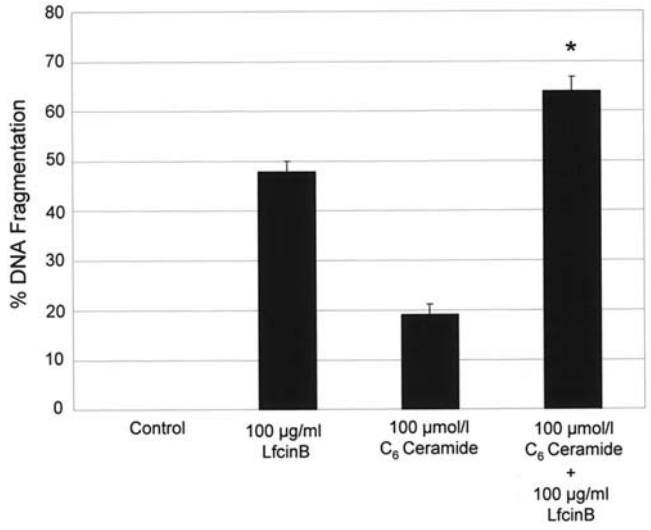

Figure 4. Additive cytotoxic effect by LfcinB and C_6 ceramide on breast cancer cells. MDA-MB-435 breast carcinoma cells were cultured for 24 h in the absence or presence of LfcinB (100 μg/ml) and/or C_6 ceramide (100 μmol/l). DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *Indicates a statistically significant difference ($p<0.001$) in comparison to the single agent-treated cells by the Tukey-Kramer multiple comparisons test.

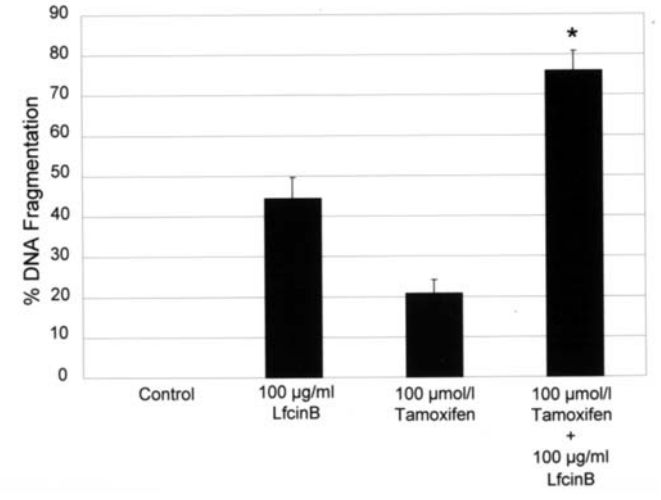

Figure 5. Tamoxifen enhances LfcinB-induced DNA fragmentation in breast cancer cells. MDA-MB-435 breast carcinoma cells were cultured for 24 h in the absence or presence of LfcinB (100 μg/ml) and/or tamoxifen (100 μmol/l). DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *Indicates a statistically significant difference ($p<0.001$) in comparison to the single agent-treated cells by the Tukey-Kramer multiple comparisons test.

second messenger in apoptosis signaling (18), we determined whether the cytotoxic effect of LfcinB was enhanced in the presence of C_6 ceramide. Fig. 4 shows an additive increase in DNA fragmentation when breast cancer cells were cultured for 24 h in the presence of LfcinB and C_6 ceramide.

Tamoxifen causes DNA fragmentation and enhances the cytotoxic response to LfcinB. Tamoxifen is an estrogen receptor antagonist that has also been shown to increase intracellular ceramide by preventing the conversion of ceramide to glucosyl-

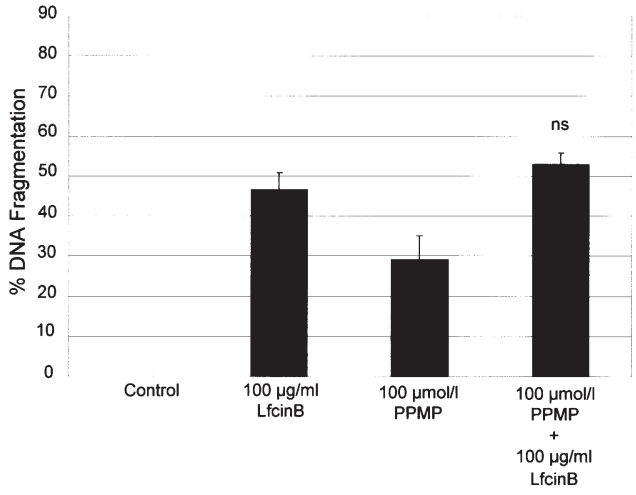

Figure 6. PPMP fails to enhance DNA fragmentation in LfcinB-treated breast cancer cells. MDA-MB-435 breast carcinoma cells were cultured for 24 h in the absence or presence of LfcinB (100 μg/ml) and/or PPMP (100 μmol/l). DNA fragmentation was then measured by JAM assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. Failure to achieve statistical significance in comparison to LfcinB-treated cells by the Tukey-Kramer multiple comparisons test ($p>0.05$) is indicated by ns.

ceramide by glucosylceramide synthase (19). Fig. 5 shows that 24 h exposure to tamoxifen caused DNA fragmentation in estrogen receptor-negative MDA-MB-435 breast carcinoma cells. Moreover, breast cancer cells that were treated with LfcinB in combination with tamoxifen exhibited a dramatic increase in DNA fragmentation that was greater than the sum of DNA fragmentation caused by either agent alone.

Inhibition of glucosylceramide synthase causes DNA fragmentation, but does not enhance LfcinB-induced apoptosis. MDA-MB-435 breast carcinoma cells were treated with the selective glucosylceramide synthase inhibitor PPMP (20), alone or in combination with LfcinB to confirm the cytotoxic effect of tamoxifen. Fig. 6 shows that 24 h culture in the presence of PPMP caused DNA fragmentation in breast cancer cell cultures. However, breast cancer cells that were treated with LfcinB in combination with PPMP exhibited DNA fragmentation that was not significantly greater than that achieved with LfcinB alone, indicating that glucosylceramide synthase inhibition did not enhance the cytotoxic effect of LfcinB.

Discussion

LfcinB shows considerable potential as a novel anti-cancer agent because the peptide is able to trigger apoptosis in a wide range of human cancer cell lines without harming untransformed lymphocytes, fibroblasts, or endothelial cells (2). In the present study, we showed that 24 h culture in the presence of LfcinB caused death to human MDA-MB-435 breast carcinoma cells by apoptosis. In contrast, the viability of normal mammary epithelial cells was unaffected by LfcinB treatment. LfcinB was therefore selectively cytotoxic for breast cancer cells. In comparison to non-tumorigenic cells, human tumor cell lines exhibit elevated cell-surface expression of negatively-charged phosphatidylserine (21). A similar

increase in cell membrane phosphatidylserine content has been reported in a majority of freshly isolated colorectal carcinoma tissue samples (22). Furthermore, many cancer cells are characterized by membrane expression of O-glycosylated mucins that confer an additional negative charge to the surface of the cancer cell (23,24). On the other hand, healthy eukaryotic cells carry a neutral charge due to the predominant expression of zwitterionic phosphatidylcholine in their outer membrane leaflet (25). We therefore postulate that the strong positive charge carried by LfcinB allows the peptide to enter into an electrostatic interaction with negatively charged cancer cells, but not with the neutral outer membrane leaflet of untransformed cells. In support of this hypothesis, we have observed rapid and substantial binding of LfcinB to human leukemia cells, but not to healthy lymphocytes (unpublished observations). Following binding to negatively charged cancer cell membranes, LfcinB is believed to exert a membrane-destabilizing effect that allows for spontaneous transfer of the peptide across the membrane (25). This is consistent with the pore formation reported in LfcinB-treated Meth A fibrosarcoma, B16F10 melanoma, and C26 colon carcinoma cells (4). Following LfcinB treatment, human T leukemia cells generate reactive oxygen species and exhibit a rapid loss of mitochondrial transmembrane potential that leads to the activation of caspase-3 and cell death by apoptosis (2).

LfcinB has been shown to inhibit the progression of several different transplanted murine tumors when tumor-bearing mice are treated with LfcinB (4,5). However, relatively high concentrations of LfcinB are needed to kill cancer cells *in vitro* and *in vivo*, which may place limits on the feasibility of using LfcinB in a clinical setting. Interestingly, the accumulation of intracellular ceramide enhances the cytotoxic effect of certain chemotherapeutic drugs (10,13). Since MDA-MB-435 breast cancer cells underwent apoptosis in the presence of exogenous cell-permeable C_6 ceramide, we hypothesized that elevated endogenous ceramide levels would potentiate the apoptosis-inducing activity of LfcinB. Indeed, combined treatment with LfcinB and C_6 ceramide caused more DNA fragmentation in cultures of MDA-MB-435 breast carcinoma cells than treatment with either LfcinB or C_6 ceramide alone. Cell-permeable ceramide analogs have been packaged in liposomes for targeted *in vitro* delivery to human breast cancer cell lines, resulting in a significantly greater accumulation of intracellular ceramide and a concomitant increase in tumor cell death by apoptosis (9). Moreover, systemic liposomal delivery of C_6 ceramide to immunodeficient mice bearing MDA-MB-231 breast carcinoma cell xenografts elicits a dramatic reduction in tumor size, thereby demonstrating that cell-permeable ceramide analogs have anti-tumor activity *in vivo* (26). Our present data suggest that targeted liposomal delivery of LfcinB in combination with C_6 ceramide might be an effective strategy to cause maximal tumor cell death *in vivo*. In this regard, we have already successfully delivered LfcinB to human T leukemia cells *in vitro* via liposomes that have been specifically designed to fuse with target cell membranes (27). Ongoing studies seek to develop fusogenic liposomes for the targeted *in vivo* delivery of LfcinB in combination with other apoptogenic molecules such as C_6 ceramide to human tumors grown as xenografts in immunodeficient mice.

Intracellular ceramide can be converted into various metabolites by four major pathways, one of which involves the conversion of ceramide to glucosylceramide by glucosylceramide synthase (28). Glucosylceramide is consistently present at high levels in multiple-drug resistant cancers and absent, or present only at very low levels, in drug-sensitive cancer cells (29). In addition, inhibition of ceramide glycosylation increases the sensitivity of adriamycin-resistant MCF-7 breast carcinoma cells to adriamycin treatment (13). These findings led us to determine whether interfering with the activity of glucosylceramide synthase enhanced LfcinB-induced apoptosis. Tamoxifen, an anti-estrogen that also inhibits glucosylceramide synthase (19), caused MDA-MB-435 breast cancer cells to undergo apoptosis. The glucosylceramide synthase inhibitor PPMP also triggered apoptosis in MDA-MB-435 breast cancer cells (20). These data suggested that conversion of ceramide to glucosylceramide was a major pathway of ceramide metabolism in MDA-MB-435 breast cancer cells, and inhibition of glucosylceramide synthase resulted in the accumulation of apoptogenic levels of intracellular ceramide in this breast carcinoma cell line. Although tamoxifen dramatically enhanced LfcinB-induced DNA fragmentation in MDA-MB-435 breast cancer cell cultures, combination treatment with LfcinB and PPMP did not result in greater cytotoxicity than treatment with LfcinB alone. Since selective inhibition of glucosylceramide synthase inhibition did not potentiate cellular sensitivity to LfcinB, we concluded that the enhancing effect of tamoxifen on LfcinB-induced apoptosis in MDA-MB-435 breast carcinoma cells must involve another mechanism. MDA-MB-435 breast cancer cells are estrogen receptor-negative (14), which ruled out any effect due to the interaction of tamoxifen with estrogen receptors. However, tamoxifen has been suggested to induce apoptosis in estrogen-nonresponsive breast cancer cell lines via multiple mechanisms that include induction of c-myc overexpression (30), oxidative stress-related activation of JNK signaling (31), and up-regulation of Fas ligand expression by Fas-bearing tumor cells (32). Elucidation of the precise mechanism(s) by which tamoxifen potentiated LfcinB-induced apoptosis in human breast cancer cell cultures may reveal additional strategies to further enhance the anti-cancer activity of LfcinB.

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