

Modulation of ceramide metabolism in T-leukemia cell lines potentiates apoptosis induced by the cationic antimicrobial peptide bovine lactoferricin

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Abstract. Bovine lactoferricin (LfcinB) is a cationic antimicrobial peptide that selectively induces apoptosis in several different types of human cancer cells. However, the potential use of LfcinB as an anticancer agent is presently limited by the need for relatively high concentrations of the peptide to trigger apoptosis. Ceramide is a membrane sphingolipid that is believed to function as a second messenger during apoptosis. In this study, we investigated the role of ceramide in LfcinB-induced apoptosis in CCRF-CEM and Jurkat T-leukemia cell lines. Exposure to LfcinB caused nuclear condensation and fragmentation, poly(ADP-ribose) polymerase (PARP) cleavage, and DNA fragmentation in CCRF-CEM and Jurkat T-cell acute lymphoblastic leukemia cell lines. Treatment with C₆ ceramide, a cell-permeable, short-chain ceramide analog, also induced apoptotic nuclear morphology, PARP cleavage, and DNA fragmentation in T-leukemia cells. Although LfcinB treatment did not cause ceramide to accumulate in CCRF-CEM or Jurkat cells, the addition of C₆ ceramide to LfcinB-treated T-leukemia cells resulted in increased DNA fragmentation. Furthermore, modulation of cellular ceramide metabolism either by inhibiting ceramidases with D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol or N-oleoylethanolamine, or by blocking glucosylceramide synthase activity with 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, enhanced the ability of LfcinB to trigger apoptosis in both Jurkat and CCRF-CEM cells. In addition, LfcinB-induced apoptosis of T-leukemia cells was enhanced in the presence of the antiestrogen tamoxifen, which has multiple effects on cancer cells, including inhibition of glucosylceramide

synthase activity. We conclude that manipulation of cellular ceramide levels in combination with LfcinB therapy warrants further investigation as a novel strategy for the treatment of T cell-derived leukemias.

Introduction

Acute lymphoblastic leukemia (ALL) of T-cell or B-cell origin is the most common leukemia diagnosed in children and adolescents, whereas ALL occurs less frequently in adults (1). Fortunately, 70-83% of children with ALL can be cured by chemotherapy (2); recent improvements to treatment regimens suggest that a cure rate approaching 90% will soon be possible for childhood ALL (3). In contrast, only 24% of adults with ALL survive for 5 years following standard chemotherapy (4). Although the survival rate of adult ALL patients has improved with the advent of allogeneic hematopoietic stem cell transplantation (2), a substantial proportion of transplant recipients fail to achieve long-term disease-free survival (5). Novel therapeutic strategies are therefore needed for the treatment of ALL patients who cannot be cured by chemotherapy or allogeneic hematopoietic stem cell transplantation.

Bovine lactoferricin (LfcinB) is a cationic antimicrobial peptide consisting of 25 amino acids that is generated by acid-pepsin hydrolysis of bovine lactoferrin (6). LfcinB possesses potent *in vitro* and *in vivo* anticancer activity. *In vitro* exposure to LfcinB causes apoptosis in several human cancer cell lines, including THP-1 human monocytic leukemia cells and MDA-MB-435 breast carcinoma cells, without having any cytotoxic effect on untransformed cells (7-9). We have recently demonstrated that LfcinB-induced apoptosis is initiated by the production of reactive oxygen species and the subsequent activation of caspase-2, which leads to mitochondrial membrane permeabilization, release of cytochrome *c*, and the sequential activation of caspase-9 and caspase-3 (8). Importantly, intratumoral injections of LfcinB inhibit the growth of murine Meth A fibrosarcoma, B16F10 melanoma, and C26 colon carcinoma cells in syngeneic mice (10), as well as human SH-SY-5Y neuroblastoma cells in nude rats (11). In addition, subcutaneous injections of LfcinB prevent the metastasis of murine L5178Y-ML25 lymphoma cells and B16-BL6 melanoma cells in syngeneic mice (12). Although

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LfcinB shows potential for use in the treatment of several human cancers, including leukemias, the feasibility of LfcinB-based therapy is at present limited because of the need to employ high concentrations (typically, 100–200 $\mu\text{g/ml}$ *in vitro*) of the peptide in order to obtain an optimal cytotoxic effect.

Ceramide is a bioactive membrane sphingolipid implicated in apoptosis induced by diverse stimuli, including death receptor ligation, exposure to chemotherapeutic agents, ionizing radiation, and hypoxia (13,14). Cellular ceramide accumulates prior to the onset of apoptosis as a result of *de novo* ceramide synthesis or the hydrolysis of sphingomyelin by activated sphingomyelinase. Although the mechanism(s) by which ceramide promotes apoptosis remain under investigation, ceramide is known to interact with a number of proteins involved in apoptosis induction, including protein phosphatase 2A (15), Raf-1 and extracellular signal-regulated kinase (p44/p42) (16), and cathepsin D (17). In addition, ceramide treatment of T cell lines causes caspase-2-dependent activation of caspase-8, followed by the loss of mitochondrial membrane potential, activation of caspase-9 and caspase-3, and cell death by apoptosis (18). Recently, ceramide has been suggested to form channels in the outer membrane of the mitochondrion, leading to the release of proapoptotic mitochondrial proteins such as cytochrome *c* (19). Interestingly, ceramide augments paclitaxel-mediated cytotoxicity in cultures of human leukemia cells and head and neck squamous carcinoma cells, suggesting that ceramide and chemotherapeutic agents may engage converging pathways during the induction of apoptosis (20,21). Recently, we have shown that LfcinB-induced apoptosis of MDA-MB-435 breast carcinoma cells is enhanced in the presence of C_6 ceramide, which is a cell-permeable, short-chain, analogue of ceramide (9). However, it is not known whether exposure to LfcinB itself causes cellular ceramide levels to increase in human leukemia cells.

On the basis that ceramide plays a central role in apoptosis, pharmacologic modulation of ceramide metabolism in order to cause the accumulation of cellular ceramide has been proposed as a novel strategy to increase the effectiveness of cancer chemotherapy (22,23). Enzymes involved in ceramide metabolism include acidic and/or alkaline ceramidases and glucosylceramide synthase. Deacylation of ceramide by alkaline and acidic ceramidases produces sphingosine (24,25), which can then be phosphorylated to yield sphingosine-1-phosphate, a molecule that inhibits ceramide-mediated apoptosis and promotes cell survival and proliferation (26,27). Glucosylceramide synthase converts ceramide to glucosylceramide (28), thereby promoting cellular proliferation and preventing apoptosis (29). It is noteworthy that acidic ceramidase is overexpressed in prostate carcinoma cells (30), while overexpression of glucosylceramide synthase is associated with the development of chemoresistance in HL-60 promyelocytic leukemia and MCF-7 breast carcinoma cell lines (31,32). Drugs that inhibit ceramidases or glucosylceramide synthase may therefore increase the effectiveness of apoptosis-inducing anticancer agents. However, the effect of ceramidase or glucosylceramide synthase inhibitors on LfcinB-induced apoptosis of human leukemia cells has not been determined.

In this study, we investigated the role of cellular ceramide in LfcinB-induced apoptosis of Jurkat and CCRF-CEM T-

leukemia cells, which are cell lines derived from human acute lymphoblastic T-leukemia cells (33,34). We also determined whether inhibition of ceramidases or glucosylceramide synthase involved in ceramide metabolism might increase the cytotoxic effect of LfcinB on T-leukemia cells. Our findings reveal that inhibition of ceramidases or glucosylceramide synthase is an effective strategy to enhance the apoptosis-inducing activity of LfcinB, even though LfcinB by itself does not cause cellular ceramide to accumulate in T-leukemia cells.

Materials and methods

Cell lines. CCRF-CEM T-leukemia cells were generously provided by Dr W. Gati (University of Alberta, Edmonton, Alberta, Canada). Jurkat T-leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich Canada, Oakville, Ontario, Canada), supplemented with 100 $\mu\text{g/ml}$ streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 5 mM *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 5% CO_2 humidified atmosphere. Cell cultures were passaged every second day or as required.

Reagents. LfcinB (amino acid sequence: FKCRRWQWRMK KLGAPSITCVRRAF) was synthesized in linear form with a purity of >95% by Sigma Genosys (The Woodlands, TX). LfcinB was dissolved in serum-free RPMI-1640 medium and stored at -80°C. Hoescht 33342 trihydrochloride dye was from Sigma-Aldrich. C_6 ceramide, C_6 dihydroceramide, DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), *N*-oleoylethanolamine (NOE), D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP), and tamoxifen were from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Stock solutions of C_6 ceramide, C_6 dihydroceramide, MAPP, NOE, and tamoxifen were prepared in dimethyl sulfoxide and stored at -20°C. A 10-mM stock solution of PPMP was prepared in water and stored at -20°C. Tritiated-thymidine ($[^3\text{H}]\text{TdR}$) and $[\gamma\text{-}^{32}\text{P}]\text{-adenosine 5'-triphosphate}$ were obtained from MP Biomedicals (Irvine, CA) and Perkin-Elmer (Mississauga, Ontario, Canada), respectively. Anti-human poly(ADP-ribose) polymerase (PARP) monoclonal antibody (mAb), anti-human actin mAb, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

$[^3\text{H}]\text{TdR}$ -release assay. DNA fragmentation was measured using a $[^3\text{H}]\text{TdR}$ -release assay (35). Briefly, T-leukemia cells were labeled with $[^3\text{H}]\text{TdR}$ (5 $\mu\text{Ci/ml}$) for 4 h at 37°C in a 5% CO_2 humidified atmosphere. Following 3 washes, radio-labeled cells were resuspended in RPMI-1640 medium and added in quadruplicate (5×10^4 cells/well) to 96-well flat-bottom tissue culture plates (Sarstedt Inc., St. Laurent, Quebec, Canada). All experiments were performed in RPMI-1640 medium containing 0.5% FCS since LfcinB has maximum

cytotoxic activity at low serum concentrations (7). T-leukemia cells were cultured under the desired conditions for 12 h at 37°C in a 5% CO₂ humidified atmosphere. DNA was then harvested onto glass fiber filters using a multiple sample harvester (Skatron Instruments, Sterling, VA) and radioactivity in counts per minute (cpm) was measured by liquid scintillation counting. Percent DNA fragmentation was calculated by the formula $[(C_{\text{cpm}} - E_{\text{cpm}})/C_{\text{cpm}}] \times 100$, where E is intact DNA from treated cells and C is intact DNA from control cells.

Hoechst staining. T-leukemia cells with apoptotic nuclear morphology were distinguished from normal cells by staining with DNA-specific Hoescht 33342 trihydrochloride dye (36). T-leukemia cells were resuspended in RPMI-1640 medium containing 0.5% FCS and added in duplicate (5×10^5 cells/well) to 24-well flat-bottom plates (Sarstedt Inc.). T-leukemia cells were cultured under the desired conditions for 12 h at 37°C in a 5% CO₂ humidified atmosphere, then harvested and resuspended in a 4% paraformaldehyde solution. Aliquots of T-leukemia cells were placed onto silanated slides, air-dried, and stained with 10 µg/ml Hoescht 33342 dye for 10 min. Slides were then washed with phosphate-buffered saline (PBS) and coverslips were mounted with 10% glycerol/phosphate-buffered saline solution. Nuclear condensation and fragmentation were visualized by fluorescence microscopy.

Detection of PARP cleavage. Western blotting was used to measure PARP cleavage, which is an early marker of apoptosis (37). T-leukemia cells in RPMI-1640 medium containing 0.5% FCS were cultured in the absence or presence of LfcinB (50 µg/ml) or C₆ ceramide (50 µM) for 6 and 12 h. Cells were then lysed in ice-cold lysis buffer [50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 50 mM Na₂HPO₄, 0.25% sodium deoxycholate (w/v), 0.1% Nonidet P-40 (v/v), 1 mM Na₃VO₄, 1 mM NaF, 5 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] containing freshly-added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin). The protein content of cell lysates was determined by Bradford assay (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). T-leukemia cell lysates (5 µg) were resolved by electrophoresis across a 10% SDS polyacrylamide gel. Protein bands were transferred to nitrocellulose membranes and the resulting blots were blocked overnight with PBS-Tween-20 (0.25 M Tris at pH 7.5, 150 mM NaCl, 0.2% Tween-20 in PBS) containing 5% powdered skim milk (w/v). Blots were probed overnight with anti-PARP mAb (1:500), then washed with PBS-Tween-20 and probed for 1 h with HRP-conjugated goat anti-mouse IgG antibody (10 µg/ml). After additional washes with PBS-Tween 20, the protein bands were visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories). Blots were stripped and re-probed with anti-actin mAb (1:1000) to confirm equal protein loading.

Measurement of cellular ceramide. T-leukemia cells were resuspended in RPMI-1640 medium containing 0.5% FCS and added in triplicate (5×10^5 cells/well) to 24-well flat-bottom plates. T-leukemia cells were then cultured in the absence or

presence of LfcinB for 2-12 h at 37°C in a 5% CO₂ humidified atmosphere. T-leukemia cells were harvested and resuspended in 5:4 methanol/water (v/v). Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories). Lipids were extracted by adding 1:2 chloroform/methanol (v/v) and 0.58% NaCl, followed by centrifugation at 400 g for 5 min to separate phases. The upper aqueous phase was removed and the bottom phase was washed twice with 45:47:5 methanol/0.58% NaCl/chloroform (v/v). The bottom phase was then dried under nitrogen, and the lipids were dissolved in chloroform. Ceramide mass in the total lipid extract was then determined by the bacterial diglyceride kinase assay (38). Briefly, the lipids were solubilized in a 7.5% octyl-β-D-glucoside/5 mM cardiolipin solution by sonication. Reaction buffer (100 mM imidazole HCl, pH 6.6, 100 mM NaCl, 25 mM MgCl₂, and 2 mM EGTA), 100 mM dithiothreitol, and diluted diglyceride kinase membranes (EMD Biosciences Inc., San Diego, CA) were added to the solubilized lipids. The reaction was then initiated by the addition of 10 mM [γ -³²P]-adenosine 5'-triphosphate and was terminated by adding 1:2 chloroform/methanol (v/v) and 1% HClO₄ after incubation at 25°C for 45 min. Lipids were extracted and the lower chloroform phase was dried under nitrogen. Lipids were dissolved in 2:1 chloroform/methanol (v/v) and spotted onto a thin layer chromatography plate (silica gel 60), which was then developed using 65:15:5 chloroform/methanol/acetic acid (v/v). Phosphorylated lipids were visualized by radioautography and the area corresponding to phosphorylated ceramide was scraped off and radioactivity was measured by liquid scintillation counting. Total ceramide mass from the original sample was determined using a standard curve established using known concentrations of ceramide.

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.

Results

LfcinB induces apoptosis in Jurkat and CCRF-CEM T-leukemia cells. Fig. 1A shows that culture for 12 h in the presence of 25, 50, or 100 µg/ml LfcinB caused dose-dependent DNA fragmentation in Jurkat and CCRF-CEM T-leukemia cell lines. A subsequent kinetics analysis indicated that LfcinB-induced DNA fragmentation in Jurkat and CCRF-CEM cells was maximal at the 12 h time-point (data not shown). To confirm that LfcinB-induced DNA fragmentation in T-leukemia cells was the result of apoptosis, Jurkat and CCRF-CEM cells were cultured in the absence or presence of 50 µg/ml LfcinB for 12 h, and then stained with DNA-specific Hoechst 33342 dye. Alternately, T-leukemia cells were cultured in the absence or presence of 50 µg/ml LfcinB for 6 or 12 h, after which PARP cleavage was measured by Western blotting. LfcinB treatment of Jurkat and CCRF-CEM cells resulted in nuclear condensation and fragmentation (Fig. 1B), as well as PARP cleavage (Fig. 1C), indicating that LfcinB-induced cell death was by apoptosis.

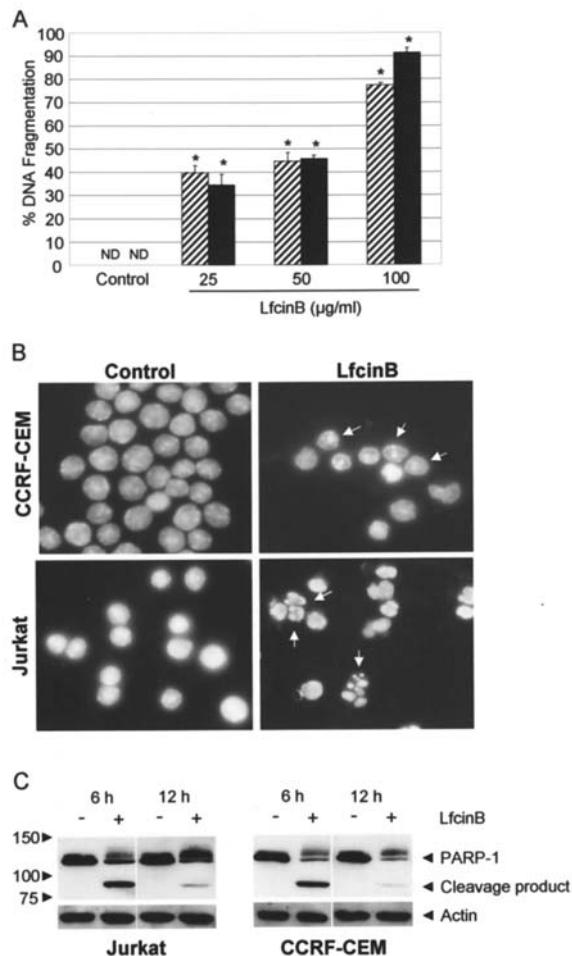


Figure 1. LfcinB triggers apoptosis in T-leukemia cells. (A) Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured in the absence (vehicle only) or presence of the indicated concentrations of LfcinB for 12 h. DNA fragmentation was then measured by [3 H]TdR-release assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation \pm SD. * P <0.001 in comparison to the medium control by the Tukey-Kramer multiple comparisons test. (B) Jurkat or CCRF-CEM cells were cultured in the absence (vehicle only) or presence of 50 μ g/ml LfcinB for 12 h. Cells were then fixed, stained with DNA-specific Hoescht 33342 dye, and visualized by fluorescence microscopy. Intense staining indicates nuclear condensation. Arrows indicate cells showing nuclear fragmentation. (C) Jurkat or CCRF-CEM cells were cultured in the absence (vehicle only) or presence of 50 μ g/ml LfcinB for 6 or 12 h. Cell lysates were then prepared and subjected to Western blot analysis for the detection of PARP cleavage. Blots were stripped and re-probed for actin expression to confirm equal protein loading.

LfcinB-induced apoptosis is not associated with the accumulation of cellular ceramide. We next compared cellular ceramide levels in control and LfcinB-treated Jurkat and CCRF-CEM T-leukemia cells because ceramide has been identified as an important second messenger during apoptosis (13,14). Ceramide measurements were performed 2, 4, 8, and 12 h after exposing T-leukemia cells to 50 μ g/ml LfcinB. Fig. 2 shows that control and LfcinB-treated Jurkat and CCRF-CEM cells had equivalent cellular ceramide content, indicating that LfcinB treatment did not cause cellular ceramide levels to become elevated in T-leukemia cells.

C₆ ceramide induces apoptosis in Jurkat and CCRF-CEM T-leukemia cells. To determine whether T-leukemia cells were sensitive to the cytotoxic effect of cellular ceramide

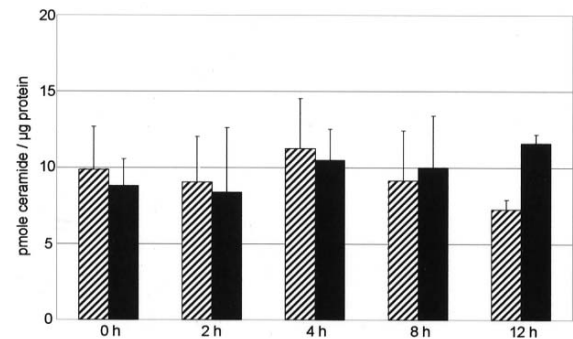


Figure 2. LfcinB does not induce ceramide accumulation in T-leukemia cells. Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured for the indicated times in the absence (vehicle only) or presence of 50 μ g/ml LfcinB. Lipids were extracted from harvested cells and ceramide mass was determined as described in Materials and methods. Data are the mean of three independent experiments \pm SEM.

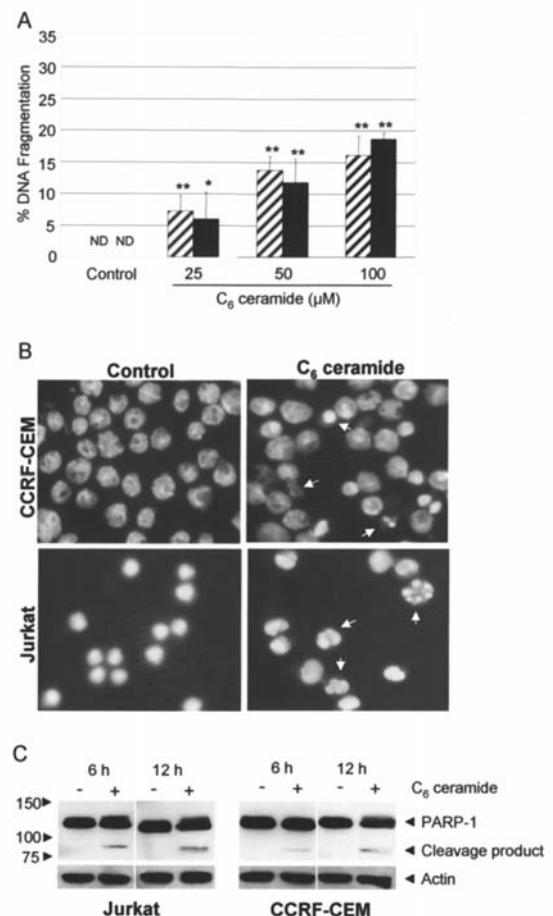


Figure 3. C₆ ceramide triggers apoptosis in T-leukemia cells. (A) Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured in the absence (vehicle only) or presence of the indicated concentrations of C₆ ceramide for 12 h. DNA fragmentation was then measured by [3 H]TdR-release assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation \pm SD. * P <0.01; ** P <0.001 in comparison to the vehicle control by the Tukey-Kramer multiple comparisons test. (B) Jurkat or CCRF-CEM cells were cultured in the absence (vehicle only) or presence of 100 μ M C₆ ceramide for 12 h. Cells were then fixed, stained with DNA-specific Hoescht 33342 dye, and visualized by fluorescence microscopy. Intense staining indicates nuclear condensation. Arrows indicate cells showing nuclear fragmentation. (C) Jurkat or CCRF-CEM cells were cultured in the absence (vehicle only) or presence of 50 μ M C₆ ceramide for 6 or 12 h. Cell lysates were then prepared and subjected to Western blot analysis for the detection of PARP cleavage.

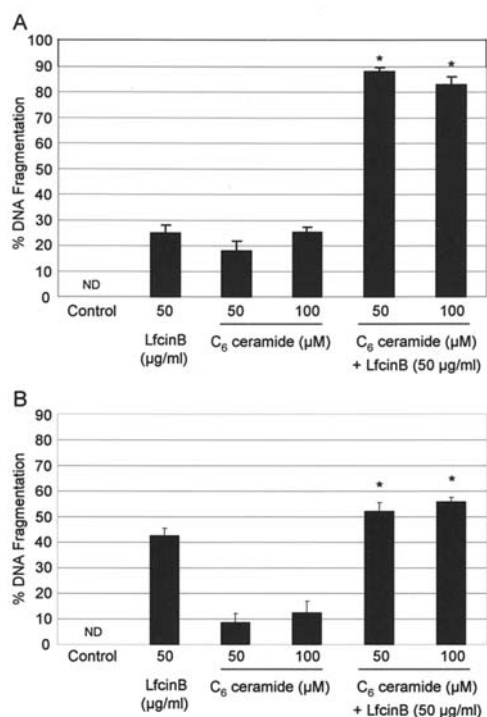


Figure 4. C₆ ceramide enhances LfcinB-induced DNA fragmentation in T-leukemia cells. (A) Jurkat or (B) CCRF-CEM T-leukemia cells were cultured for 12 h in the absence (vehicle only) or presence of LfcinB and/or C₆ ceramide at the indicated concentrations. DNA fragmentation was then measured by [³H]TdR-release assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *P<0.001 in comparison to cells treated with LfcinB or C₆ ceramide alone by the Tukey-Kramer multiple comparisons test.

accumulation, Jurkat and CCRF-CEM cells were cultured for 12 h in the absence or presence of a 25, 50, or 100 µM concentration of C₆ ceramide, which is a cell-permeable, short-chain analog of ceramide. DNA fragmentation was then measured by [³H]TdR-release assay. The intracellular accumulation of cell-permeable ceramide analogs mimics the apoptosis-inducing effect of elevated cellular ceramide (39). Fig. 3A shows that exposure to C₆ ceramide caused dose-dependent DNA fragmentation in both T-leukemia cell lines. In addition, nuclear condensation and fragmentation (Fig. 3B), as well as PARP cleavage (Fig. 3C) were observed in C₆ ceramide-treated Jurkat and CCRF-CEM cells, indicating that exposure to C₆ ceramide caused T-leukemia cells to die by apoptosis. In contrast, the viability of Jurkat and CCRF-CEM cells was not reduced following treatment with a 25, 50, or 100 µM concentration of C₆ dihydroceramide, which is an inactive analog of C₆ ceramide (data not shown).

Combination treatment with LfcinB and C₆ ceramide leads to increased cytotoxicity. Since T-leukemia cells were sensitive to LfcinB and C₆ ceramide individually, we decided to investigate the effect of combined LfcinB and C₆ ceramide treatment on cell viability. Fig. 4 shows that combined treatment with LfcinB and C₆ ceramide had a greater cytotoxic effect on Jurkat and CCRF-CEM cells than was seen when either agent was used alone. Jurkat cells showed a synergistic increase in DNA fragmentation following exposure to

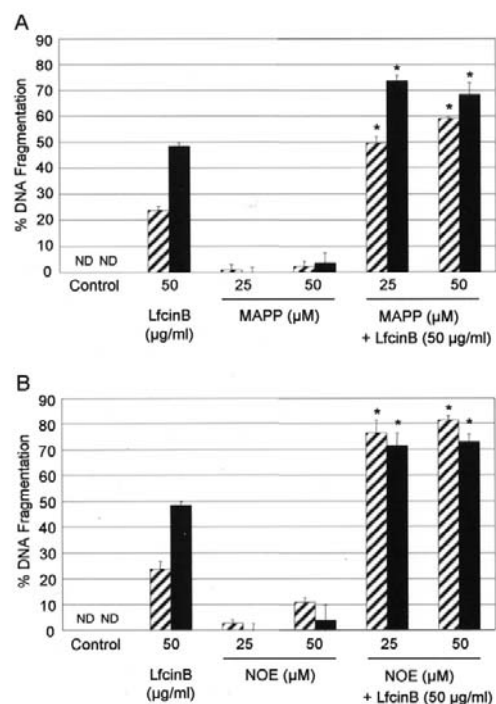


Figure 5. Ceramidase inhibitors enhance LfcinB-induced DNA fragmentation in T-leukemia cells. Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured for 12 h in the absence (vehicle only) or presence of LfcinB and/or (A) MAPP or (B) NOE at the indicated concentrations. DNA fragmentation was then measured by [³H]TdR-release assay. Data from a representative experiment (n=3) are expressed as % DNA fragmentation ± SD. *P<0.001 in comparison to the single agent-treated cells by the Tukey-Kramer multiple comparisons test.

combined LfcinB and C₆ ceramide (Fig. 4A) whereas CCRF-CEM cells exhibited an additive increase in DNA fragmentation when cultured in the presence of combined LfcinB and C₆ ceramide (Fig. 4B).

Inhibition of ceramidases enhances the cytotoxic effect of LfcinB. Since combination treatment with LfcinB and cell-permeable C₆ ceramide had a greater apoptosis-inducing effect than either agent alone, we hypothesized that inhibition of enzymes that normally prevent the accumulation of cellular ceramide might enhance LfcinB-induced T-leukemia cell death. Fig. 5A shows that concomitant treatment of Jurkat and CCRF-CEM cells with LfcinB and the acidic ceramidase inhibitor MAPP (25) resulted in significantly greater DNA fragmentation (P<0.001) than was evident following treatment with LfcinB alone. Fig. 5B shows that a significant increase (P<0.001) in DNA fragmentation over that caused by LfcinB alone was also observed when Jurkat and CCRF-CEM cells were cultured in the presence of LfcinB plus the alkaline ceramidase inhibitor NOE (24). Neither MAPP nor NOE alone had any substantial cytotoxic effect on Jurkat or CCRF-CEM cells. These data indicated that inhibition of acidic or alkaline ceramidases within T-leukemia cells enhanced the apoptosis-inducing activity of LfcinB.

Inhibition of glucosylceramide synthase increases the cytotoxic effect of LfcinB. We also cultured T-leukemia cells in the absence or presence of LfcinB, with or without the glucosyl-

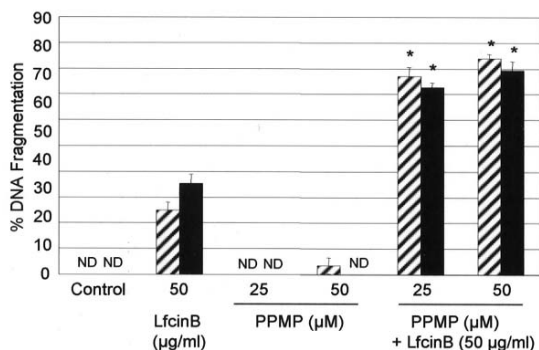


Figure 6. Glucosylceramide synthase inhibition enhances LfcinB-induced DNA fragmentation in T-leukemia cells. Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured for 12 h in the absence (vehicle only) or presence LfcinB and/or PPMP at the indicated concentrations. DNA fragmentation was then measured by [3 H]TdR-release assay. Data from a representative experiment ($n=3$) are expressed as % DNA fragmentation \pm SD. * $P<0.001$ in comparison to the single agent-treated cells by the Tukey-Kramer multiple comparisons test.

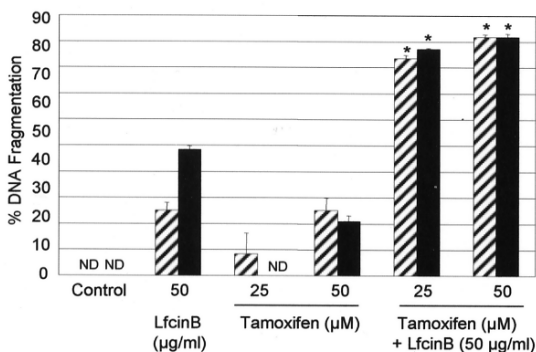


Figure 7. Tamoxifen enhances LfcinB-induced DNA fragmentation in T-leukemia cells. Jurkat (hatched) or CCRF-CEM (solid) T-leukemia cells were cultured for 12 h in the absence (vehicle only) or presence LfcinB and/or tamoxifen at the indicated concentrations. DNA fragmentation was then measured by [3 H]TdR-release assay. Data from a representative experiment ($n=3$) are expressed as % DNA fragmentation \pm SD. * $P<0.001$ in comparison to the single agent-treated cells by the Tukey-Kramer multiple comparisons test.

ceramide synthase inhibitor PPMP to prevent the conversion of endogenous ceramide to glucosylceramide (28). Fig. 6 shows that combination treatment with LfcinB and PPMP was far more effective than LfcinB alone in causing Jurkat and CCRF-CEM cells to die by apoptosis. Treatment with PPMP alone did not have any significant cytotoxic effect on Jurkat or CCRF-CEM cells. Interestingly, similar results were obtained when Jurkat and CCRF-CEM cells were exposed to LfcinB in combination with tamoxifen (Fig. 7), which has multiple apoptosis-promoting effects on cancer cells (40), including inhibition of ceramide glucosylation (41). Tamoxifen by itself had a modest cytotoxic effect on T-leukemia cells when used at the highest concentration tested (50 μ M). Taken together, these data indicated that blockade of glucosylceramide synthase activity within T-leukemia cells enhanced the apoptosis-inducing activity of LfcinB.

Discussion

We have shown potent induction of apoptosis, as indicated by DNA fragmentation, nuclear condensation and fragmentation, and PARP cleavage, following LfcinB treatment of two different T-leukemia cell lines. However, LfcinB-induced apoptosis was not associated with increased cellular ceramide content, which was in contrast to numerous reports that have implicated ceramide as an important second messenger during apoptosis caused by death receptor ligation or cellular stress due to exposure to chemotherapeutic agents or ionizing radiation (13,14). This apparent discrepancy might be explained by our recent finding that LfcinB causes limited damage the cytoplasmic membrane of T-leukemia cells, leading to LfcinB uptake without any evidence of necrosis (42). LfcinB that gains access to the cytoplasmic compartment colocalizes with mitochondria, resulting in mitochondrial membrane permeabilization, the release of cytochrome *c*, and apoptosis induction. The ability of LfcinB to trigger apoptosis by direct disruption of mitochondrial membrane integrity might circumvent any need for apoptotic signaling via the ceramide pathway. Although T-leukemia cells failed to accumulate cellular ceramide in response to LfcinB treatment, both Jurkat and CCRF-CEM cells were sensitive to the cytotoxic effect of elevated cellular ceramide since exposure of these T-leukemia cell lines to cell-permeable C_6 ceramide resulted in DNA fragmentation, PARP cleavage, and the appearance of nuclear morphology that was consistent with apoptosis. The intracellular accumulation of C_6 ceramide has an apoptosis-inducing effect comparable to that of an increased level of endogenous ceramide (39). Ceramide-induced apoptosis is known to involve the sequential activation of caspase-2 and -8, leading to mitochondrial membrane permeabilization and the activation of caspase-9 and caspase-3 (18). In addition, recent evidence suggests that ceramide is able to directly cause the loss of mitochondrial membrane integrity (19). However, the ability of ceramide analogues to trigger apoptosis may vary depending on the cellular context since C_2 ceramide (*N*-acetyl-*D*-erythro-sphingosine) induces apoptosis in HL-60 promyelocytic leukemia cells but causes B-chronic lymphocytic leukemia cells to die by a non-apoptotic mechanism (43).

Although LfcinB shows potential as a novel cytotoxic agent for the treatment of several human cancers, the fact that relatively high concentrations of LfcinB are required for optimal induction of cancer cell apoptosis may place limits on its therapeutic utility. The finding that LfcinB can be combined with C_6 ceramide to create a greater apoptosis-inducing effect than that of either agent alone on T-leukemia cells suggested that the mechanism of LfcinB-induced apoptosis was convergent with the apoptotic pathway triggered by the accumulation of cellular ceramide. Combination treatment with LfcinB and drugs that cause cellular ceramide to accumulate through the inhibition of endogenous ceramide metabolism might therefore be a feasible strategy to improve the anticancer activity of LfcinB. In this regard, we show here for the first time that LfcinB-induced apoptosis of T-leukemia cells was enhanced when acidic or alkaline ceramidase-mediated ceramide metabolism was blocked with MAPP or NOE, respectively, or ceramide glucosylation was inhibited

by PPMP; even though LfcinB-induced apoptosis in Jurkat and CCRF-CEM cells was not associated with the accumulation of cellular ceramide. It is possible that ceramidase and glucosylceramide synthase activity were heightened in T-leukemia cells in response to mitochondrial damage caused by LfcinB, resulting in increased sensitivity of T-leukemia cells to agents that block these pathways of ceramide metabolism. Our findings are in line with reports that killing of solid tumor cell lines by the synthetic retinoid N-(4-hydroxyphenyl) retinamide, which, like LfcinB (8), promotes mitochondrial membrane permeabilization and the release of cytochrome *c* (44), is also enhanced in the presence of agents that inhibit ceramidase or glucosylceramide synthase function (45,46). Elevated cellular ceramide levels caused by ceramidase or glucosylceramide synthase inhibition in LfcinB-treated T-leukemia cells may lead to a further loss of mitochondrial membrane integrity and increase in cytochrome *c*-dependent apoptosome formation, thereby augmenting LfcinB-induced apoptosis. Inhibition of acidic or alkaline ceramidases by MAPP or NOE, respectively, or glucosylceramide synthase by PPMP in the absence of LfcinB did not have any substantial cytotoxic effect on Jurkat or CCRF-CEM cells, suggesting that ceramidase-mediated deacylation of ceramide to sphingosine and glucosylceramide synthase-mediated conversion of ceramide to glucosylceramide are not normally major pathways of cellular ceramide metabolism in T-leukemia cells. Baseline ceramide metabolism within T-leukemia cells and other types of cancer cells is apparently different since inhibition of acidic ceramidase by NOE elevates cellular ceramide levels and increases apoptosis in prostate cancer cells (47) while inhibition of glucosylceramide synthase by PPMP causes cellular ceramide accumulation and apoptosis in colon carcinoma cells (48).

In addition to ceramide metabolism by ceramidases and glucosylceramide synthase, cellular ceramide can be reversibly converted to sphingomyelin by sphingomyelin synthase (49), acylated by 1-*O*-acylceramide synthase to form 1-*O*-acylceramide (50), or phosphorylated by ceramide kinase to yield ceramide-1-phosphate (51). Although there is at present no information on 1-*O*-acylceramide levels in human cancer cells, ceramide kinase activity has been detected in human HL-60 promyelocytic leukemia cells (51). In addition, inhibition of sphingomyelin synthase activity by tricyclodecan-9-yl-xanthogenate (D609) causes human U937 monocytic leukemia cells to die by apoptosis via a mechanism that is associated with a substantial increase in cellular ceramide content (52). It is therefore reasonable to speculate that ceramide kinase and/or sphingomyelin synthase inhibitors might be combined with ceramidase and glucosylceramide synthase inhibitors to further enhance LfcinB-induced apoptosis in human T-leukemia cells.

It is noteworthy that LfcinB-induced apoptosis in Jurkat and CCRF-CEM cells was also enhanced in the presence of the antiestrogen tamoxifen, which mediates its anticancer activity via multiple mechanisms (40), including inhibition of ceramide glucosylation (41). We also observed that a 50 μ M concentration of tamoxifen alone had a modest apoptosis-inducing effect on T-leukemia cells. Tamoxifen-induced cytotoxicity was most likely not related to the inhibitory effect of tamoxifen on glucosylceramide synthase since the glucosyl-

ceramide synthase inhibitor PPMP by itself did not induce apoptosis in Jurkat or CCRF-CEM cells. In addition to inhibiting ceramide glucosylation, tamoxifen has been reported to trigger apoptosis by mechanisms that include Fas death receptor signaling (53), c-myc overexpression (54), and oxidative stress-related activation of c-Jun NH₂-terminal kinase (55). Additional study is required to determine which, if any, of these mechanisms contribute to the ability of tamoxifen to enhance the cytotoxic effect of LfcinB on T-leukemia cells.

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