Biochemical and Biophysical Research Communications 388 (2009) 736–741

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Intracellular delivery of bovine lactoferricin's antimicrobial core (RRWQWR) kills T-leukemia cells

Angela Richardson [a], Roberto de Antueno [a], Roy Duncan [a,b], David W. Hoskin [a,c,d,*]

[a] Department of Microbiology & Immunology, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada
[b] Department of Pediatrics, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada
[c] Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada
[d] Department of Surgery, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada

ARTICLE INFO

Article history:
Received 13 August 2009
Available online 21 August 2009

Keywords:
Antimicrobial peptide
Bovine lactoferricin
Cancer
Caspase
Cathepsin B
Fusogenic liposome

ABSTRACT

Bovine lactoferricin (LfcinB) is a cationic antimicrobial peptide with potent cytotoxic activity against cancer cells. The antimicrobial activity of LfcinB resides in its RRWQWR amino acid sequence (referred to here as LfcinB6); however, the anticancer activity of LfcinB6 is not known. Here, we show that free LfcinB6 did not kill T-leukemia or breast cancer cells but LfcinB6 was strongly cytotoxic when delivered to the cytosolic compartment by fusogenic liposomes. LfcinB6 bound weakly to isolated mitochondria but, unlike LfcinB, did not permeabilize mitochondria or cause cytochrome *c* to be released. Cathepsin B and caspase activity were important for cytotoxicity caused by intracellular LfcinB6 whereas reactive oxygen species were not involved. The mechanism of LfcinB6-induced cytotoxicity is therefore different from that of LfcinB. We suggest that LfcinB6, in combination with a fusogenic liposome delivery system that selectively targets malignant cells, has potential as a novel anticancer agent.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Bovine lactoferricin (LfcinB) is a cationic antimicrobial peptide that consists of amino acid residues 17–41 (FKCRRWQWRMKKL GAPSITCVRRAF) from the N-terminus of bovine lactoferrin [1], an 80 kDa iron-binding glycoprotein stored in neutrophil granules and present in secretions such as tears, saliva and milk [2,3]. LfcinB, which is generated by acid–pepsin hydrolysis of bovine lactoferrin [1], exhibits a range of biological activities, including cytotoxic activity against various microorganisms [1,4,5] and cancer cells [6–8]. Depending on the cancer cell type, LfcinB kills by either apoptosis induction [6,7] or membrane lysis [8], without causing harm to healthy cells [7]. Subcutaneous administration of LfcinB to mice inhibits lymphoma cell metastasis to the liver and lungs, as well as lung metastasis by melanoma cells [8].

LfcinB contains five arginine and three lysine residues that confer a net charge of +8 on the peptide, as well as two tryptophan and two phenylalanine residues that are both aromatic and hydrophobic [1]. LfcinB possesses a cyclic structure created by the disulfide bond that links its two cysteine residues. In aqueous solution, LfcinB adopts an amphipathic β-sheet configuration in which the hydrophobic residues line up on one face of the peptide and the basic residues comprise the opposing face [10], which interacts with anionic components of cell membranes of microorganisms and cancer cells [11]. Replacement of arginine with lysine and tryptophan with phenylalanine or tyrosine results in LfcinB derivatives with considerably reduced antimicrobial activity [12,13]. Arginine residues are more important than lysine residues for antimicrobial activity because arginine forms multiple hydrogen bonds with anionic structures [11] while bulky tryptophan mediates peptide partitioning into biological membranes, leading to membrane disruption [14]. The antimicrobial core of LfcinB, referred to hereafter as LfcinB6, consists of the amino acid sequence RRWQWR [15,16]. This six-amino acid peptide adopts a well-defined amphipathic structure when bound to sodium dodecyl sulfate (SDS) micelles [17].

Abbreviations: Ab, antibody; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horse radish peroxidase; HSP 70, heat shock protein 70; HUVEC, human umbilical vein endothelial cell; LfcinB, bovine lactoferricin; LfcinB6, antimicrobial core of LfcinB; $\Delta\psi_m$, mitochondrial transmembrane potential; mAb, monoclonal Ab; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline-Tween-20; $[^3H]$TdR, tritiated-thymidine.

* Corresponding author. Address: Department of Microbiology & Immunology, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, NS, Canada B3H 1X5. Fax: +1 902 494 5125.
E-mail address: d.w.hoskin@dal.ca (D.W. Hoskin).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.083

Although LfcinB shows potential as a novel cytotoxic agent for cancer treatment [6–9], no studies to date have investigated the effect of the antimicrobial core of LfcinB on cancer cells. The aim of this study was to determine the in vitro anticancer activity of LfcinB6. Neither T-leukemia nor breast cancer cells were killed by free LfcinB6; however, fusogenic liposome-mediated delivery of LfcinB6 into the cytosolic compartment resulted in extensive DNA fragmentation that was dependent on cathepsin B and caspase activation. We suggest that LfcinB6, in combination with a tumor-targeting fusogenic liposome delivery system, warrants further investigation as a possible novel approach to cancer treatment.

Materials and methods

Reagents and antibodies. Fluorescein isothiocyanate (FITC)-dextran (4 kDa), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *N*-acetyl-L-cysteine (NAC) were from Sigma–Aldrich Canada (Oakville, ON). Z-VAD-FMK (pan-caspase inhibitor), CA-074-Me (cathepsin B inhibitor), and glutathione (GSH) were from EMD Biosciences, Inc. (San Diego, CA). FITC-streptavidin was from Cedarlane Laboratories (Hornby, ON). 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) was from Molecular Probes (Eugene, OR). Tritiated-thymidine ([^{3}H]TdR) was from ICN Biomedicals (Irvine, CA). All lipids were from Avanti Polar Lipids (Alabaster, AL). Mouse anti-cytochrome *c* monoclonal antibody (mAb) was from Upstate Biotechnology (Charlottesville, VA). Mouse anti-mitochondrial heat shock protein 70 (HSP 70) mAb was from Affinity BioReagents (Golden, CO). Goat anti-mouse IgG-horseradish peroxidase (HRP) antibody (Ab) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. Jurkat T-leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). CCRF-CEM T-leukemia cells were a gift from Dr. W. Gati (University of Alberta, Edmonton, AB). T-leukemia cell lines were maintained at 37 °C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.4 (all from Invitrogen, Burlington, ON). MDA-MB-231 breast carcinoma cells were a gift from Dr. S. Drover (Memorial University of Newfoundland, St. John's, NL) and were maintained at 37 °C in a 10% CO_2 humidified atmosphere in complete Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich Canada) supplemented with 5% heat-inactivated FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 5 mM HEPES buffer at pH 7.4. Human umbilical vein endothelial cells (HUVECs) were from Cambrex (Walkersville, MD) and were maintained at 37 °C in a 5% CO_2 humidified atmosphere in EGM™ Complete Endothelial Cell Medium (Cambrex).

Peptides. LfcinB (FKCRRWQWRMKKLGAPSITCVRRAF), LfcinB6 (RRWQWR), biotinylated-LfcinB6, and control peptide (NNGQGN) were synthesized in linear form at >95% purity by Dalton Pharma Services (Toronto, ON). Biotinylated-LfcinB was synthesized in linear form at >95% purity by Sigma Genosys (The Woodlands, TX). Lyophilized peptides were dissolved in FCS-free RPMI 1640 medium (Sigma–Aldrich Canada) and aliquots were stored at −80 °C.

Fusogenic liposomes. Reptilian reovirus p14 protein was isolated and used to prepare fusogenic liposomes as previously described [18]. An equivalent of 9 μM LfcinB6 was entrapped in liposomes (60:30:8:2% molar ratio of DOPC:DOPE:cholesterol:DC-cholesterol) by repeated freeze–thawing with liquid N_2 and a 37 °C water bath. Fusogenic liposomes were then washed with Hank's buffered saline and resuspended in fully-supplemented RPMI 1640 medium minus FCS to give a final concentration of 33.3 μM lipid.

Cell viability assays. DNA fragmentation, which is an indicator of cell death, was determined by the JAM test [19]. Briefly, radiolabeled (5 μCi/ml [^{3}H]TdR for 4 h) cells were plated in quadruplicate (5×10^4 cells/well) in 96-well flat-bottom tissue culture plates (Sarstedt, Inc., St. Laurent, QC). After 18 h, DNA was harvested onto fiberglass filter mats using a Titertek® cell harvester (Skatron Instruments, Sterling, VA) and [^{3}H]TdR content of the trapped DNA was measured by liquid scintillation counting. Percent cell death was calculated using the formula $[(cpm_{control} - cpm_{experimental})/cpm_{control}] \times 100$. Alternatively, cell viability was determined by MTT assay [20]. Cells were plated in quadruplicate (2×10^4 cells/well) in 96-well flat-bottom tissue culture plates. After 18 h, MTT was added at a final concentration of 500 μg/ml. After incubation for an additional 4 h, supernatant was removed, cells were solubilized in 0.1 ml dimethyl sulfoxide, and optical density (OD) was measured at 492 nm. Percent cell death was calculated using the formula $(1 - OD_{experimental}/OD_{control}) \times 100$. All experiments were performed in fully-supplemented RPMI 1640 medium containing 0.5% FCS because LfcinB exhibits optimal cytotoxic activity at lower serum concentrations [6].

Mitochondria isolation. Jurkat cells were washed in phosphate-buffered saline (PBS) and placed in ice-cold mitochondria isolation buffer (0.2 mM EDTA, 0.25 M sucrose, and 10 mM Tris–HCl, pH 7.8) for 30 min, followed by homogenization using a pre-cooled glass homogenizer. Cell lysates were cleared by centrifugation at 1000*g* for 10 min at 4 °C and the resulting supernatant was centrifuged at 12,000*g* for 10 min at 4 °C. Pelleted mitochondria were resuspended in ice-cold mitochondria isolation buffer containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μM sodium orthovanadate, 10 mM sodium fluoride and 10 μM phenylarsine oxide).

Peptide binding. Cells or mitochondria were incubated at 4 °C with biotinylated-peptides for 1 h or 10 min, respectively. After extensive washing with PBS, cells or mitochondria were incubated with 1:1000 FITC-streptavidin for 30 min at 4 °C. Samples were then washed and analyzed by flow cytometry.

FITC-dextran and PI uptake. Jurkat cells (5×10^5 cells/well) in fully-supplemented RPMI 1640 medium were cultured for 1 h in the absence or presence of 31 μM LfcinB or LfcinB6 in a 24-well flat-bottom tissue culture plate (Sarstedt, Inc.). FITC-dextran or PI was then added to cells at a final concentration of 10 μg/ml and 5 min later cells were analyzed by flow cytometry.

Mitochondrial transmembrane potential ($\Delta\psi_m$) measurement. The fluorescent probe $DiOC_6$ was used to measure mitochondria membrane permeabilization [21]. Briefly, mitochondria (5 μg in 0.1 ml mitochondria isolation buffer) were incubated for 10 min in the absence or presence of 31 μM LfcinB or LfcinB6. $DiOC_6$ was then added at a final concentration of 20 nM and 20 min later mitochondria were analyzed by flow cytometry.

Western blotting. Mitochondria (50 μg) in mitochondria isolation buffer were incubated for 10 min at 37 °C in the absence or presence of 31 μM LfcinB or LfcinB6. Mitochondria were then pelleted by centrifugation at 12,000*g* for 10 min at 4 °C and the supernatant was collected. Pelleted mitochondria were resuspended in ice-cold lysis buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate [w/v], 0.1% Nonidet P-40 [v/v], 5 mM ethylenediaminetetraacetic acid, and 5 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) containing previously described protease inhibitors. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories Ltd., Mississauga, ON). Samples were boiled for 5 min in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (200 mM Tris–HCl [pH 6.8], 30% glycerol, 6% SDS, 15% β-mercaptoethanol, and 0.01% bromophenol blue), and equal amounts of mitochondria pellet protein (5 μg) and a corresponding volume of supernatant were resolved on 12% and 15% SDS–PAGE gels, respectively. Protein was transferred onto a nitrocellulose mem-

brane and the resulting blots were blocked for 1 h at room temperature with Tris-buffered saline–Tween-20 (TBST; 20 mM Tris-HCl at pH 7.6, 150 mM NaCl, 0.2% Tween-20 in water) containing 5% powdered skim milk. Membranes were then washed with TBST and incubated overnight at 4 °C with 1:500 anti-cytochrome *c* mAb (mitochondria supernatant samples) or anti-mitochondrial HSP 70 mAb (mitochondria pellet samples) to confirm equal loading of supernatant samples. Membranes were washed with TBST and incubated with 1:1000 goat anti-mouse IgG-HRP Ab for 1 h at room temperature. Following additional TBST washes, protein bands were visualized by enhanced chemiluminescence detection (Bio-Rad Laboratories Ltd.).

Results and discussion

LfcinB6 does not bind to or damage cancer cells

DNA fragmentation was measured to determine the effect of LfcinB6 on the viability of T-leukemia and breast cancer cell lines that are sensitive to LfcinB [7]. Although LfcinB was strongly cytotoxic to Jurkat and CCRF-CEM T-leukemia cells, as well as MDA-MB-231 breast carcinoma cells, an equal concentration of LfcinB6 failed to kill any of these cancer cell lines (Fig. 1A). Since LfcinB must bind to the cell membrane in order to kill cancer cells [22], we next determined whether LfcinB6 interacted with cancer cells. Fig. 1B shows that biotinylated-LfcinB6 did not bind to Jurkat cells, as determined by flow cytometry. In contrast, the same concentration of biotinylated-LfcinB bound strongly to Jurkat cells. Furthermore, LfcinB disrupted the cell membrane of Jurkat cells, allowing uptake of FITC-dextran (Fig. 1C) and PI (data not shown). In contrast, exposure of Jurkat cells to an equivalent concentration of LfcinB6 did not cause cell membrane damage because there was no uptake of either FITC-dextran (Fig. 1C) or PI (data not shown). Since the net charge of +3 that is conferred upon LfcinB6 by its three arginine residues does not allow the peptide to interact with the cell membrane of cancer cells, it is not surprising that Jurkat cells remained intact in the presence of extracellular LfcinB6. Our results are consistent with reports that lactoferrin-derived peptides have more stringent structural requirements for anticancer activity than for antimicrobial activity, i.e., a net charge of +7 is required for strong cytotoxic activity against cancer cells [23] whereas a net charge of +3 is sufficient for killing of microorganisms [16]. Interestingly, the anticancer activity of lactoferrin-derived peptides with a net charge less than +7 can be enhanced by the addition of a bulky tryptophan residue [23]. Inadequate hydrophobicity due to the presence of only two tryptophan residues may therefore also contribute to the inability of extracellular LfcinB6 to interact with and destabilize the cell membrane of cancer cells.

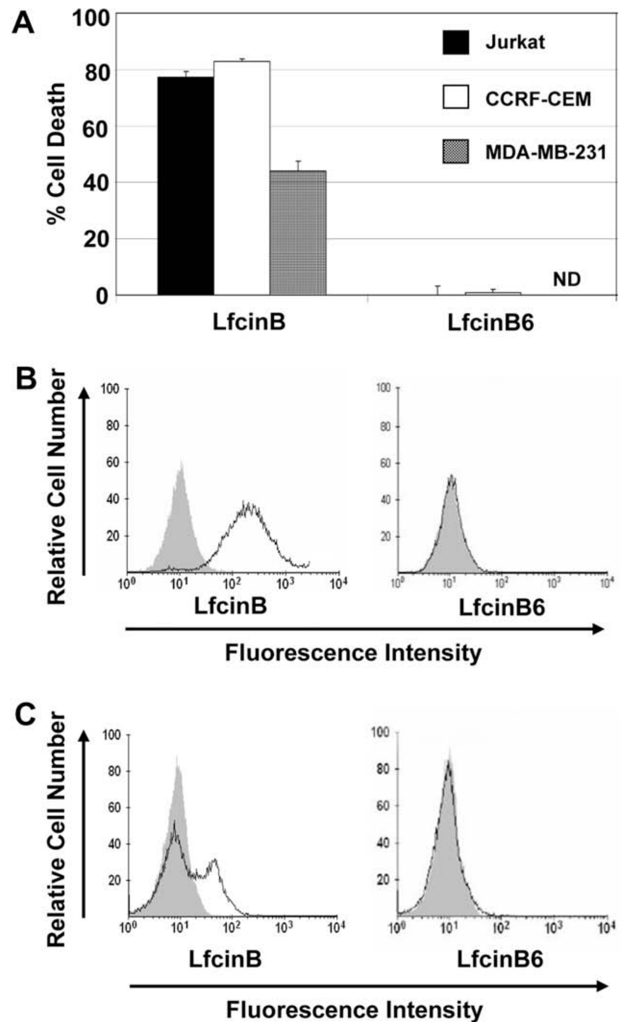

Fig. 1. Differential effect of LfcinB and LfcinB6 on T-leukemia and breast cancer cell viability. (A) Jurkat T-leukemia, CCRF-CEM T-leukemia or MDA-MB-231 breast carcinoma cells were cultured in the absence or presence of 31 μM LfcinB or LfcinB6. Cytotoxicity was measured after 18 h by DNA fragmentation assay. ND denotes none detected. Data are shown as % cell death ± SD of quadruplicate samples. (B) Jurkat cells were treated with medium alone or with 31 μM biotinylated-LfcinB or biotinylated-LfcinB6 for 1 h, stained with FITC-streptavidin and analyzed by flow cytometry. Open peaks indicate staining by biotinylated-LfcinB or biotinylated-LfcinB6 while filled peaks indicate background fluorescence of untreated cells. (C) Jurkat cells were treated with medium alone or with 31 μM LfcinB or LfcinB6 in the absence or presence of 10 μg/ml FITC-dextran (4 kDa) for 1 h, then analyzed by flow cytometry. Open peaks indicate FITC-dextran uptake while filled peaks indicate background fluorescence of untreated cells. All cytotoxicity and flow cytometry data are from one experiment that is representative of at least three independent experiments.

Intracellular LfcinB6 is cytotoxic

Since extracellular LfcinB6 failed to interact with or damage the cell membrane of cancer cells, we asked whether intracellular LfcinB6 might cause cell death. We have previously shown that LfcinB must enter the cytosolic compartment to cause apoptosis [22]. Fusogenic liposomes composed of 400 nm unilamellar vesicles with reptilian reovirus p14 fusion-associated small transmembrane protein incorporated into their lipid bilayer [18], which efficiently deliver native LfcinB into the cytosolic compartment of Jurkat cells [22], were used to deliver LfcinB6 directly into the cytoplasm of Jurkat cells. Stable entrapment of LfcinB6 by fusogenic liposomes and entrapped peptide concentrations were determined by high performance liquid chromatography. Fig. 2A shows that intracellular delivery of LfcinB6 resulted in substantial cytotoxicity. In contrast, a control peptide (NNGQGN) that did not contain any basic or hydrophobic residues was not cytotoxic, either in solution or encapsulated in fusogenic liposomes. In addition, HUVEC viability was also substantially diminished following treatment with LfcinB6-containing fusogenic liposomes (Fig. 2B), indicating that intracellular LfcinB6 was cytotoxic to both untransformed and malignant cells. In vivo delivery of LfcinB6 to the cytosolic compartment of cancer cells will therefore require the development of fusogenic liposomes that specifically target neoplastic cells. Importantly, preliminary studies show that the C-terminus of p14 can be coupled to peptides that interact with structures on the surface of cancer cells in order to create tumor-targeted fusogenic liposomes [R. de Antueno and R. Duncan, unpublished]. One targeting peptide for possible future consideration is LARLLT, which targets conventional liposomes to epidermal growth factor

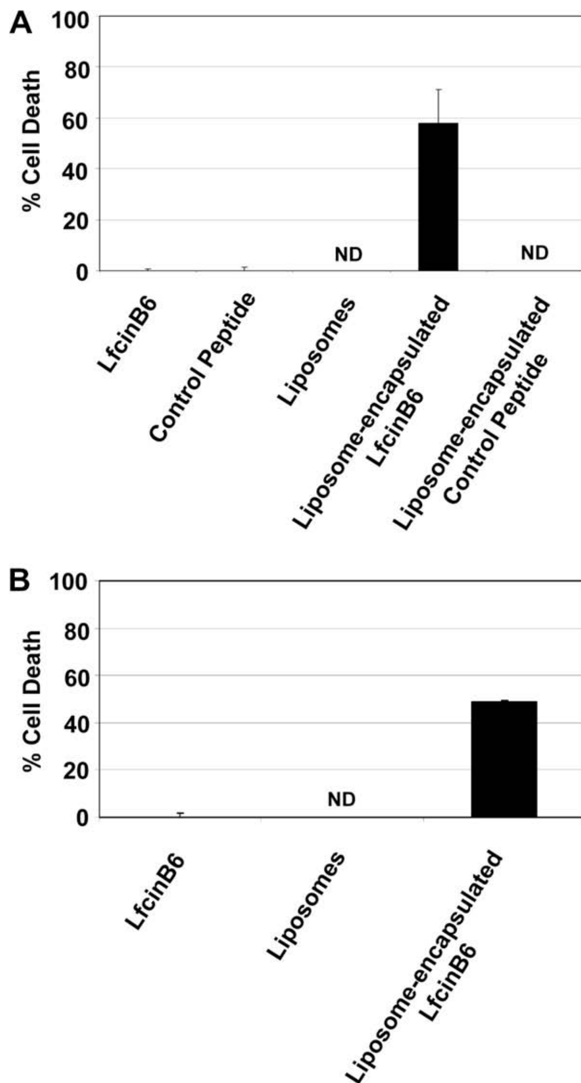

Fig. 2. Fusogenic liposome-encapsulated LfcinB6 is cytotoxic. (A) Jurkat T-leukemia cells or (B) HUVECs were cultured in the presence of medium alone, empty fusogenic liposomes, or 31 μM free or fusogenic liposome-encapsulated LfcinB6 (9 μM). Jurkat cells were also exposed to 31 μM free or fusogenic liposome-encapsulated control peptide (9 μM NNGQGN)). Cytotoxicity was measured after 18 h by (A) DNA fragmentation assay or (B) MTT assay. ND denotes none detected. Data are shown as % cell death ± SD of quadruplicate samples. All data are from one experiment that is representative of at least three independent experiments.

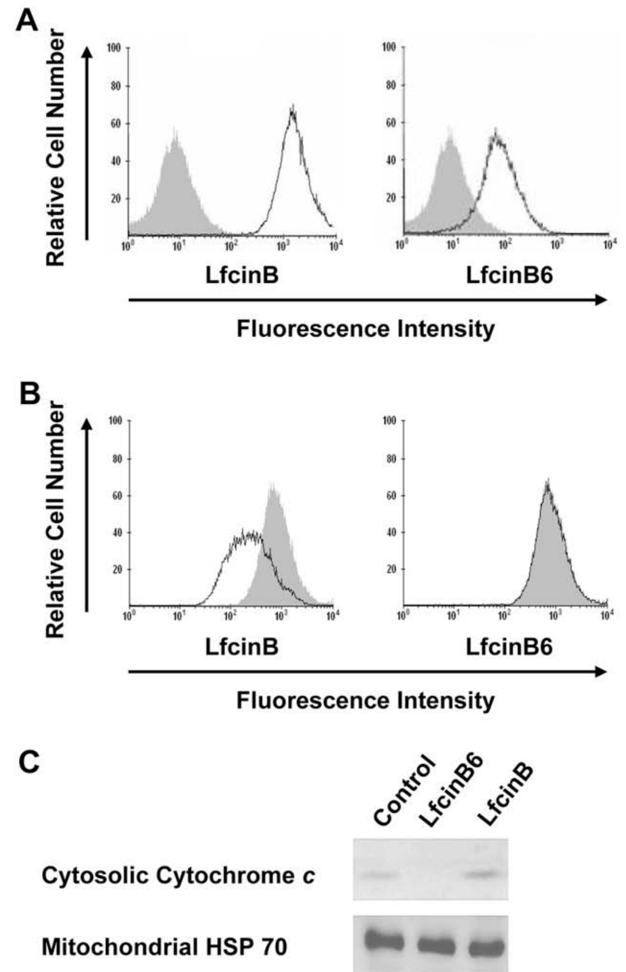

Fig. 3. LfcinB6 interacts with but does not damage mitochondria. (A) Mitochondria were isolated from Jurkat T-leukemia cells and exposed to medium alone, 31 μM biotinylated-LfcinB, or 31 μM biotinylated-LfcinB6 for 1 h, stained with FITC-streptavidin, and analyzed by flow cytometry. Open peaks indicate staining by biotinylated-LfcinB or biotinylated-LfcinB6 while filled peaks indicate background fluorescence of untreated mitochondria. (B) Mitochondria were isolated from Jurkat cells and treated with medium alone, 31 μM LfcinB, or 31 μM LfcinB6 for 10 min, stained with 20 nM $DiOC_6$, and analyzed by flow cytometry. Loss of $\Delta\psi_m$ is seen as a leftward shift in fluorescence by peptide-treated mitochondria (open peak) in comparison to untreated mitochondria (filled peak). (C) Mitochondria were isolated from Jurkat cells and treated with medium alone, 31 μM LfcinB, or 31 μM LfcinB6 for 10 min. Mitochondria-free supernatants were then collected and cytochrome *c* content was determined by Western blotting. Mitochondrial HSP 70 content in corresponding volumes of mitochondria pellet lysates was determined to confirm equal sample loading. All flow cytometry and Western blot data are from one experiment that is representative of at least three independent experiments.

receptor high-expressing cancer cells [24]. In addition, encapsulation of LfcinB6 within tumor-targeted liposomes will protect the peptide from proteolysis and neutralization by anionic serum proteins, as well as enhance liposome accumulation in and peptide delivery to tumor sites [25].

LfcinB6 binds mitochondria but does not reduce $\Delta\psi_m$

Mitochondria are negatively charged due to anionic cardiolipin in their outer membrane [26]. We therefore expected LfcinB6 to interact with and damage mitochondria, as indicated by a loss of $\Delta\psi_m$, in a fashion similar to LfcinB [22]. Fig. 3A shows that LfcinB6 bound to mitochondria obtained from Jurkat cells, albeit to a lesser extent than LfcinB. Relatively poor binding of LfcinB6 to mitochondria in comparison to LfcinB likely reflects the difference in net charge (+3 versus +8) between the shorter and the longer peptide. Mitochondria that were exposed to LfcinB6, unlike LfcinB, did not show any reduction in $\Delta\psi_m$ (Fig. 3B), nor did LfcinB6-treated mitochondria release cytochrome *c* (Fig. 3C). LfcinB6 that interacts with mitochondria is therefore unable to damage the outer mitochondrial membrane. These data suggest that the mode of cytotoxic action of intracellular LfcinB6 is different from that of LfcinB, which triggers the intrinsic pathway of apoptosis in Jurkat cells by permeabilizing the outer membrane of mitochondria, resulting in the release of pro-apoptotic cytochrome *c* and the sequential activation of caspase-9 and caspase-3 [22].

Cathepsin B and caspases, but not reactive oxygen species (ROS), are involved in LfcinB6-induced cytotoxicity

Caspase activation and ROS generation are crucial for LfcinB-induced apoptosis in Jurkat cells [7] while lysosomal cathepsin B has caspase-processing activity [27]. Jurkat cells that were exposed to

fusogenic liposome-encapsulated LfcinB6 exhibited substantial DNA fragmentation that was prevented in the presence of the pan-caspase inhibitor Z-VAD-FMK or the cathepsin B inhibitor CA-074-Me, implicating caspases and cathepsin B in the cytotoxic process (Fig. 4A). In contrast, the antioxidants NAC and GSH did not significantly diminish LfcinB6-induced cytotoxicity (Fig. 4B), excluding a cytotoxic role for ROS. Intracellular LfcinB6 therefore presumably mediates cytotoxicity by triggering the release of lysosomal cathepsin B, possibly by direct damage to lysosomes or by inducing Ca^{2+} release from the endoplasmic reticulum, leading to cytosolic calpain activation and the subsequent calpain-mediated release of cathepsin B from lysosomes [28]. In turn, cathepsin B activity is associated with the activation of initiator and executioner caspases involved in apoptosis [29,30]. Although cathepsin B activation has been linked to mitochondrial membrane permeabilization [29], this does not seem to be the case with LfcinB6-induced cytotoxicity because LfcinB6 did not cause any reduction in $\Delta\psi_m$ or trigger cytochrome *c* release. Nevertheless, cathepsin B- and caspase-dependent DNA fragmentation in Jurkat cells that were exposed to intracellular LfcinB6 is consistent with apoptosis induction.

The need to reduce production costs, immunogenicity, and toxicity, as well as enhance the stability of peptide-based drugs has spurred the identification and development of small peptides with potent biological activity [31,32]. Peptides that consist of six or fewer amino acids are of potential clinical interest because they are relatively inexpensive to synthesize, minimally immunogenic, and less susceptible to degradation by endopeptidases. We believe that these advantages make LfcinB6, in combination with a tumor-targeted intracellular delivery system based on p14-containing fusogenic liposomes, an attractive candidate for further development as a novel small peptide-based anticancer agent.

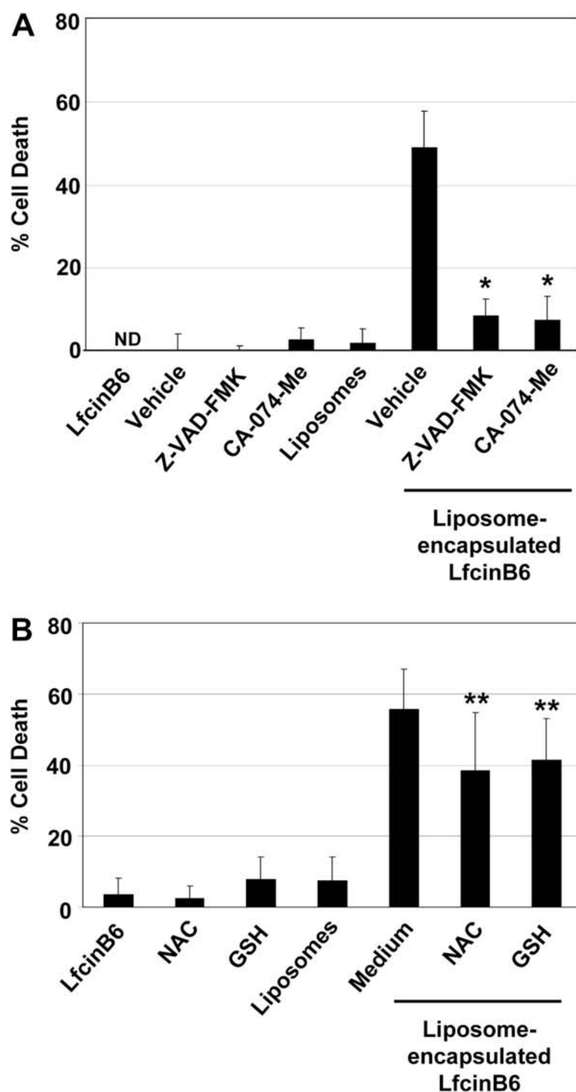

Fig. 4. Cytotoxicity caused by intracellular LfcinB6 is caspase- and cathepsin B-dependent but does not involve ROS. Jurkat T-leukemia cells were cultured without or with 31 μM free or fusogenic liposome-encapsulated LfcinB6 (9 μM) in the absence or presence of (A) 20 μM Z-VAD-FMK (pan-caspase inhibitor) or 25 μM CA-074-Me (cathepsin B inhibitor), or (B) the antioxidants NAC or GSH (both at 5 mM). Cytotoxicity was measured after 18 h by DNA fragmentation assay. ND denotes none detected. Data are shown as % cell death ± SD of quadruplicate samples. Statistical significance relative to the (A) vehicle control or (B) medium control was determined by the Tukey–Kramer multiple-comparisons test; $^{*}p < 0.001$, $^{**}p > 0.05$. All data are from one experiment that is representative of at least three independent experiments.

Acknowledgments

A. Richardson was supported by a Trainee Award from the Cancer Research Training Program with funding from the Canadian Cancer Society. This work was supported by a grant to D. Hoskin from the Leukemia and Lymphoma Society of Canada and to R. Duncan from the Canadian Institutes of Health Research.

References

[1] W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, M. Tomita, Identification of the bactericidal domain of lactoferrin, Biochim. Biophys. Acta 1121 (1992) 130–136.
[2] P.L. Masson, J.F. Heremans, E. Schonne, Lactoferrin, an iron-binding protein in neutrophilic leukocytes, J. Exp. Med. 130 (1969) 643–658.
[3] E.M. Jones, A. Smart, G. Bloomberg, L. Burgess, M.R. Millar, Lactoferricin, a new antimicrobial peptide, J. Appl. Bacteriol. 77 (1994) 208–214.
[4] W. Bellamy, H. Wakabayashi, M. Takase, K. Kawase, S. Shimamura, M. Tomita, Killing of *Candida albicans* by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin, Med. Microbiol. Immunol. 182 (1993) 97–105.
[5] M.I. van der Kraan, J. van Marle, K. Nazmi, J. Groenink, W. van't Hof, E.C. Veerman, J.G. Bolscher, A.V. Nieuw Amerongen, Ultrastructural effects of antimicrobial peptides from bovine lactoferrin on the membranes of *Candida albicans* and *Escherichia coli*, Peptides 26 (2005) 1537–1542.
[6] Y.C. Yoo, R. Watanabe, Y. Koike, M. Mitobe, K. Shimazaki, S. Watanabe, I. Azuma, Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species, Biochem. Biophys. Res. Commun. 237 (1997) 624–628.
[7] J.S. Mader, J. Salsman, D.M. Conrad, D.W. Hoskin, Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines, Mol. Cancer Ther. 4 (2005) 612–624.
[8] L.T. Eliassen, G. Berge, A. Leknessund, M. Wikman, I. Lindin, C. Lokke, F. Ponthan, J.I. Johnsen, B. Sveinbjornsson, P. Kogner, T. Flaegstad, O. Rekdal, The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo, Int. J. Cancer 119 (2006) 493–500.
[9] Y.C. Yoo, S. Watanabe, R. Watanabe, K. Hata, K. Shimazaki, I. Azuma, Bovine lactoferrin and lactoferricin inhibit tumor metastasis in mice, Adv. Exp. Med. Biol. 443 (1998) 285–291.
[10] P.M. Hwang, N. Zhou, X. Shan, C.H. Arrowsmith, H.J. Vogel, Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin, Biochemistry 37 (1998) 4288–4298.
[11] H.J. Vogel, D.J. Schibli, W. Jing, E.M. Lohmeier-Vogel, R.F. Epand, R.M. Epand, Towards a structure–function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides, Biochem. Cell Biol. 80 (2002) 49–63.
[12] J.H. Kang, M.K. Lee, K.L. Kim, K.S. Hahm, Structure–biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin, Int. J. Pept. Protein Res. 48 (1996) 357–363.
[13] M.B. Strom, O. Rekdal, J.S. Svendsen, Antibacterial activity of 15-residue lactoferricin derivatives, J. Pept. Res. 56 (2000) 265–274.
[14] K. Kachel, E. Asuncion-Punzalan, E. London, Anchoring of tryptophan and tyrosine analogs at the hydrocarbon-polar boundary in model membrane vesicles: parallax analysis of fluorescence quenching induced by nitroxide-labeled phospholipids, Biochemistry 34 (1995) 15475–15479.
[15] M. Tomita, M. Takase, W. Bellamy, S. Shimamura, A review: the active peptide of lactoferrin, Acta Paediatr. Jpn. 36 (1994) 585–591.
[16] E. Ueta, T. Tanida, T. Osaki, A novel bovine lactoferrin peptide, FKCRRWQWRM, suppresses *Candida* cell growth and activates neutrophils, J. Pept. Res. 57 (2001) 240–249.

[17] D.J. Schibli, P.M. Hwang, H.J. Vogel, The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles, FEBS Lett. 446 (1999) 213–217.
[18] D. Top, R. de Antueno, J. Salsman, J. Corcoran, J. Mader, D. Hoskin, A. Touhami, M.H. Jericho, R. Duncan, Liposome reconstitution of a minimal protein-mediated membrane fusion machine, EMBO J. 24 (2005) 2980–2988.
[19] P. Matzinger, The JAM test. A simple assay for DNA fragmentation and cell death, J. Immunol. Methods 145 (1991) 185–192.
[20] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[21] L.B. Chen, Fluorescent labeling of mitochondria, Methods Cell Biol. 29 (1989) 103–123.
[22] J.S. Mader, A. Richardson, J. Salsman, D. Top, R. de Antueno, R. Duncan, D.W. Hoskin, Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria, Exp. Cell Res. 313 (2007) 2634–2650.
[23] N. Yang, M.B. Strom, S.M. Mekonnen, J.S. Svendsen, O. Rekdal, The effects of shortening lactoferrin derived peptides against tumour cells, bacteria and normal human cells, J. Pept. Sci. 10 (2004) 37–46.
[24] S. Song, D. Liu, J. Peng, H. Deng, Y. Guo, L.X. Xu, A.D. Miller, Y. Xu, Novel peptide ligand directs liposomes toward EGF-R high-expressing cancer cells in vitro and in vivo, FASEB J. 23 (2009) 1396–1404.
[25] V.P. Torchilin, Recent approaches to intracellular delivery of drugs and DNA and organelle targeting, Annu. Rev. Biomed. Eng. 8 (2006) 343–375.
[26] A.I. de Kroon, D. Dolis, A. Mayer, R. Lill, B. de Kruijff, Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the mitochondrial outer membrane?, Biochim Biophys. Acta 1325 (1997) 108–116.
[27] K. Vancompernolle, F. Van Herreweghe, G. Pynaert, M. Van de Craen, K. De Vos, N. Totty, A. Sterling, W. Fiers, P. Vandenabeele, J. Grooten, Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity, FEBS Lett. 438 (1998) 150–158.
[28] T. Yamashima, Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain–cathepsin cascade' from nematodes to primates, Cell Calcium 36 (2004) 285–293.
[29] C. Paris, J. Bertiglio, J. Bréard, Lysosomal and mitochondrial pathways in miltefosine-induced apoptosis in U937 cells, Apoptosis 12 (2007) 1257–1267.
[30] A.M. Giammarioli, A. Maselli, A. Casagrande, L. Gambardella, A. Gallina, M. Spada, A. Giovannetti, E. Proietti, W. Malorni, M. Pierdominici, Pyrimethamine induces apoptosis of melanoma cells via a caspase and cathepsin double-edged mechanism, Cancer Res. 68 (2008) 5291–5300.
[31] M.B. Strom, B.E. Haug, M.L. Skar, W. Stensen, T. Stiberg, J.S. Svendsen, The pharmacophore of short cationic antibacterial peptides, J. Med. Chem. 46 (2003) 1567–1570.
[32] K. Hilpert, R. Volkmer-Engert, T. Walter, R.E. Hancock, High-throughput generation of small antibacterial peptides with improved activity, Nat. Biotechnol. 23 (2005) 1008–1012.