Anticancer activities of bovine and human lactoferricin-derived peptides

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Anticancer activities of bovine and human lactoferricin-derived peptides

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Abstract

Lactoferrin (LF) is a mammalian host defense glycoprotein with diverse biological activities. Peptides derived from the cationic region of LF possess cytotoxic activity against cancer cells *in vitro* and *in vivo*. Bovine lactoferricin (LFcinB), a peptide derived from bovine LF (bLF), exhibits broad-spectrum anticancer activity, while a similar peptide derived from human LF (hLF) is not as active. In this work, several peptides derived from the N-terminal regions of bLF and hLF were studied for their anticancer activities against leukemia and breast cancer cells, as well as normal peripheral blood mononuclear cells. The cyclized LFcinB-CLICK peptide, which possesses a stable triazole linkage, showed improved anticancer activity, while short peptides hLF11 and bLF10 were not cytotoxic to cancer cells. Interestingly, hLF11 can act as a cell penetrating peptide (CPP), and when combined with the antimicrobial core sequence of LFcinB (RRWQWR) through either a Pro or Gly-Gly linker, toxicity to Jurkat cells increased. Together, our work extends the library of LF-derived peptides tested for anticancer activity, and identified new chimeric peptides with high cytotoxicity towards cancerous cells. Additionally, these results support the notion that short CPPs and antimicrobial peptides can be combined to create new adducts with increased potency.

Keywords

Lactoferrin, lactoferricin, anticancer, leukemia, breast cancer

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein (~80 kDa) that is found in most exocrine secretions of mammals, including milk, tears, saliva, and sweat (Baker and Baker 2009, Alexander et al. 2012, Vogel 2012). LF is also found at high concentrations in neutrophils where it is stored in secondary granules and can be secreted in response to infection (Legrand et al. 2005). LF is involved in the modulation of the inflammatory response, making it an important part of the innate immune system of the host (Legrand et al. 2005, Latorre et al. 2012). In addition, LF exhibits direct antimicrobial, antiviral and anticancer activities (Valenti and Antonini 2005, Jenssen and Hancock 2009, Wakabayashi et al. 2014). Accordingly, LF preparations from both human (hLF) and bovine (bLF) origins have been extensively studied for their antitumor properties (Vogel 2012, Yin et al. 2013). Various *in vitro* experiments showed that hLF inhibits the growth of breast and colon cancer cell lines (Damiens et al. 1999). Similarly, bLF inhibits the growth of HeLa cells (Zemann et al. 2010). These *in vitro* activities also correlate with a study that demonstrated that bLF inhibits tumour progression *in vivo* by preventing tumor growth and metastasis in mice (Yoo et al. 1997a). Additionally, recombinant human LF (talactoferrin) has been tested in phase II clinical trials against non-small-cell lung cancer (NSCLC) in patients where previous anticancer therapies had failed, showing an apparent improvement (Parikh et al. 2012). Unfortunately, a subsequent trial did not identify such an improvement after administration of talactoferrin in patients with advanced NSCLC persisting after two regimens of anticancer therapy (Ramalingam et al. 2013). Nonetheless, a distinct clinical trial, in this case testing the oral administration of bLF, showed that high doses of bLF delayed the growth of adenomatous colorectal polyps in

the colon of humans (Kozu et al. 2009). The latter results suggest potential benefits of the ingestion of bLF when combined with polyp extraction during colonoscopy as a prophylactic treatment against the development of colorectal cancer.

In addition to the anticancer properties of the full-length LF proteins, smaller segments (peptides) of LF also exhibit cytotoxic activities towards several cancer cell lines (Yin et al. 2013). LFcInB, the cationic peptide encompassing residues 17-41 of bLF (Bellamy et al. 1992), is one of the most extensively studied anticancer peptides. LFcInB-mediated cytotoxicity towards cancer cells was first described for THP-1 (acute monocytic leukemia) cells. This work showed that apoptosis was mediated by the intracellular production of reactive oxygen species (ROS) and activation of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases (Yoo et al. 1997b). Others showed that LFcInB induces mitochondrial-dependent apoptosis in leukemia, lymphoma, and breast cancer cell lines (Yoo et al. 1997b, Mader et al. 2005, 2007, Furlong et al. 2006, 2010, Chan et al. 2007). However, LFcInB-mediated cell death occurs by a lytic mechanism in neuroblastoma and fibrosarcoma cells (Eliassen et al. 2002, 2006).

One of the structural characteristics of LFcInB is the presence of a single disulfide bridge between residues Cys-3 and Cys-20. This bridge confers a cyclic beta-hairpin structure to the peptide (Hwang et al. 1998, Gifford et al. 2005). Interestingly, *in vitro* studies with Jurkat and other cancerous cell lines suggests that LFcInB cyclization is not required for its cytotoxic activity (Mader et al. 2005). However, others report that the cyclic structure is absolutely required for the anticancer activity of LFcInB against fibrosarcomas *in vitro* (Eliassen et al. 2002). Therefore, the role of the disulphide bridge in mediating the anticancer activity of LFcInB remains unclear.

Similarly to bLF and its potential use as a treatment for some cancers, peptides derived from LFcinB could provide new alternatives to existing clinical cancer therapies. Currently, Lytix Biopharma is developing a new oncolytic immunotherapy that is based on the LTX-315 peptide. The design of this new peptide was originally derived from LFcinB and was the result of extensive structure-activity relationship studies (Haug et al. 2016). In terms of hLF, the peptide derived from the first 11 residues (hLF11) has been extensively studied for its antimicrobial activity against several bacterial and fungal strains (Lupetti et al. 2000, Nibbering et al. 2001). The mechanisms of action was proposed to involve direct membrane permeabilization and the targeting of energized mitochondria (Lupetti et al. 2000, Nibbering et al. 2001). In contrast, *in vivo* studies using mouse infection models suggested that the mechanism of action against bacterial infections was related to the activation/modulation of the immune system (Nibbering et al. 2001, Dijkshoorn et al. 2004). This proposed mechanism was based on the ability of hLF11 to induce an immune response by entering eukaryotic cells (van der Does et al. 2012). The latter results demonstrate that hLF11 can be considered as part of the family of cell penetrating peptides (CPPs) (Henriques et al. 2006). The main characteristic of CPPs is their ability to enter into the cytoplasm of eukaryotic cells. In several cases, CPPs are able to aid in the internalization of impermeable molecules, peptide/proteins, or nucleic acids (Copolovici et al. 2014). In addition, CPPs share several structural and biochemical properties with antimicrobial peptides (AMPs) (Epand and Vogel 1999, Vogel et al. 2002, Henriques et al. 2006). In contrast, the main characteristic of an AMP is the ability to kill different microorganisms, in most cases through a membranolytic mechanism (Nguyen et al. 2011).

In this work, the cytotoxicity of several peptides derived from bovine and human LF towards cancer and normal cells was assessed. First, a modified version of LFcinB was studied, where the disulfide bridge was replaced with a stable non-reducible linkage. The “click chemistry” method creates a triazole linkage, which preserves the cyclic beta-hairpin characteristics of the LFcinB. Second, the cytotoxicity of hLF11 and its bovine counterpart (bLF10) were studied, in order to determine if these peptides exhibit anticancer activities. In addition, several modified versions of both peptides were prepared and analyzed to identify the structural features that can give rise to increased anticancer activities *in vitro*. These modifications included mutation of Cys residues to Ser, cyclization and dimerization of the peptides. Moreover, the hexapeptide Trp and Arg-rich ‘antimicrobial core’ sequence of LFcinB (RRWQWR; LFcinB6) was C-terminally amidated to remove its negative charge, and the anticancer activity of the resulting peptide was tested. It was previously shown that fusogenic liposomal delivery of LFcinB6 results in cytotoxic activities towards cancer and normal cells (Richardson et al. 2009). Therefore, two new chimera peptides were created by combining the peptide sequences of hLF11 and RRWQWR. These two peptides were designed to take advantage of the cell penetrating potential of hLF11 and the potent anticancer activity of LFcinB’s core sequence.

Materials and Methods

Cell lines and Reagents

Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and heat inactivated fetal bovine serum (FBS) were

purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Thiazolyl blue tetrazolium bromide (MTT) and calcein were acquired from Sigma-Aldrich (St. Louis, MO, USA). Phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Jurkat T-leukemia cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and the breast cancer cell line (MDA-MB-231) were kindly provided by Dr. David Hoskin (Dalhousie University). Jurkat cells were grown in RPMI-1640 supplemented with 5% FBS, while MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS. All media also contained 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 5 mM HEPES. The cells were cultured at 37°C in a 5% CO₂ (Jurkats) or 10% CO₂ (MDA-MB-231) humidified atmosphere. In order to maintain optimal growth, the cells were routinely passaged as required. Only cultures with > 90% viable cells were used for cytotoxicity assays.

Peptides

Most peptides were purchased from GenScript, Inc. (Piscataway, NJ, USA), AnaSpec, Inc. (Fremont, CA, USA) and/or 21st Century Biochemicals (Malborough, MA, USA), as indicated in Table 1. These peptides were synthesized by routine solid phase methods; the purity ($\geq 95\%$) and molecular weights were confirmed by HPLC and mass spectrometry, respectively. Several peptides with Cys to Ser mutations were considered in these studies to prevent spontaneous dimerization of peptides during the lengthy incubations. The

peptide LFcInB-CLICK was synthesized and purified at the Academic Centre for Dentistry Amsterdam (ACTA) at the University of Amsterdam and VU University of Amsterdam, as described previously (Arias et al. 2014b). Melittin, purified from honeybee venom, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The peptides were weighed and resuspended in sterile water. The molecular weight for each peptide was used to establish the concentrations, assuming that 70% of the dry weight corresponded to actual peptide mass. For the cytotoxicity assays, the peptides were diluted in sterile water to a concentration of 20X as described previously, and all experiments were performed at a final FBS concentration of 2.5%, unless otherwise stated (Douglas et al. 2014).

Peripheral blood mononuclear cell (PBMC) and red blood cell (RBC) isolation

Whole venous blood from healthy, consenting volunteers was collected in BD vacutainer[®] tubes with sodium heparin as the anticoagulant (BD Bioscience, Mississauga, ON, Canada), using protocols approved by UBCs Clinical Research Ethics Board. To isolate RBCs, a fraction of whole blood was clarified by centrifugation (1400 xg for 5 min) and was washed with PBS. RBCs were collected again by centrifugation, and concentrated red blood cells were diluted with PBS to a final hematocrit concentration of 5%.

To isolate PBMCs, whole blood was diluted (1:2) in PBS, and the cell suspension was layered on to Lymphoprep density gradient medium (STEMCELL Technologies, Vancouver, BC). Diluted blood was clarified by centrifugation (1400 xg for 20 min), and the PBMC-containing buffy coat was collected. PBMCs were then washed twice with

PBS, and were resuspended in RPMI-1640 (5% FBS). Cells were rested, in 96 well flat-bottom tissue culture plates, for 1 h prior to peptide exposure.

MTT assay

Cytotoxicity assays were performed as previously described (Douglas et al. 2014).

Briefly, MDA-MB-231 cells (2×10^4 cells/well) were seeded in 96-well flat bottom tissue culture treated plates, and were incubated at 37°C in a 10% CO₂ humidified atmosphere for 24 hours, allowing for cell adhesion. After 24 h the media was changed (5% FBS), and peptide was added, bringing the final FBS concentration down to 2.5%. In contrast, Jurkats (5×10^4 cells/well) and PBMCs (2×10^5 cells/well) were exposed to peptide immediately, or after a 1 h resting period, respectively. Peptides were added to a final concentration of 10, 20 and 40 µM. In all cases, serum free media and/or peptide (in water) was added, bringing the FBS concentration down to 2.5%. Plates were incubated at 37°C in a 5% (Jurkats and PBMCs) or 10% (MDA-MB-231) CO₂ humidified atmosphere. Negative controls included medium and water, while the positive control contained 2% Triton X-100 [v/v]. All controls were prepared in triplicate. Cell viability was determined using the MTT assay after 24 h (Mosmann 1983). For this, MTT (100 µg) was added to each well, and the plates were incubated for 2 h at 37°C. Formazan crystals were clarified by centrifugation (1400 xg for 5 min), the supernatant was discarded, and the crystals were dissolved in DMSO (100 µl). Absorbance (490 nm) was registered on an Epoch microplate spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT) for each well. Threshold cell viability was established by the absorbance of the negative (water) and positive controls (Triton X-100) defined as 0% and 100%, respectively.

Hemolysis assay

RBCs were exposed to 10, 20 or 40 μM peptide in 96-well flat bottom plates as described previously (Douglas et al. 2014). The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 4 h. After incubation, the plates were centrifuged at 1400 $\times g$ for 5 min, and the supernatant was transferred to a new 96-well flat bottom plate. The presence of hemoglobin in the supernatant was then registered by measuring the absorbance at 490 nm using an Epoch microplate spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT). The absorbance for 100% hemolysis was established by incubation of RBCs with Triton X-100 (2% [v/v]).

Large unilamellar vesicles (LUVs) preparation

LUVs were prepared by the extrusion method as described previously (Rekdal et al. 2012, Arias et al. 2014a). Briefly, phospholipids dissolved in chloroform and methanol (2:1) were combined in a glass vial, dried under a gentle stream of nitrogen gas, and then placed under vacuum overnight to remove all trace of the organic solvents. The dry lipid films were hydrated with 70 mM calcein dissolved in leakage buffer (HEPES 10 mM, NaCl 150 mM and EDTA 1mM, pH 7.5) and multilamellar lipid vesicles (MLVs) were formed by vigorous vortexing. The MLVs suspension was then freeze-thawed seven times with liquid nitrogen and warm water ($\sim 40^\circ\text{C}$). LUVs (100 nm in diameter) were prepared from the MLVs suspension by the extrusion method using a mini-extruder (Avanti lipids, Inc.) and 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA). Free calcein was removed from the LUVs suspension by size exclusion chromatography on a Sephadex G-50 column previously equilibrated with leakage buffer.

The phospholipid concentration of the final LUVs suspension was measured by the Ames assay (Ames et al. 1966), performed in triplicate. LUVs composed of POPC and POPC:POPS (2:1) were used as model systems for the neutrally charged eukaryotic (normal) membranes and the negatively charged membrane of cancerous cells (Rekdal et al. 2012), respectively.

Calcein leakage experiments

The ability of the LFcInB-derived peptides to induce calcein leakage from calcein-loaded LUVs was assessed by the calcein leakage experiments. The fluorescence of calcein released from the LUVs was registered in an Eppendorf PlateReader AF2200 (Eppendorf, Mississauga, ON, Canada). The LUVs were incubated in 96-well non-binding surface, flat bottom, black plates (Corning® 3991) for 15 min at 37°C with 30 sec shaking every 2 min until the baseline fluorescence was stabilized. Peptides were then added to each well at different concentration 0 – 0.25 μ M, while the final concentration of LUVs phospholipids in each well was 2.5 μ M. The fluorescence of released calcein was then measured by excitation and emission wavelengths of 435 and 535 nm, respectively, every ~ 2 min for 15 min, under the same temperature and shaking conditions described above. LUVs in the presence of melittin 0.25 μ M was used as positive control, and the fluorescence intensity in this condition was considered as maximum (100%) calcein leakage. LUVs incubated with water were used as negative control (0% calcein leakage). Results were calculated as percentage of the maximum leakage.

Results

Cytotoxicity towards Jurkat cells

Several of the peptides killed Jurkat T-leukemia cells in a concentration-dependent manner. However, for the majority of the active peptides, only the highest concentration used (40 μ M) killed more than 50% of the cells. In contrast, the highly cytotoxic peptide melittin exhibited 100% killing at a peptide concentration of 10 μ M. LFcInB exhibited the highest cytotoxic activity of all LF-derived peptides that were evaluated in this study (Fig. 1). LFcInB (40 μ M) killed ~80% of the Jurkat cells. Similarly, the CLICK version of LFcInB retained the same cytotoxic activity as the parent peptide. Interestingly, the short hexapeptide version of LFcInB, LFcInB1 (Table 1), did not display any appreciable anticancer activity towards Jurkat cells.

In contrast to the high activity of LFcInB, the peptides derived from the first 11 residues of human LF did not exhibit cytotoxic activity toward Jurkat cells under these experimental conditions. Similarly, peptides derived from the first 10 and 16 residues of bovine LF (bLF10 and bLF16) showed less than 20% cytotoxicity (Fig. 1). Mutation of the Cys to a Ser did not increase the killing potential of either hLF11C10S or bLF10C9S. Additionally, cyclization of the short human and bovine LF-derived peptides did not change the anti-Jurkat activity of the peptides, even though others have shown that cyclization can promote the antimicrobial and anticancer activities of similar peptides (Dathe et al. 2004, Wessolowski et al. 2004, Nguyen et al. 2010, Tørfoss et al. 2012). Dimerization of the hLF11 peptide resulted in a peptide with slightly decreased potency, while the bLF10 dimer was almost twice as active as the bLF10 monomer (Fig. 1).

Interestingly, the linear combination of hLF11 and LFcInB1, which were joined as a single polypeptide chain by either introducing a Pro or a Gly-Gly linker,

considerably increased Jurkat-mediated cytotoxicity. The peptide linked by two Gly residues (hLF11-GG-LFcinB1) showed an anti-Jurkat activity of 65% at 40 μ M, while the peptide linked by a single Pro residue (hLF11-P-LFcinB1) exhibited 47% killing activity (Fig. 1).

Cytotoxicity towards PBMCs and RBCs

All LF-derived peptides were minimally toxic to PBMCs, even at the highest peptide concentrations tested (40 μ M) (Fig. 2). Most peptides showed killing percentages below 10%. In contrast, the highly cytotoxic peptide melittin induced 100% killing of PBMCs already at 10 μ M. The only LF-derived peptide with any appreciable cytotoxic activity towards PBMCs was the chimera hLF11-P-LFcinB1, which killed 30% of PBMCs at a concentration of 40 μ M (Fig. 2). In the case of RBCs none of the LF-derived peptides caused any appreciable hemolysis, with values lower than 2% measured for all peptides (Fig. 3). As expected, melittin retained its hemolytic activity with 90% hemolysis observed at a peptide concentration of 10 μ M (Fig. 3).

Cytotoxicity towards breast cancer (MDA-MB-231) cells

Almost all of the LF-derived peptides used in this study showed only weak cytotoxic activity towards the adherent breast cancer cell line MDA-MB-231, even at the highest concentration (40 μ M) tested (Fig. 4). In contrast, the cytotoxic control peptide melittin was able to induced 100% killing at the lowest (10 μ M) concentration tested. Of all of the LF-derived peptides tested only the LFcinB-CLICK peptide caused any appreciable killing, with 45% cytotoxicity observed at 40 μ M (Fig. 4).

Permeabilization of phospholipids model membranes

The ability of selected peptides to permeabilize the membranes of LUVs was assessed by performing calcein leakage experiments. In these experiments, calcein-loaded LUVs composed of POPC or POPC:POPS (2:1) were incubated in the presence of different peptide concentrations (Fig. 5). Only peptides that displayed considerable cytotoxicity were considered in these assays. Neither LFcinB nor its derivatives induced calcein leakage in the neutrally charged LUVs composed of POPC, when compared to the highly membrane permeabilizing control peptide melittin (Fig. 5B). However, in the presence of negatively charged phospholipid head groups, such as POPS, some of the peptides induced membrane permeabilization (Fig. 5A). When mixed with LUVs composed of POPC:POPS (2:1), LFcinB caused a relatively low level of calcein release (14 %) at high P/L ratios, while the peptides LFcinB1 and hLF11 did not induce any considerable leakage (Fig. 5A). Interestingly, the chimera peptides formed by LFcinB1 and hLF11 linked by either Pro or Gly-Gly exhibited enhanced calcein leakage abilities from the anionic LUVs, resulting in 24% and 37% calcein release, respectively (Fig. 5A).

Discussion

The study of the antimicrobial activities of bovine LF has resulted in the characterization of several LF-derived peptides with broad-spectrum activity against Gram-negative and Gram-positive bacteria (Gifford et al. 2005, Vogel 2012, Sinha et al. 2013). Interestingly, the peptides derived from hLF are consistently less active than their bovine counterparts in antimicrobial assays (Bellamy et al. 1992, Vorland et al. 1998, Haney et al. 2009).

Nonetheless, a peptide derived from the first 11 residues of human LF (hLF11) has gained considerable attention due to its antimicrobial and antifungal activity *in vitro* (Lupetti et al. 2000, Nibbering et al. 2001), in addition to its immunomodulatory effects that help reduce bacterial counts in mouse infection models (Nibbering et al. 2001, Dijkshoorn et al. 2004). In an attempt to develop novel anticancer agents with fewer side effects than those currently used, we decided to evaluate the potential of these peptides against cancer cells. In the current study, several bovine and human LF-derived peptides were tested against two different cancer cell lines and normal blood cells as controls. In addition, several strategies (i.e., site-specific mutations, cyclization, dimerization or formation of peptide adducts) aimed at improving the cytotoxicity of these peptides were also implemented and tested.

In this study, the cytotoxic activity of hLF11 and bLF10, its direct bovine counterpart (encompassing residues 1-10 of bLF), were evaluated against Jurkat T leukemia and MDA-MB-231 breast cancer cells, as well as PBMCs and RBCs as normal cell controls for the Jurkat cells. These cancer cells lines were selected in order to evaluate the anticancer potential of LF-derived peptides against two drastically different types of cancer. First, a T cell acute lymphoblastic leukemia cell line (Jurkat) cells, and secondly, an aggressive highly metastatic breast carcinoma cell line. In addition to hLF11 and bLF10, several modified versions of these peptides were produced by Cys-to-Ser mutations, head-to-tail cyclization, and dimerization, and their anticancer activity was investigated. The breast cancer cells, PBMCs and RBCs were largely unaffected by this collection of LF-derived peptides (Figs. 2-4). However, Jurkat cells were susceptible to three peptides, namely hLF11, dimer bLF10 and bLF16 (Fig. 1), with the dimer bLF10

being the most potent, exhibiting 32% killing at a concentration of 40 μ M. These results indicate that most of the peptides derived from the first residues of bovine and human LF do not possess direct anticancer activity toward the cells tested here.

LFcinB, one of the best studied anticancer peptides, is cytotoxic towards several human cancer cells *in vitro* (Yoo et al. 1997b, Mader et al. 2005, 2007, Eliassen et al. 2006). LFcinB reduces the growth of tumors and inhibits metastasis in mouse models (Yoo et al. 1997a, Eliassen et al. 2002, 2006). In this work, a new version of LFcinB was tested for its cytotoxic activity against Jurkat and MDA-MB-231 cells. LFcinB-CLICK was generated using “click chemistry” in order to replace the disulfide bridge between residues Cys-3 and Cys-20 in LFcinB (Arias et al. 2014b) with a triazole linkage that allows the peptide to retain the cyclic hairpin characteristics of the original peptide. Our results indicate that LFcinB and LFcinB-CLICK were similarly toxic to Jurkat cells (Fig. 1), suggesting that the triazole linkage can be used as a stable substitute for the relative labile disulfide bridge. Similar results have been reported for the antimicrobial activity of LFcinB-CLICK in comparison to LFcinB against Gram-positive and Gram-negative bacterial (Arias et al. 2014b). Interestingly, in contrast to LFcinB, 40 μ M LFcinB-CLICK killed nearly 50% of the MDA-MB-231 breast cancer cells (Fig. 4), suggesting that stable cyclization chemistries may improve the cytotoxic activity of LFcinB-derived peptides toward more refractory cancer cells.

Previous studies identified a cluster of 6 amino acids (RRWQWR), called LFcinB6, as the antimicrobial core of LFcinB (Tomita et al. 1994, Schibli et al. 1999, Mader et al. 2005). In comparison to LFcinB, LFcinB6 was minimally toxic to Jurkat and

MDA-MB-231 cells (Mader et al. 2005, Richardson et al. 2009). Here, we tested a modified version of LFcInB6, called LFcInB1, where its N-terminal end was acetylated and the C-terminal end was amidated (Nguyen et al. 2010). These two modification strategies were previously shown to increase the antimicrobial activity of this peptide against Gram-negative and Gram-positive bacteria, even in the presence of human serum (Nguyen et al. 2010). Additionally, when combined, these two modifications improved the resistance of the peptide to protease degradation in human plasma (Nguyen et al. 2010). We decided to test LFcInB1 for cytotoxic activity towards Jurkat and MDA-MB-231 cells, as well as PBMCs and RBCs, to see whether this trend of increased activity held true for the toxicity toward eukaryotic cells. Unfortunately, LFcInB1 was not toxic to any of these cells (Fig. 1 – 4), indicating that the acetylation and amidation of LFcInB6 do not promote its anticancer activity. These results indicate that the same modification strategies that enhance antimicrobial activities will not necessarily enhance the anticancer activity of the peptide.

Studies by Richardson et al. (Richardson et al. 2009) showed that intracellular delivery of LFcInB6 using fusogenic liposomes resulted in high cytotoxic activity towards Jurkat cells. Taking into account these results, Hilchie et al. (Hilchie et al. 2013) attempted to promote internalization of LFcInB6 by covalently attaching a cell-penetrating Arg-rich sequence (R₇) using a Gly-Gly linker. In this case, the new peptide chimera (R₇-GG-LFcInB6) exhibited potent cytolytic activity toward Jurkat cells (Hilchie et al. 2013). A similar approach has been investigated where the antimicrobial peptides P7-27 and (KLAKLAK)₂ were linked to the same R₇ cell penetrating sequence using a Gly-Gly linker (Lemeshko 2013). In these experiments the new peptides were highly

cytotoxic to Jurkat cells. Together, these experiments suggest that linear combinations of a CPP, such as R₇, and AMPs could result in the creation of selective cytotoxic peptides.

Interestingly, the peptide hLF11 has been reported to enter eukaryotic cells where it can interact with the intracellular myeloperoxidase enzyme (van der Does et al. 2012). This behavior would suggest that hLF11 has the capacity to behave as a CPP. Therefore, hLF11 was used in this study as a potential CPP to promote the internalization of LFcInB6 into cancer cells. Thus, two new chimera peptides were created by making linear adducts of hLF11 and LFcInB1 through two different linkers (Pro or Gly-Gly). The Gly-Gly linker has previously been used in other CPP-AMP chimeras as described above (Hilchie et al. 2013, Lemeshko 2013), while a Pro linker has been used by other research groups to create other dimer or retro-dimer peptides with improved cancer cell toxicity (Riedl et al. 2015). In the presence of both hLF11-LFcInB1 chimeras, Jurkat cell killing was substantially increased compared to either component peptide alone (Fig. 1). These results are in agreement with the previously reported increases in anticancer activity of P7-27 and LFcInB6 after attachment of a N-terminal R₇ cell penetrating sequence (Hilchie et al. 2013, Lemeshko 2013). Unfortunately, hLF11-P-LFcInB1 and hLF11-GG-LFcInB1 killed 30% and 12% of PBMCs, respectively (Fig. 2). However, it is noteworthy that neither peptide exhibited significant hemolytic activity (Fig. 3). Together, these results demonstrate that the combination of short potent AMPs and CPPs consistently improves the anticancer activity of peptides. In addition, the chimera peptide hLF11-GG-LFcInB1 provided some selectivity for transformed T-lymphocytes according to our results. However, when tested against the breast cancer cells, these chimeric peptides did not exhibit potent anticancer activities (Fig. 4), suggesting that the difference in the

characteristics between the Jurkats and MDA-MB-231s cells are important for the anticancer activity of the LF-derived peptides. It is notable that the linker itself seems to play an important role regarding the cytotoxicity toward PBMCs and MDA-MB-231s. The Pro linker conferred higher toxicity to the peptide to PBMCs in comparison to the Gly-Gly linker, making the latter the preferred choice, because of its improved selectivity and activity for Jurkat cells. Differences in the structure and flexibility of the two chimera peptides could be related to this behavior. A future detailed study of the structure of these two chimera peptides could provide some insights into the role that structure and/or flexibility may play in their mechanism of cytotoxic activity.

In this study, simple model membrane systems were used to gain some insight into the mechanism of action of the chimeras. For this, we used LUVs composed of POPC:POPS (2:1) and POPC, respectively, to represent the negatively charged surface of malignant cells and the neutrally charged surface of normal eukaryotic cells (Rekdal et al. 2012). LFcInB1 and hLF11 alone did not induce any permeabilization of the POPC or POPC:POPS LUVs, whereas the peptide chimeras both resulted in higher levels of permeabilization for the POPC:POPS membranes (Fig. 5). The results obtained in these assays suggest that the potency of the chimeric peptides could be related to an increase in their membranolytic activities. These results are in agreement with the findings reported for R₇-GG-LFcInB6 and R₇-GG-(KLAKLAK)₂, which also caused increased cytoplasmic and mitochondrial membrane lysis (Hilchie et al. 2013, Lemeshko 2013). In addition, the selectivity exhibited by the chimera hLF11-GG-LFcInB1 can be explained by the differences in the membrane composition of normal blood cells and transformed/cancerous cells. The increase of the overall negative charge of cancerous

cells could enhance the attraction and consequently the cytotoxicity of certain positively charged peptides. The presence of phospholipids with negatively charged phosphatidylserine (PS) headgroup on the outer surface of the cell membrane is a common feature shared by numerous cancer cells (Utsugi et al. 1991, Riedl et al. 2011), including Jurkat cells (Schroder-Borm et al. 2005). This characteristic suggests that the PS phospholipid headgroup can act as a selective target for anticancer peptides (Riedl et al. 2011). However, other negatively charged molecules, such as heparan-sulfate proteoglycans, glycoproteins and glycolipids could also contribute to the increased activity of anticancer peptides (Harris et al. 2011). The contribution of these other membrane components, although out of the scope of the current study, could constitute future research projects. It is important to note that the increase in the overall positive charge of the peptides is not directly correlated with the increase in the anticancer activity, as we did not observe any cytotoxicity for the homo-dimer version of hLF11 (net charge +8) while we did see enhanced activity for the chimeric peptides (net charge +7).

In conclusion, several LF-derived peptides were tested for their anticancer activity towards leukemia and breast cancer cells *in vitro*. Among these peptides, LFcInB, LFcInB-CLICK and the chimeras composed of hLF11 and LFcInB1 exhibited interesting anticancer activities with considerable selectivity for the cancerous cells. These results highlight the relevance of LFcInB as a potential source of novel anticancer agents, and they also support the notion that linear combinations of CPPs and AMPs can give rise to more potent anticancer peptides.

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Tables

Table 1. Lactoferrin derived peptides

Peptide	Sequence	Charge	Source
LFcinB	FK C RRWQWRMKKLGAPSI T CVRRAF	+ 8	GenScript
LFcinB-CLICK	FK * RRWQWRMKKLGAPSI * VRRAF	+ 8	ACTA
LFcinB1	Ac-RRWQWR-NH ₂	+ 3	AnaSpec
hLF11	GRRRRSVQWCA	+ 4	GenScript
hLF11C10S	GRRRRSVQWSA	+ 4	21 st Century Bio
Cyclo-hLF11	~GRRRRSVQWCA~	+ 4	21 st Century Bio
Cyclo-hLF11C10S	~GRRRRSVQWSA~	+ 4	21 st Century Bio
Dimer hLF11	GRRRRSVQW CA GRRRRSVQW CA	+ 8	GenScript
bLF10	APRKNVRWCT	+ 3	21 st Century Bio
bLF10C9S	APRKNVRWST	+ 3	21 st Century Bio
Cyclo-bLF10	~APRKNVRWCT~	+ 3	21 st Century Bio
Dimer bLF10	APRKNVRW CT APRKNVRW CT	+ 6	21 st Century Bio
bLF16	APRKNVRWCTISQPEW	+ 2	21 st Century Bio
hLF11-P-LFcinB1	GRRRRSVQWCA-P-RRWQWR-NH ₂	+7	GenScript
hLF11-GG-LFcinB1	GRRRRSVQWCA-GG-RRWQWR-NH ₂	+ 7	GenScript
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	+ 6	Sigma-Aldrich

* represents a triazole linkage

Figure captions

Figure 1. Cytotoxicity of LF-derived peptides to Jurkat leukemia cells. Jurkat cells were incubated in the presence or absence of 10 (white), 20 (gray) and 40 μM (black) peptides for 16 h. Percent cytotoxicity was established using the MTT assay, where vehicle- and Triton X-100-treated cells served as negative and positive controls, respectively. Results represent mean $\pm$ SEM ($n = 3$).

Figure 2. Cytotoxicity of LF-derived peptides to primary PBMCs. PBMCs were incubated in the presence or absence of 10 (white), 20 (gray) and 40 μM (black) peptides for 16 h. Percent cytotoxicity was established using the MTT, where vehicle- and Triton X-100-treated cells served as negative and positive controls, respectively. Results represent mean $\pm$ SEM ($n = 3$).

Figure 3. Cytotoxicity (hemolysis) of LF-derived peptides to RBCs. RBCs were incubated in the presence or absence of 10 (white), 20 (gray) and 40 μM (black) peptides for 4 h. Percent hemolysis was established using the hemolysis assay, where vehicle- and Triton X-100-treated cells served as negative and positive controls, respectively. Results represent mean $\pm$ SEM ($n = 3$).

Figure 4. Cytotoxicity of LF-derived peptides to MDA-MB-231 breast cancer cells. MDA-MB-231 cells were incubated in the presence or absence of 10 (white), 20 (gray) and 40 μM (black) peptides for 16 h. Percent cytotoxicity was established using the MTT assay, where vehicle- and Triton X-100-treated cells served as negative and positive controls, respectively. Results represent mean $\pm$ SEM ($n = 3$).

Figure 5. Permeabilization of LUVs membranes induced by LF-derived peptides, followed by calcein leakage percentage. LUVs (2.5 μM) POPC:POPS (2:1) (**A**) and POPC (**B**) incubated with different concentration of peptides (0 – 0.25 μM) for 15 min at 37°C. Peptides are depicted as follow: LFcinB (●), LFcinB1 (◇), hLF11 (△), hLF11-P-LFcinB1 (○), hLF11-GG-LFcinB1 (□) and melittin (■). Results represent mean $\pm$ S.D. ($n = 4$).

%Cytotoxicity

100
75
50
25
0

LFcinB

LFcinB-CLICK

LFcinB1

hLF11

hLF11C10S

Cyclo-hLF11

Cyclo-hLF11C10S

Dimer hLF11

bLF10

bLF10C9S

Cyclo-bLF10

Dimer bLF10

bLF16

hLF11-P-LFcinB1

hLF11-GG-LFcinB1

Melittin

%Cytotoxicity

100

75

50

25

0

LFcinB

LFcinB-CLICK

LFcinB1

hLF11

hLF11C10S

Cyclo-hLF11

Cyclo-hLF11C10S

Dimer hLF11

bLF10

bLF10C9S

Cyclo-bLF10

Dimer bLF10

bLF16

hLF11-P-LFcinB1

hLF11-GG-LFcinB1

Melittin

%Hemolysis

100
75
50
25
0

LFcinB

LFcinB-CLICK

LFcinB1

hLF11

hLF11C10S

Cyclo-hLF11

Cyclo-hLF11C10S

Dimer hLF11

bLF10

bLF10C9S

Cyclo-bLF10

Dimer bLF10

bLF16

hLF11-P-LFcinB1

hLF11-GG-LFcinB1

Melittin

Draft

%Cytotoxicity

100
75
50
25
0

LFcinB

LFcinB-CLICK

LFcinB1

hLF11

hLF11C10S

Cyclo-hLF11

Cyclo-hLF11C10S

Dimer hLF11

bLF10

bLF10C9S

Cyclo-bLF10

Dimer bLF10

bLF16

hLF11-P-LFcinB1

hLF11-GG-LFcinB1

Melittin

ND