APOPTOGENIC AND ANTI-ANGIOGENIC ACTIVITY OF THE BOVINE MILK-DERIVED PEPTIDE, LACTOFERRICIN

by

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ABSTRACT

Bovine lactoferricin (LfcinB) is a 25 amino acid peptide released from the N-terminal portion of its parent protein, lactoferrin by acid-pepsin hydrolysis. Lactoferrin is found in the intracellular granules of neutrophils and in biological fluids, including saliva and milk. There is abundant evidence that lactoferrin is a major component in antimicrobial host defense. Most of the antimicrobial activity of lactoferrin has been attributed to LfcinB, which kills a wide variety of bacteria, viruses and fungi through a mechanism involving membrane destabilization. LfcinB also displays potent anticancer activity; however the extent of this activity and the mechanism(s) by which it occurs has not been investigated. In this study, I found that LfcinB killed a wide variety of human cancer cell lines, with no apparent cytotoxic effect on normal human cells. LfcinB triggered the mitochondria-dependent pathway of apoptosis, through a reactive oxygen species (ROS)-, and caspase-2-dependent loss of mitochondrial transmembrane potential, release of mitochondrial cytochrome c, and sequential caspase-9 and caspase-3 activation. LfcinB interacted with Jurkat T leukemia cells, most likely through initial electrostatic interactions, followed by formation of membrane-spanning pores that allowed LfcinB to enter the cell. In addition, immune-deficient SCID/beige mice bearing disseminated Ramos B-lymphoma cells showed a 1.9-fold increase in median survival, as well as an increased proportion (60%) of long-term survivors after treatment with LfcinB on days 1 and 3 post lymphoma cell injection. LfcinB also inhibited blood vessel development, which is required for tumour growth beyond 1-2 mm in diameter. LfcinB bound to the surface of human umbilical vein endothelial cells (HUVECs) through an interaction with heparin-like proteoglycans, thereby inhibiting heparin-binding growth factors from interacting with their cell-surface receptors. LfcinB blocked bFGF- and $VEGF_{165}$-induced HUVEC proliferation and migration *in vitro*, and bFGF- and $VEGF_{165}$-induced angiogenesis *in vivo* using the Matrigel model. This study has allowed me to understand the multifunctional anticancer activity of LfcinB. I have determined that LfcinB kills human tumour cells both *in vitro* and *in vivo*, through a mechanism involving membrane binding and destabilization, and the induction of apoptosis. I have also found that LfcinB displays potent antiangiogenic activity.

List of Abbreviations and Symbols Used

AIF	apoptosis-inducing factor
Amino Acids	
A	alanine
C	cysteine
F	phenylalanine
G	glycine
I	isoleucine
K	lysine
L	leucine
M	methionine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Ang	angiopoietin
ANOVA	one way analysis of variance
ANT	adenine nucleotide translocator
Apaf-1	apoptosis activating factor-1
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
Bak	Bcl-2-homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
bFGF	basic fibroblast growth factor
BH3	Bcl-2 homology domain 3
Bid	BH3-interacting domain death agonist

Biotin-LfcinB	biotinylated LfcinB
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
Ca^{2+}	calcium ion
CAD	caspase-activated deoxyribonuclease
CAP	cationic antimicrobial peptide
Caspase	cysteine-dependent aspartate specific protease
CD36	collagen type I receptor, thrombospondin receptor
cFLIP	cellular FLICE-inhibitory protein
CHAPs	(3-[(3-cholamidopropyl)dimethylammonio]- 1-propanesulfonate)
CO_2	carbon dioxide
cpm	counts per minute
^{51}Cr	chromium
dATP	2'-deoxyadenosine 5'-triphosphate
DD	death domain
DED	death effector domain
$DiOC_6$	3, 3'- dihexyloxacarbocyanine iodide
diPG	diphosphatidylglycerol
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DR	death receptor

ECGS	endothelial cell growth supplement
ENDO G	endonuclease G
ERK	extracellular signal-regulated kinase
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	(ethylenebis(oxyethylenenitrilo)) tetra-; ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
FADD	Fas-associated death domain
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like interleukin-1β converting enzyme
GAG	glycosaminoglycans
GSH	glutathione
h	hours
HBSS	hanks buffered salt solution
HS	heparan sulfate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNP	human neutrophil peptide α-defensin
HRP	horse radish peroxidase
HSPG	heparan sulfate proteoglycan
^{3}H-TdR	tritiated thymidine
HtrA2	high temperature requirement A2
HUVEC	human umbilical vein endothelial cell
IAP	inhibitor of apoptosis protein

ICAD	inhibitor of caspase-activated DNase
IF	immunofluorescence
INF	interferon
IL	interleukin
IP	intraperitoneal
JNK	jun N-terminal kinase
$\Delta\Psi_m$	dissipation in mitochondrial membrane potential
LAK	lymphokine activated killer
LfcinB	bovine lactoferricin
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MDR	multiple drug resistance
min	minutes
MMP	matrix metalloproteinase
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAC	*N*-acetyl-*L*-cysteine
NF-κB	nuclear factor kappa beta
NK	natural killer cells
p53	protein 53 kilodaltons in size
PARP	poly (ADP-ribose) polymerase

PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PG	phosphatidylglycerol
PLP	periodate-lysine-paraformaldehyde
pO_2	partial pressure of oxygen
PS	phosphatidylserine
PT	permeability transition
RNA	ribonucleic acid
ROS	reactive oxygen species
RRV	reptilian reovirus
Tc	transcription
SC	subcutaneous
SCID	Severe Combined Immunodeficiency
SDS	sodium dodecyl sulphate
Smac/ DIABLO	second mitochondria-derived activator of caspase/direct IAP-binding protein with a low isoelectric point
TNF	tumour necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand
TSP	thrombospondin
VDAC	voltage dependent anion channel

VEGF	vascular endothelial cell growth factor
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1

Introduction

Cancer has become a growing problem worldwide, especially in many countries of the western world where the demographic trend is that of an aging population. Treatment of many cancers often involves the use of individual or a combination of chemotherapeutic drugs. However, one of the major obstacles limiting the efficacy of cancer chemotherapy is the development of multiple drug resistance (MDR). MDR can occur by a number of mechanisms, including the overexpression of drug transporters (Gottesman *et al.*, 2002), and mutations in downstream cellular processes such as cell cycle checkpoint control, DNA damage repair, and death signals (Kroemer and Reed, 1997; Bunz *et al.*, 1999; Johnstone *et al.*, 2002). In addition, the efficacy of chemotherapeutic drugs is limited by their requirement for the target cell to be in a proliferative state, therefore, dormant or slowly proliferating tumour cells are not susceptible to these agents (Naumov *et al.*, 2003). Furthermore, many chemotherapeutic drugs are non-specific in their action, killing or damaging normal healthy cells in addition to tumour cells, which results in a plethora of toxic side effects (Kobayashi *et al.*, 2002; Sehouli *et al.*, 2002; Hickman *et al.*, 1994). Due to the existence of chemotherapeutic drug resistance and lack of target specificity, it has become necessary to explore other more effective and possibly less toxic alternatives to conventional cancer treatment.

Cationic Antimicrobial Peptides

Background and Antimicrobial Activity

Cationic antimicrobial peptides (CAPs) are a group of microbicidal peptides expressed in many species, including amphibians, insects, and mammals, where they are an important part of innate immunity (Zasloff, 2002; Hancock and Diamond, 2000).

CAPs tend to be small in size (< 40 amino acids) and have broad spectrum antimicrobial activity, killing a wide variety of Gram negative and Gram positive bacteria, while also displaying antifungal, antiviral, and antiparasitic activity (Leuschner and Hansel, 2004; Martin *et al.*, 1995; Bellamy *et al.*, 1992). One of the hallmarks of microbicidal peptides is their ability to permeabilize bacterial membranes, leading to cellular destabilization and death (Gazit *et al.*, 1994; Pouny *et al.*, 1992; Ellerby *et al.*, 1999; Zasloff, 2002). This ability is believed to be directly related to their net charge and amphipathic secondary structure (Leuschner and Hansel, 2004). Due to a net positive charge, these peptides initially interact with anionic membrane phospholipids in bacterial membranes through electrostatic interactions, while hydrophobic interactions between the lipid head groups and the cationic peptide determine the extent of membrane destabilization (Vorland *et al.*, 1999a). The two main mechanisms by which CAPs interact with the microbial membrane are termed the barrel-stave and carpet models (Ye *et al.*, 2004; Ehrenstein and Lecar, 1977; Monaco *et al.*, 1999). A number of CAPs, including magainin 2 isolated from the skin of the African clawed frog, *Xenopus laevis* (Zasloff, 2002; Matsuzaki, 1997), follow the barrel-stave model of interaction (Haukland *et al.*, 2001), whereby multiple peptides insert into the hydrophobic bacterial membrane forming transient transmembrane pores that allow subsequent peptide entry into the cell (Ehrenstein and Lecar, 1997; Ye *et al.*, 2004). Model membrane studies have determined that magainin 2 permeabilizes the outer and inner bacterial membrane in *Escherichia coli* and *Acinetobacter calcoaceticus* (Matsuzaki, 1997), while further analysis found that magainin 2 crosses the cytoplasmic membrane and enters the cytosol of *Escherichia coli* (Haukland *et al.*, 2001). The carpet model of membrane interaction involves the binding of CAPs to the anionic head groups

of membrane phospholipids, followed by alignment of the hydrophilic surface of the peptide monomers parallel to the cell surface. This gives way to membrane destabilization by disrupting membrane curvature without actual insertion of the peptide monomers into the hydrophobic core of the lipid bilayer (Monaco *et al.*, 1999). An intermediate step may involve transient pore or channel aggregate formation, known as the two-state or toroidal pore formation model (Matsuzaki, 1999; Zasloff, 2002). Peptides that utilize this method include the defensin family of cysteine- and arginine-rich CAPs, which are isolated from insect and mammal species (Marshall and Arenas, 2003). In general, amphipathic peptides conform to the barrel-stave model, while hydrophobic peptides tend to destabilize the cell membrane through the carpet method (Ye *et al.*, 2004). The specificity of many CAPs for microbial cells over eukaryotic host cells may be due to the increased content of negatively charged phospholipids in bacterial cell membranes. The different mechanisms of membrane destabilization are illustrated in Figure 1.1.

Anticancer Activity of CAPs

In addition to their antimicrobial activity, many CAPs have recently been shown to display anticancer activity (Farkas-Himsley *et al.*, 1995; Chen *et al.*, 2005; Mader *et al.*, 2005). Magainin 2 permeabilizes and enters human cancers cells, such as HeLa human cervical carcinoma cells, through the formation of transient toroidal pores of 2-3 nm in diameter in an energy-independent and non-receptor-driven manner (Takeshima *et*

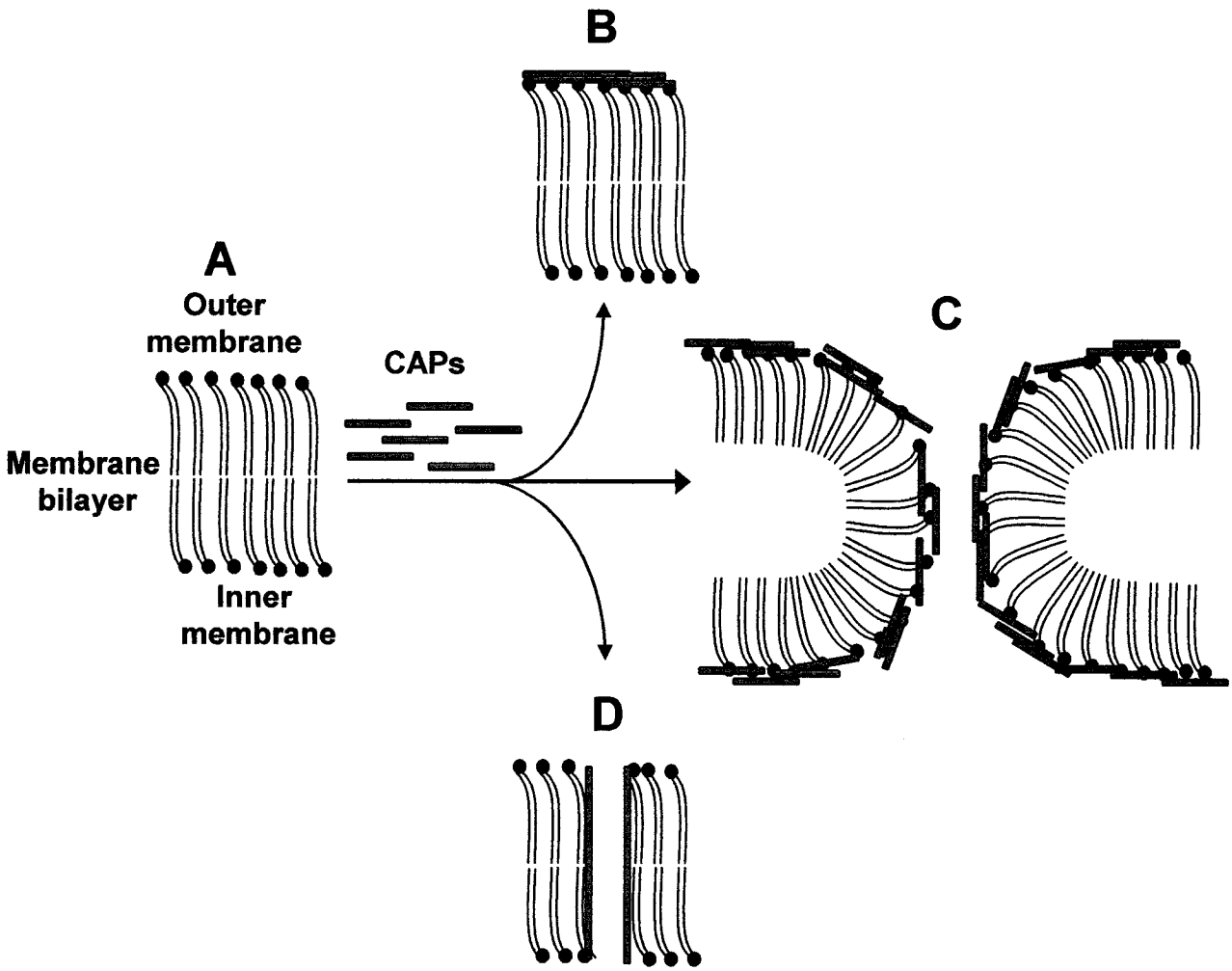


Figure 1.1 *Schematic representation of the different mechanisms of membrane destabilization by CAPs.* Peptides bind initially through electrostatic interactions and align parallel to the outer membrane (A). The carpet model of membrane destabilization postulates increasing CAP concentrations at the cell surface (B), followed by membrane disintegration (C). The peptides remain in contact with the lipid headgroups during this process. The barrel-stave model (D) postulates that CAPs insert into the hydrophobic core of the membrane bilayer and polymerize in order to form transmembrane pores.

al., 2003). This 23 amino acid peptide displays selective cytotoxicity, killing many tumour cell lines at concentrations 5-10 times lower than those necessary to kill untransformed human cells (Jacob *et al.*, 1994). Both human and rabbit defensins are cytotoxic to a variety of human and murine tumour targets (Lichtenstein *et al.*, 1986). IM-9 myeloma cells, and Wil-2 and Raji human B cell lymphoma lines were most susceptible to the cytotoxic effects of human neutrophil defensins HNP-1, -2, and -3; killing is detected as early as three hours following exposure to the defensins.

Relatively little is known about the mechanism by which CAPs selectively target and kill tumour cells. Scientists believe that the anticancer activity may be similar to the antimicrobial activity of these peptides, involving membrane binding and destabilization (Ellerby *et al.*, 1999). Like bacteria, many tumour cell membranes display a high proportion of anionic phospholipids, resulting in a negative net charge, while untransformed human cells generally have a neutral membrane charge (Chan *et al.*, 1998). Studies have shown that cancer cells can display up to eight-fold more anionic phospholipids on their membrane surface than normal cells (Conner *et al.*, 1989; Utsugi *et al.*, 1991). Furthermore, glycoproteins, such as *O*-glycosylated mucins, are overexpressed in many tumour types, such as breast and prostate carcinomas, which adds to the negative charge found on the tumour cell surface (Rakha *et al.*, 2005; Moniaux *et al.*, 2004). In addition to anionic phospholipids, the protein content and fluidity of many tumour cell membranes tend to be different from those of normal human cells (Matsuzaki, 1995), potentially increasing their susceptibility to CAPs. Non-malignant vertebrate cell membranes contain a high concentration of cholesterol, which protects cells from CAP-induced membrane destruction through an alteration in membrane

fluidity (Silvestro *et al.*, 1997). Moreover, tumour cells tend to express high levels of microvilli on their surface (Ren, 1991), which can increase the effective cell surface area, thus allowing for a greater degree of peptide binding.

Together, these differences between tumour and normal cell membrane composition, as well as differences in membrane fluidity may explain why, unlike chemotherapeutic drugs, the tumouricidal activity of CAPs is often selective, having no adverse effect on untransformed cells. CAPs are also superior anticancer agents due to their ability to kill cancer cells through membrane destabilization, which is independent of the proliferative state of the cell (Naumov *et al.*, 2003). Dormant or slowly proliferating cancer cells are therefore susceptible to the cytotoxic effects of CAPs.

Of interest, the common ancestry of prokaryotic cells and the mitochondria of eukaryotic cells has led researchers to hypothesize that CAPs, upon entering the cell, may target and lyse mitochondrial membranes (Gray *et al.*, 1999; Risso *et al.*, 2002). Both prokaryotic cells and mitochondrial membranes have large transmembrane potentials and are composed of a high proportion of anionic phospholipids, including diphosphatidyl-glycerol (diPG), which is not found in the plasma membrane of eukaryotic cells (Dekroon *et al.*, 1997; Matsuzaki, 1995). The ability of CAPs to lyse mitochondrial membranes may be another mechanism by which these peptides mediate cytotoxicity in eukaryotic cells.

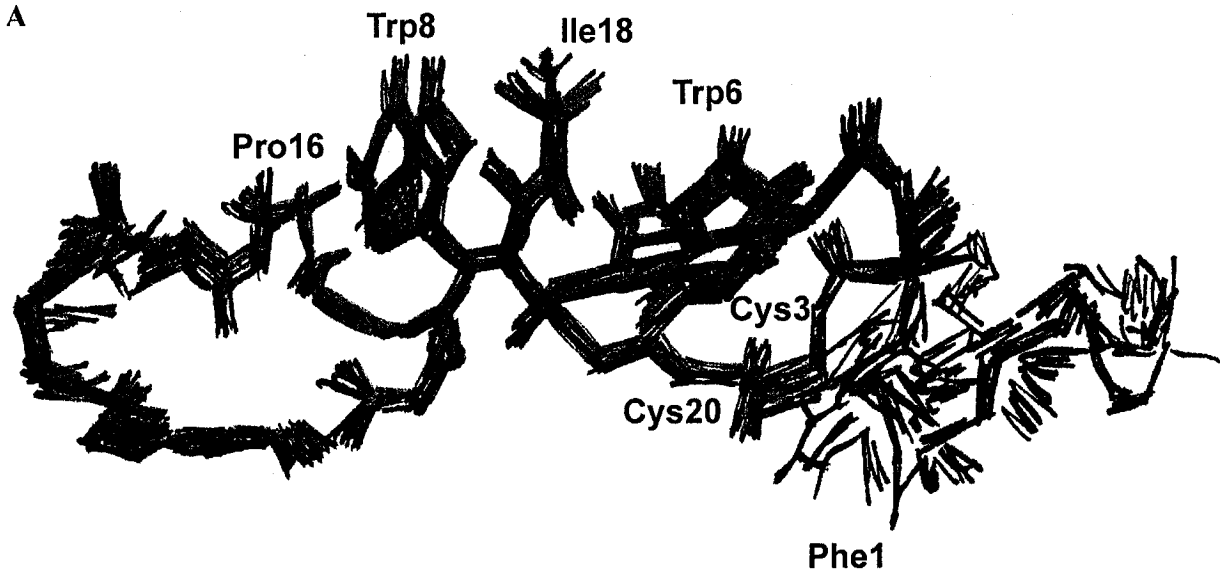
Bovine Lactoferricin

Bovine lactoferricin (LfcinB) is a 25 amino acid CAP produced by acid-pepsin hydrolysis of its parent protein, lactoferrin (Bellamy *et al.*, 1992; Hwang *et al.*, 1998).

Lactoferrin is an 80 kDa single-chain glycoprotein found in the secretory granules of neutrophils, and at significant levels in biological fluids that include saliva, and milk (Masson *et al.*, 1969; Arnold *et al.*, 1997; Gahr *et al.*, 1991; Brock, 2002). LfcinB, which has the amino acid sequence FKCRRWQWRMKKLGAPSITCVRRAF, assumes an α-helical conformation when contained within lactoferrin. However, upon enzymatic release from the parent protein, LfcinB acquires an amphipathic, antiparallel β-sheet structure (Hwang *et al.*, 1998; Yoo *et al.*, 1997a). This structural alteration may be responsible for the disparity observed between the cleavage peptide and the parent protein in terms of their cytotoxic activity (Yoo *et al.*, 1997a).

Antimicrobial Activity of Lactoferrin and Lfcin

Lactoferrin is a major component of antimicrobial host defense, because the protein inhibits the growth of a wide variety of microbes through its ability to bind and sequester iron, which is an essential nutrient for invading microorganisms, as well as through its ability to bind directly to the surface of various microbes thus leading to membrane destabilization and cell death by direct membrane damage independent of iron-binding capacity. (Baker *et al.*, 2002; Arnold *et al.*, 1997; Ward *et al.*, 2002). The majority of the antimicrobial activity of bovine milk-derived lactoferrin can be attributed to the N-terminal peptide fragment composed of amino acid residues 17-41, termed bovine lactoferricin (LfcinB) (Vorland *et al.*, 1999b; Bellamy *et al.*, 1992). The antimicrobial activity of LfcinB is much greater than that of an equimolar concentration of intact bovine lactoferrin, as Bellamy *et al.* (1992) revealed that LfcinB displayed a



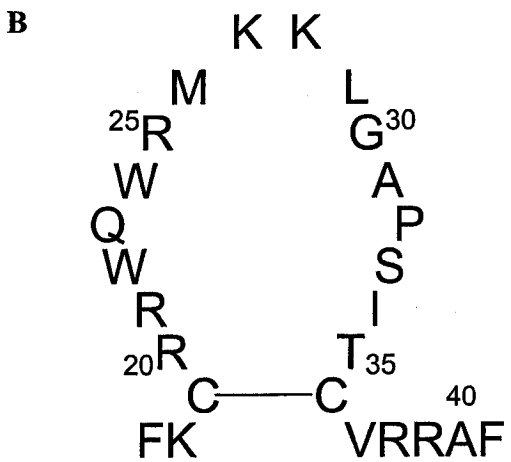


Figure 1.2 A. *Structure of LfcinB in aqueous solution.* The Trp, Phe, Ile, and Pro residues form the hydrophobic face of the amphipathic structure. The Lys and Arg sidechains on the opposite face of the structure (not shown) make up the positively charged surface. (*adapted from Hwang et al., 1998*). **B.** *Cyclic amino acid structure of LfcinB.* (*adapted from Bellamy et al., 1992*).

minimum inhibitory concentration in *Escherichia coli* up to 30 times less than that of the parent protein. LfcinB kills a variety of gram negative and gram positive bacteria (Tomita *et al.*, 1992; Yamauchi *et al.*, 1993), as well as displaying antiviral (Anderson 2001; Di Biase 2003; Anderson 2004), and antifungal activity (Wakabayashi *et al.*, 1996; Vorland *et al.*, 1999b). LfcinB does not contain iron-binding tyrosine or histidine residues, and therefore does not have the ability to bind and sequester iron (Bellamy *et al.*, 1992). Rather, LfcinB is believed to interact with, and damage, microbial membranes leading to cytoplasmic membrane depolarization, loss of pH gradient, and cell death (Wakabayashi *et al.*, 1996; Ulvatne *et al.*, 2001).

The interaction between LfcinB and the microbial membrane is not well defined. However, the distinct cationic, amphipathic, β-sheet structure of LfcinB, which contains multiple tryptophan (Trp) residues and displays a high proportion of asymmetrically clustered basic amino acid residues is believed to be integral for its cytotoxic activity (Schibli *et al.*, 2002). When *Escherichia coli* are exposed to LfcinB, the peptide is thought to initially interact with negatively-charged lipopolysaccharide (LPS) on the *Escherichia coli* surface, which leads to the disorganization of the outer membrane structure and facilitates the interaction between the amphipathic, Trp-containing region of LfcinB and the hydrophobic portion of the membrane bilayer, which destabilizes the packing of membrane phospholipids (Farnaud *et al.*, 2004). The two Trp residues contained within LfcinB have individually been shown to be essential for the antimicrobial activity of a 15 amino acid fragment of LfcinB in that substitution with Ala residues dramatically decreases the antimicrobial potency of the peptide (Strøm *et al.*, 2002). Human Lfcin, which contains only one Trp residue, does not have equivalent

antimicrobial or anticancer activity compared to LfcinB (Haug *et al.*, 2001). Of interest, recent studies have shown that neither the hydrogen-bonding activity nor the amphipathicity are necessary for the effect seen with Trp. Instead, the size, shape, and aromatic character of Trp are the most important features for its activity (Haug *et al.*, 2001). The requirement of both Arg and Trp residues in the antibacterial function of LfcinB highlights the importance of both electrostatic and hydrophobic interactions. A similar clustering of basic amino acids has been observed in other CAPs that have a high affinity for biological membranes, including magainins (Zasloff, 2002), defensins (Marshall 2003), and cecropins (Van Hofsten *et al.*, 1985; Moore *et al.*, 1994; Chen *et al.*, 1997). These cationic peptides all kill sensitive microorganisms by disrupting membrane integrity.

Researchers have recently determined that LfcinB can cross the cytoplasmic membrane of microbes such as *Candida albicans* and *Escherichia coli* to reside in the cytosol, suggesting the barrel-stave model of membrane interaction (Haukland *et al.*, 2001; Van Der Kraan *et al.*, 2005). Experiments performed with the N-terminal portion of human Lfcin have shown that, in the presence of LPS, the peptide can self-associate in an ordered manner forming an array that disrupts the bacterial outer membrane, resulting in the translocation of Lfcin peptides across the cytoplasmic membrane (Chapple *et al.*, 2004). In contrast, another group has shown that LfcinB does not damage the integrity of the cytoplasmic membrane in bacteria, but does cause the loss of transmembrane electrochemical and pH gradients (Aguilera *et al.*, 1999).

Short peptide fragments isolated from LfcinB are believed to contain the required antimicrobial residues of the 25 amino acid peptide. Tomita *et al.* (1994) determined that

a 6 amino acid peptide fragment of LfcinB, RRWQWR-NH_2, has equivalent antimicrobial activity to that of full length LfcinB, and is thus termed the antimicrobial centre. Consistent with this observation, Kang *et al.* (1996) found that the 11 amino acid fragment, RRWQWRMKKLG, has microbicidal action equivalent to full length LfcinB, and that the antimicrobial activity of the peptide is dependent on both the hydrophobic and basic residues. Both the 6-mer and the 11-mer form of LfcinB have the same minimum inhibitory concentration (MIC) as that of the full length peptide when treating *Escherichia coli* and *Staphylococcus aureus* (Tomita *et al.*, 1994; Kang *et al.*, 1996), which is believed to be due to membrane interactions orchestrated by the amphipathic tryptophan and basic arginine residues (Schibli *et al.*, 1999).

Bovine lactoferrin and LfcinB are recognized as a potent inhibitors of virus infectivity, including human cytomegalovirus (Andersen *et al.*, 2001), herpes simplex viruses (Marchetti *et al.*, 1998), and adenovirus (Di Biase *et al.*, 2003). Di Biase *et al.* (2003) demonstrated that the N-terminal portion of bovine lactoferrin is responsible for its antiadenoviral activity, inhibiting viral attachment to HEp-2 cells by competition for cell-surface glycosaminoglycans (GAG), including heparin and heparan sulfate. GAGs are long, polyanionic carbohydrate chains consisting of repeating disaccharide units containing sulfate residues that are termed proteoglycans when they are covalently linked to a protein core. Proteoglycans are important in cell attachment, proliferation, migration, and receptor-mediated endocytosis (Ho *et al.*, 1997). More recent reports demonstrated that LfcinB does indeed bind to cell-surface GAGs, including heparin and heparan sulfate (Shimazaki *et al.*, 1998; Andersen *et al.*, 2001), and that this binding is necessary to inhibit viral infection (Andersen *et al.*, 2004).

In addition to its effects at the microbial membrane, LfcinB may exert antimicrobial activity by interacting with intracellular molecules (Haukland *et al.*, 2001). Bovine lactoferrin is a highly cationic molecule, displaying a pI of approximately 9, which allows lactoferrin to bind anionic molecules such as nucleic acids and heparin (Baker *et al.*, 1994; van Berkel *et al.*, 1997). Lactoferrin is spontaneously internalized in K562 human myelogenous leukemia cells where it can interact directly with nuclear DNA (He and Furmanski, 1995). Of interest, the DNA-binding properties of bovine lactoferrin have been shown to reside within the N-terminal region containing LfcinB (Kanyshkova *et al.*, 1999). Ulvatne *et al.* (2004) determined that treatment of *Bacillus subtilis* with sublethal concentrations of LfcinB leads to inhibition of DNA, RNA and protein synthesis. However, it is not known whether LfcinB enters human tumour cell lines, or whether this peptide interacts with eukaryotic nucleic acids to inhibit protein synthesis. The ability to enter microbial cells and inhibit protein synthesis is seen in other CAPs, such as indolicidin, a 13-residue CAP isolated from the cytoplasmic granules of bovine neutrophils (Subbalakshmi and Sitaram, 1998). Magainin 2 is also known to target intracellular compounds such as nucleic acids, shutting down RNA and protein synthesis which leads to cell death (Park *et al.*, 1998).

Cellular Effects and Immunomodulatory Functions of Lactoferrin and LfcinB

Lactoferrin is believed to play an important role in the regulation of cell growth and differentiation (Kanyshkova *et al.*, 2001). Lactoferrin has been reported to activate natural killer (NK) cells and lymphokine activated killer (LAK) cells *in vitro* and *in vivo* (Mantel *et al.*, 1994; Shau *et al.*, 1992; Iigo *et al.*, 1999), which may play a role in

antitumour defense (Horwitz *et al.*, 1984; Garre *et al.*, 1992). Several studies determined that lactoferrin can also cause macrophage activation, and induce the production of CXCL8, and tumour necrosis factor (TNF) (Sorimachi *et al.*, 1997). This activity was shown to be independent of the iron-binding ability of lactoferrin, which suggests that it may be related to the LfcinB fragment of lactoferrin. The immunomodulatory function of lactoferrin may be a result of lactoferrin binding to specific DNA sequences, and regulating transcription of genes bearing these sequences in their promoter region (He and Furmanski, 1995). Additionally, lactoferrin induces rapid proliferation and differentiation of crypt enterocytes in the human intestine (Nichols *et al.*, 1990), potentially due to interactions with lactoferrin receptors on adult and fetal duodenal brush-border membranes (Cox *et al.*, 1979; Kawakami and Lonnerdal, 1991). Lactoferrin can directly regulate the inflammatory response by binding bacterial endotoxin, LPS, which is a major inflammatory response mediator during bacterial infections (Miyazawa *et al.*, 1991). The ability of neutrophils to inactivate LPS, thus limiting inflammation and tissue destruction, is believed to be directly related to lactoferrin, which is secreted from neutrophil granules during the inflammatory response (Wang *et al.*, 1995). The high-affinity LPS binding site is found within the N-terminal sequence of bovine lactoferrin (Elas-Rochard *et al.*, 1995), which suggests that LfcinB may have equivalent LPS-binding activity to that of the intact parent protein. Lactoferrin may also downregulate LPS-induced cytokine expression by entering inflammatory cells. In this regard, lactoferrin has been shown to enter THP-1 human monocytic leukemia cells, localize to the nucleus, and bind to the important cytokine transcription factor NF-κB (Haversen *et al.*, 2002). Other studies have shown that lactoferrin can influence cutaneous immune and

inflammatory processes by inhibiting allergen-induced Langerhan cell migration (Griffiths *et al.*, 2001; Kimber *et al.*, 2002). Additionally, bovine lactoferrin and LfcinB bind to prokaryotic CpG-containing oligodeoxynucleotides through a charge-charge interaction, thus inhibiting CpG immunostimulatory effects on human B cells (Britigan *et al.*, 2001). By binding to, and in turn neutralizing these molecules, LfcinB prevents the activation of mononuclear cells, and the subsequent production of proinflammatory cytokines, such as TNF and IL-1β. LfcinB may bind to extracellular cytokines, such as IL-6, further inhibiting the inflammatory response (Mattsby-Baltzer *et al.*, 1996). Finally, bovine lactoferrin and pepsin-generated LfcinB are known to enhance the phagocytic activity of human neutrophils; however, this enhancing activity may be due to an opsonin-like mechanism in which bovine lactoferrin and LfcinB bind to bacterial membranes to increase bacteria-neutrophil interactions (Miyauchi *et al.*, 1998).

Anticancer Activity of Lactoferrin and LfcinB

In addition to their microbicidal activities, both bovine lactoferrin and LfcinB are known to have *in vitro* and *in vivo* anticancer activity. One study determined that bovine lactoferrin causes a reduction in methylcholanthrene-induced murine fibrosarcoma growth, as well as experimental lung metastasis by B16-F10 murine melanoma cells in syngeneic mice through a mechanism that is independent of its iron-binding activity (Bezault *et al.*, 1994). Iigo *et al.* (1999) have demonstrated that orally administered bovine lactoferrin can inhibit colon carcinoma 26 lung metastasis in mice, while Tsuda *et al.* (1998) found that dietary bovine lactoferrin prevents aberrant crypt foci and colon carcinoma development in rats pretreated with carcinogenic azoxymethane. This activity

may be due, at least in part, to the ability of lactoferrin to stimulate NK cells (Iigo *et al.*, 1999). Of interest, oral administration of bovine lactoferrin inhibits vascular endothelial cell growth factor $(VEGF)_{165}$-induced angiogenesis in mesenteric window assays in rats, possibly as a result of the generation of peptide fragments, including LfcinB (Norrby *et al.*, 2001). Shimamura *et al.* (2004) found that oral or intraperitoneal (i.p.) administration of bovine lactoferrin inhibits tumour-induced angiogenesis *in vivo* in the dorsal air sac and chorioallantoic membrane assays, as well as basic fibroblast growth factor (bFGF)- and VEGF-induced endothelial cell proliferation *in vitro*.

LfcinB is cytotoxic for a diverse range of murine and human tumour cells, exhibiting potent *in vitro* and *in vivo* anticancer activity (Yoo *et al.*, 1997a and b; Iigo *et al.*, 1999; Roy *et al.*, 2002; Eliassen *et al.*, 2002; Mader *et al.*, 2005). LfcinB is a potent inducer of apoptosis in cultures of THP-1 human monocytic leukemia cells (Yoo *et al.*, 1997b). In contrast, intact bovine lactoferrin is unable to trigger cell death in THP-1 leukemia cells, even at 10-fold higher concentrations than LfcinB, indicating that apoptosis-inducing activity is unique to LfcinB. LfcinB is also an effective inducer of apoptosis in a broader range of human cancer cells, including more common cancers that are of epithelial origin (Mader *et al.*, 2005). However, LfcinB does not affect the viability of untransformed human cells (Mader *et al.*, 2005; Furlong *et al.*, 2006). Studies indicate that LfcinB also possesses potent *in vivo* activity against cancer cells (Yoo *et al.*, 1997a; Tsuda *et al.*, 1998). Yoo *et al.* (1997a) found that subcutaneous (s.c.) and i.p. administration of LfcinB to mice 1 day post intravenous tumour inoculation leads to a significant inhibition of L5178Y-ML25 murine lymphoma lung and liver metastasis, as well as B16-BL6 murine melanoma lung metastasis. In the same study, it was

demonstrated that LfcinB has an inhibitory effect on both tumour growth and tumour-induced angiogenesis in mice inoculated intradermally with B16-BL6 melanoma cells (Yoo *et al.*, 1997a). In addition, intratumoural injection of LfcinB inhibits the growth of murine Meth A fibrosarcoma cells grown as subcutaneous tumours in mice by a direct cytocidal mechanism resulting in extensive hemorrhagic necrosis of tumours as early as 1 day post LfcinB administration (Eliassen *et al.*, 2002). This activity is selective for tumour cells, since there is no cytotoxic effect on normal murine fibroblasts or erythrocytes. Moreover, oral administration of LfcinB to rats that had previously been injected with azoxymethane to promote colon carcinogenesis results in an impressive 83% reduction in the incidence of colon adenocarcinomas (Tsuda *et al.*, 1998). A similar effect was achieved by oral administration of intact bovine lactoferrin, which raises the intriguing possibility that LfcinB derived from dietary bovine lactoferrin may protect against colon carcinogenesis. Of interest, the consumption of milk and milk products has recently been linked to a reduced risk of colorectal cancer in humans (Cho *et al.*, 2004), as well as reduced tumour growth in mice treated with 1, 2-dimethylhydrazine to induce colon cancer (Perdigon *et al.*, 2002). While remarkably little is known about the mechanism(s) by which LfcinB exerts this activity, the available evidence favors a direct inhibitory effect of LfcinB on cancer cell growth and metastasis.

Mechanisms of Cell Death

Apoptosis is a distinct form of physiological cell death that occurs during tissue remodeling, embryonic development, cellular homeostasis, and tumour regression (Krammer, 1999; Kaufmann and Hengartner, 2001). Apoptosis can also result from non-

physiological exposure to ultraviolet radiation, and many chemotherapeutic drugs, as well as oligomerization of cell surface death receptors (Green and Reed, 1998; Ashkenazi, 2002). Apoptotic cells are characterized by number of specific morphological features, including cell shrinkage, DNA fragmentation, chromatin condensation, membrane blebbing, and cell surface expression of phosphatidylserine (PS) (Kerr *et al.*, 1972). In contrast, necrosis, which can occur as a result of acute trauma or injury to the cell, is characterized by cellular swelling, plasma membrane lysis, and release of intracellular contents into the extracellular environment (Kerr *et al.*, 1972; Nieminen *et al.*, 1990; Grooten *et al.*, 1993). Unlike apoptosis, in which membrane bound vesicles termed apoptotic bodies are taken up by professional phagocytic cells, necrosis is followed by activation of inflammatory processes which can injure or kill surrounding cells (Kerr *et al.*, 1972).

Pathways of Apoptosis

Background

The two main pathways that mediate the activation and subsequent execution of apoptosis are the extrinsic/death receptor pathway, and the intrinsic/mitochondria-dependent pathway. The extrinsic pathway is activated by ligand-induced aggregation of cell surface death receptors, such as Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 (Ashkenazi, 2002). The intrinsic pathway is triggered by radiation or cytotoxic drug-induced cellular stress (Green and Reed, 1998).

Caspase Proteins

A group of aspartate-specific cysteine proteases, known as the caspases, have important roles in the initiation and execution of both the extrinsic and intrinsic pathways of apoptosis (Earnshaw *et al.*, 1999). Caspase family members are synthesized as inactive proenzymes that are activated by enzymatic cleavage after aspartic acid residues (Alnemri *et al.*, 1996). The active enzyme is a heterodimeric complex composed of two large and two small subunits (Watt *et al.*, 1999; Blanchard *et al.*, 1999). These proteins can be divided into initiator and executioner caspases based on the presence of a large N-terminal prodomain (Salvesen *et al.*, 1999). The prodomain of initiator caspases interacts with specific adaptor molecules, which bring multiple proenzymes into close proximity, permitting autoprocessing (Fesik and Shi, 2001). Caspase-8 and caspase-10 are initiator caspases for the death receptor pathway of apoptosis (Juo *et al.*, 1998; Wang *et al.*, 2001), while caspase-2 and caspase-9 are initiator caspases for the mitochondria-dependent pathway of apoptosis (Read *et al.*, 2002; Slee *et al.*, 1999; Fesik and Shi, 2001). Activation of initiator caspases leads to the subsequent activation of executioner caspases, including caspase-3, caspase-6, and caspase-7 (Slee *et al.*, 2001). Upon activation, executioner caspases trigger the proteolysis of cytoplasmic and nuclear polypeptide substrates, including DNA repair proteins, and cytoplasmic and nuclear structural proteins, which ultimately results in cell death (Earnshaw *et al.*, 1999).

Cells also contain natural caspase inhibitors. These inhibitor of apoptosis proteins (IAPs), including X-linked inhibitor-of-apoptosis protein (XIAP), cIAP-1, and cIAP-2 block apoptosis by inactivating caspases (Verhagen *et al.*, 2002). IAPs can directly inhibit caspases, as well as target caspase family members for degradation through the

ubiquitin-proteosome pathway (Yang *et al.*, 2000). XIAP has been shown to interact with oligomerized apoptotic protease activating factor-1 (APAF-1) and/or processed caspase-9, thus influencing the activity of caspase-3, as well as binding caspase-3 in the apoptosome complex inhibiting its release (Bratton *et al.*, 2002). Cells can overcome the inhibition by IAPs through a number of mechanisms.

In cells undergoing apoptosis, caspases can be liberated from IAP blockade by binding to second mitochondria-derived activator of caspase/direct IAP-binding protein with a low isoelectric point (Smac/DIABLO). Smac/DIABLO is a protein, much like apoptosis inducing factor (AIF) and cytochrome c, that normally localizes to the intermembrane space of the mitochondria, but can be released into the cytosol during apoptosis (Hegde *et al.*, 2002). Once in the cytosol, Smac/DIABLO binds to, and prevents IAPs interaction with active caspases, thus promoting cell death (Schimmer, 2004). Similarly, the serine protease, high temperature requirement A2 (HtrA2)/Omi, also blocks the caspase-inhibitory action of IAPs upon release from the mitochondria (van Gurp *et al.*, 2003). Of interest, deletion of the amino-terminal IAP-binding motif in Omi does not rid this protein of its apoptosis-promoting ability, which suggests that Omi has additional effects on the process of apoptosis (Suzuki *et al.*, 2001; Hegde *et al.*, 2002). The bacterial Omi homologue, HtrA acts as a molecular chaperone at low temperatures and as a protease that degrades misfolded proteins at high temperatures. Therefore, it is possible that the Omi may also have a dual role as mitochondrial chaperone and proapoptotic serine protease, which may trigger apoptosis in a caspase-independent manner (Suzuki *et al.*, 2001; Verhagen *et al.*, 2002).

Mitochondria-Dependent Pathway

Mitochondria are key regulators of apoptosis following alterations in the mitochondrial transmembrane potential, which results in mitochondrial membrane permeability, and reactive oxygen species (ROS) production (Gottlieb, 2003). Dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) is an early event in apoptosis, which can be triggered by a number of stimuli, such as calcium (Ca^{2+}) and ROS, as well as by anticancer drugs and irradiation (Kim *et al.*, 2003; Hajnoczky *et al.*, 2003; Roberts *et al.*, 2003). Mitochondrial membrane permeability is believed to be a necessary step in the intrinsic pathway of apoptosis (Deshmukh *et al.*, 2000; Kroemer and Reed, 2000). Designated "the-point-of-no-return" by many researchers, mitochondrial membrane permeability appears to be virtually synonymous with cell death (Kroemer and Reed, 2000). Mitochondrial membrane permeability culminates in the complete loss of outer mitochondrial membrane barrier function, leading to the subsequent release of apoptosis-inducing proteins, such as AIF and cytochrome c from the intermembrane space of the mitochondria into the cell cytosol. Cytochrome c is a globular protein that is found in the intermembrane space of the mitochondria under normal conditions, where it serves as an electron shuttle between complex III and IV of the mitochondrial respiratory chain (Dudkina *et al.*, 2005). During cell death, cytochrome c can be released from the mitochondria into the cytosol due to mitochondrial membrane permeability. Upon entering the cytosol, the oligomerized cytosolic adaptor protein, APAF-1, together with cytochrome c and ATP/dATP recruit procaspase-9 to the apoptosome complex (Cain *et al.*, 2000). Apoptosome formation triggers the cleavage of inactive procaspase-9 into its active form (Bratton *et al.*, 2002). Upon activation, caspase-9 subsequently activates

downstream executioner caspases, such as caspase-3. Caspase-3 cleaves substrates, including Poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase-activated DNase (ICAD), leading to oligonucleosomal DNA fragmentation and apoptosis (Riedl and Shi, 2004).

Outer membrane permeability, and the release of proapoptotic proteins such as cytochrome c, and AIF from the intermembrane space of the mitochondria, is controlled by pro- and antiapoptotic members of the Bcl-2 protein family (Gross *et al.*, 1999; Kroemer and Reed, 2000; Zamzami and Kroemer, 2001). More than 20 members of the Bcl-2 family have been identified to date, including proteins that inhibit apoptosis, such as Bcl-2 and Bcl-x_L, and proteins that promote apoptosis, including Bax, Bak, and Bid (Wei *et al.*, 2001). Antiapoptotic Bcl-2 family members are found predominantly on the outer mitochondrial membrane, and these family members are also constitutively expressed at contact sites where the inner and outer membranes fuse. Antiapoptotic Bcl-2 proteins maintain mitochondrial membrane barrier function by preventing permeabilization of mitochondrial membranes, while simultaneously maintaining the exchange of small molecules, such as ADP and ATP, across the membranes (Zamzami and Kroemer, 2001). Antiapoptotic Bcl-2 proteins inhibit apoptosis by locally antagonizing proapoptotic Bcl-2 family members (Letai *et al.*, 2002). Many of the proapoptotic Bcl-2 proteins are found in the cytosol of the cell under normal conditions, and translocate to the mitochondria during apoptosis (Gross *et al.*, 1998). Alternatively, certain proapoptotic Bcl-2 family members reside in the outer membrane of the mitochondria, but undergo conformational changes during apoptosis to induce mitochondrial membrane permeability (Desagher *et al.*, 1999).

Role of Bcl-2 Family Members in Apoptosis

Deciphering the mechanism(s) by which Bcl-2 family members exert their biological activity is a very active area of apoptosis research. Although the exact mechanism by which Bcl-2 family members regulate apoptosis remains controversial, one belief is that opposing Bcl-2 proteins interact with each other and/or with proteins localized in the mitochondrial membranes to affect membrane barrier function. Some studies have shown that various pro- and antiapoptotic proteins of the Bcl-2 family antagonize one another through direct interaction (Kroemer and Reed, 2000). Other studies suggest that Bcl-2 family members interact with proteins that make up the permeability transition (PT) pores that span the inner and outer mitochondrial membranes (Crompton, 1999). Mitochondrial membranes can become permeabilized through the opening of PT pores, which are comprised of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the adenine nucleotide traslocator (ANT) located in the inner membrane (Zamzami and Kroemer, 2001). Under normal conditions, the VDAC coordinates the release of ATP from the mitochondria, while the ANT exchanges ATP for ADP across the inner membrane (Liu *et al.*, 1992). Damage of VDAC led to an increase in outer membrane permeability and the release of cytochrome c (Pastorino *et al.*, 1999), while disruptions in ANT create a conformational change leading to the formation of a membrane permeable pore in the inner membrane, resulting in $\Delta\Psi_m$ dissipation, and mitochondrial matrix swelling (Brenner *et al.*, 2000). Sustained opening of the PT pores allows for the dissipation of $\Delta\Psi_m$ and consequent rupture of the outer membrane (Pastorino *et al.*, 1999). Bax has been shown to bind to the PT pore complex where it interacts with ANT and VDAC to increase mitochondrial membrane

permeability and trigger cell death by forming a larger channel than either protein alone (Marzo *et al.*, 1998; Shimizu *et al.*, 1999). In contrast, the antiapoptotic protein, Bcl-x_L can bind to and close PT channels, inhibiting the release of cytochrome c from the mitochondria (Shimizu *et al.*, 1999). Overexpression of Bcl-2 and Bcl-x_L inhibits both $\Delta\Psi_m$ and mitochondrial membrane permeability, thereby preventing cell death (Gabriel *et al.*, 2003). Other proapoptotic Bcl-2 family members may not require VDAC opening to have proapoptotic effects. The proapoptotic BH3-only protein, Bid can mediate mitochondrial membrane permeability in a process requiring either Bax or Bak, and not the PT pore complex (Wei *et al.*, 2001; Tsujimoto and Shimizu, 2000). Through the use of inactivating antibodies targeting VDACs, Tsujimoto and Shimizu (2000) have shown that while proapoptotic family members such as Bax and Bak require VDAC interaction and opening to lead to apoptosis, BH3 only proteins, such as Bid and Bik, do not directly target the VDAC to induce cytochrome c release, therefore not requiring functional VDACs. In contrast, other researchers have reported that Bax and Bak can oligomerize to form large, ion-conducting channels in lipid bilayers, inducing cytochrome c release from isolated mitochondria without the need for other membrane proteins (Antonsson *et al.*, 2000). Alternatively, proapoptotic proteins may be released due to physical rupture of swollen mitochondria independent of Bcl-2 family members (Vander Heiden *et al.*, 1997).

Death Receptors

Members of the death receptor family, which includes Fas, and TRAIL DR4 and DR5, are part of the TNF/nerve growth factor receptor superfamily that is involved in cell

death and survival (Ashkenazi, 2002). Ligand-induced receptor crosslinking leads to the recruitment of specific proteins that form the death inducing signaling complex (DISC) (Kischkel *et al.*, 1995). The DISC associated with the Fas receptor, which has been characterized most extensively, consists of oligomerized Fas, the serine-phosphorylated adapter Fas-associated death domain protein (FADD), and procaspase-8 or -10 (Kaufmann *et al.*, 2002). FADD binds to procaspase-8 and -10 through its N-terminal death effector domain (DED), leading to the cleavage of procaspase-8 or -10 into its active form (Medema *et al.*, 1997). Active caspase-8 and -10 then dissociate from the DISC to begin the cascade of caspase activation events during the execution phase of apoptosis. Caspase activation in the DISC, and the resultant death-receptor-mediated apoptosis, can be inhibited by a cellular protein called cellular FLICE-inhibitory protein (cFLIP) (Krueger *et al.*, 2001). cFLIP is similar in structure to caspase-8, containing multiple DEDs, but lacking the active proteinase site (Irmler *et al.*, 1997). Thus, cFLIP cannot convey a death signal, even though it is recruited to the DISC.

Mitogen Activated Protein Kinase Pathways and Ceramide Signaling

The pathways that connect the induction of apoptosis by exogenous cellular stressors to mitochondrial membrane disruption are complex. The mitogen-activated protein kinase (MAPK) signaling pathway includes the subfamilies of extracellular signal-regulated kinase (ERK), jun N-terminal kinase (JNK), and p38 MAPK. MAPK members belong to a large family of serine-threonine kinases that can lead to altered gene expression in the regulation of cell death and survival, and are regulated by distinct stimuli (Dong *et al.*, 2002; Hommes *et al.*, 2003). ERK can become activated by ligation

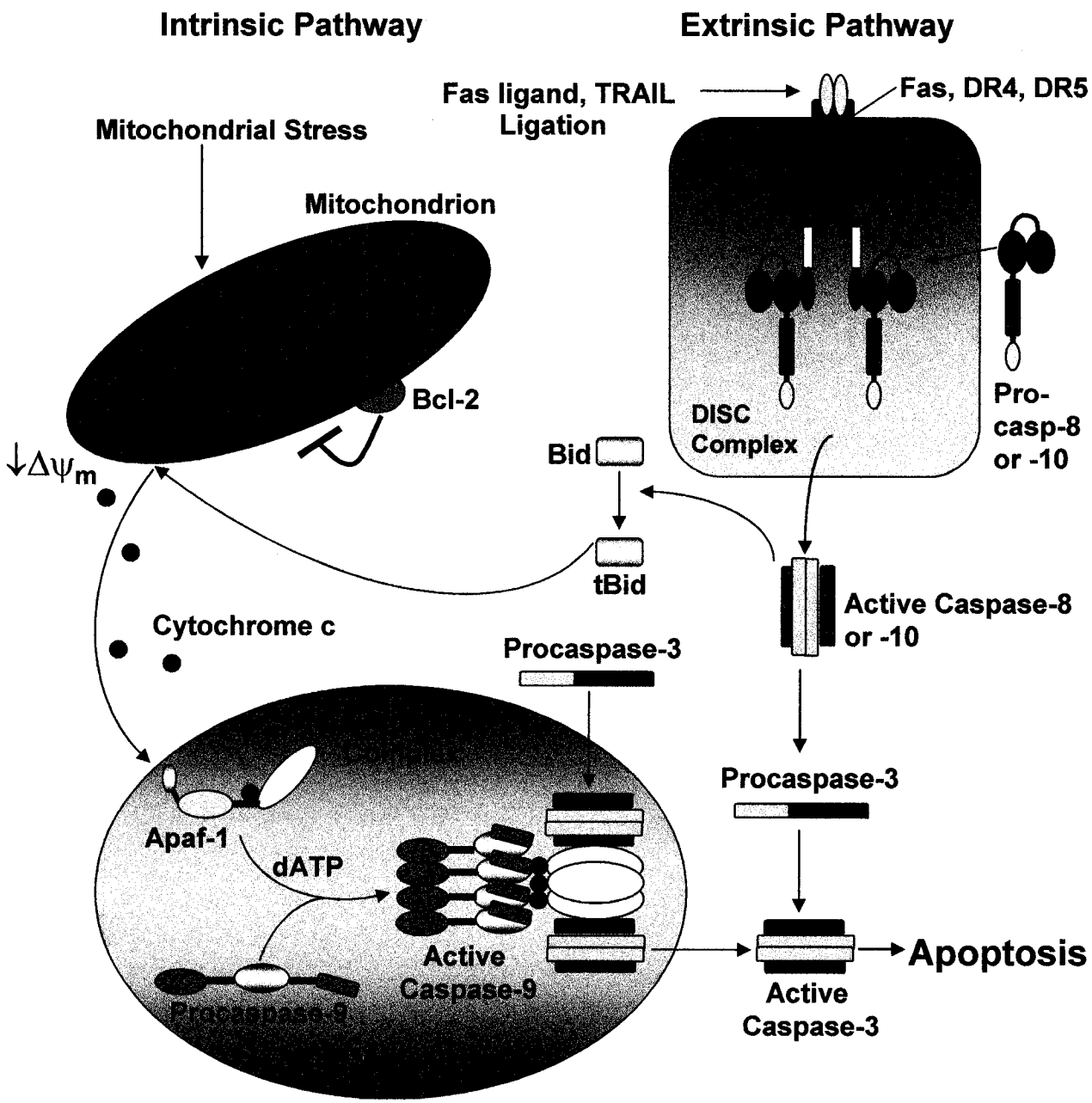


Figure 1.3 *Schematic representation of the pathways leading to apoptosis.* The extrinsic pathway becomes activated upon ligand-induced aggregation of cell surface death receptors, while the intrinsic pathway can be activated by a variety of stress-inducing stimuli. Both pathways can activate initiator caspase members, which subsequently trigger downstream executioner caspase activation, leading to the cleavage of important cellular substrates. The result is the classical biochemical and morphological changes associated with the apoptotic phenotype.

of receptor tyrosine kinase members to provide proliferation and differentiation signals to the cell (Troppmair *et al.*, 1994), while JNK and p38 MAPK can be activated by various stress stimuli, and have been implicated in cell proliferation, and stress-induced apoptosis depending on the cell type, stimuli, and context of other signals received by the cell (Ip and Davis, 1998; Xia *et al.*, 1995). JNK may contribute to transcription (Tc)-dependent death-receptor-mediated cell death by induction of Fas ligand and p53, and Tc-independent apoptosis via inactivation of antiapoptotic Bcl-2 family members and stabilization of p53 (Kharbanda *et al.*, 2000; Buschmann *et al.*, 2001). Additionally, JNK can activate proapoptotic Bcl-2 family members, such as Bid, resulting in mitochondrial membrane permeability and cytochrome c release (Deng *et al.*, 2003).

Ceramide is a membrane sphingolipid that is synthesized *de novo*, or generated through the catabolism of sphingomyelin, in response to various apoptosis-inducing stimuli (Bleicher and Cabot, 2002). These stimuli include multiple cellular stressors, such as exposure to chemotherapeutic agents and ionizing radiation, as well as signaling through the Fas receptor (Mathias *et al.*, 1998; Kolesnick and Kronke, 1998). Following conversion to ganglioside GD3 in the Golgi apparatus, ceramide can stimulate apoptosis by activating the JNK pathway (Verheij *et al.*, 1996). In addition, ceramide can directly induce mitochondrial membrane permeabilization, leading to cytochrome c release, resulting in downstream caspase activation and apoptosis (De Maria *et al.*, 1997; Scorrano *et al.*, 1999; Garcia-Ruiz *et al.*, 2000). Ceramide has been shown to form large protein permeable channels in planar phospholipid and mitochondrial outer membranes (Novgorodov *et al.*, 2005). Therefore, ceramide channel formation is a possible

mechanism for the release of proapoptotic proteins from mitochondria during the induction phase of apoptosis.

Reactive Oxygen Species

ROS are a group of molecules that are constantly produced under normal conditions as a byproduct of aerobic metabolism (Nohl and Jordan, 1980; England and Cotter, 2005). ROS include the superoxide anion, hydroxyl radical, and hydrogen peroxide. ROS display high reactivity, and thereby threaten the integrity of redox-sensitive cellular components, including lipids, proteins, and nucleic acids (Nohl and Jordan, 1980). Antioxidant systems, including reduced glutathione (GSH) and thirodoxin, exist to protect cellular components from excess ROS (McEligot *et al.*, 2005). Oxidative stress created by an imbalance between ROS and cellular antioxidants has been implicated in the induction of both death receptor- and mitochondria-dependent apoptosis (Susuki *et al.*, 1998; Zamzami *et al.*, 1995). Mitochondria are a major endogenous source of ROS, since electrons can leak out of the electron transport chain and pass to oxygen, thus generating superoxide anion (Tan *et al.*, 1998). Changes in cellular redox state due to an increase in ROS generation, or depletion in reduced GSH can lead to the opening of PT pores (Zoratti and Szabo, 1995). The ANTs that make up the PT pores express a thiol group that, when oxidized, will convert ANT into a non-specific pore (Costantini *et al.*, 2000). Interestingly, ROS are involved in LfcinB-induced apoptosis of THP-1 leukemia cells (Yoo *et al.*, 1997b), although it is not known whether the production of ROS is a proximal or distal event in the apoptotic process. ROS not only induces PT pore opening, but also can cause lipid peroxidation, and DNA cleavage (Huang *et al.*, 1999).

Caspase-Independent Apoptosis

Caspase-mediated apoptosis is the principal pathway of apoptotic cell death in many physiological situations. However, caspase-independent death processes also exist in apoptotic cells (Lockshin and Zakeri, 2002). As previously mentioned, when outer membrane barrier function becomes jeopardized, there is a release of proapoptotic factors such as cytochrome c and AIF into the cell cytosol (Susin *et al.*, 1999; Liu *et al.*, 1996). AIF is a flavoprotein that is ubiquitously expressed in both normal tissues and cancer cell lines, where it is synthesized in the cytosol and then transported into the intermembrane space of the mitochondria where the mature protein resides (Kroemer and Reed, 2000). Like cytochrome c, AIF can be released from the mitochondria into the cytosol of the cell during apoptosis. Upon its release, AIF is transported to the nucleus where it induces nuclear chromatin condensation and large scale DNA fragmentation (~50 base pair fragments) (Susin *et al.*, 1999; Cande *et al.*, 2002). Overexpression of the antiapoptotic protein Bcl-2 blocks the translocation of AIF from the mitochondria to the nucleus in mammalian cells (Daugas *et al.*, 2000), which suggests that AIF is released from the mitochondria following mitochondrial membrane permeability.

Endonuclease G (endoG) is a mitochondrial nuclease that is also released from the intermembrane space of the mitochondria during apoptosis (Li *et al.*, 2001). Similar to AIF, endoG translocates to the nucleus following its release, where it generates oligonucleosomal DNA fragments independent of caspase activation (Parrish *et al.*, 2001; Li *et al.*, 2001). AIF and endoG appear to work in parallel with cytochrome c-mediated caspase activation during apoptosis.

Angiogenesis

Background

One promising new anticancer strategy is not aimed at the tumour cells directly, but rather at the formation of the tumour-associated vascular network. Angiogenesis is a complex process whereby new blood vessels develop from pre-existing parental vessels (Folkman, 1995) in response to tumour and host-derived signals (Satchi-Fainaro *et al.*, 2004). Angiogenesis plays an essential role in physiological processes such as embryonic development, wound healing, and the normal menstrual cycle (Reynolds *et al.*, 1992; Folkman, 1995; Klagsbrun and D'Amore, 1991). However, angiogenesis is also an important pathogenic process in several disease states, such as arthritis (Colville-Nash and Scott, 1992), diabetic retinopathy (Sharp, 1995), and tumour growth (Folkman, 1971). It is a multistep process that includes controlled proteolytic degradation of the extracellular matrix (ECM), proliferation and migration of endothelial cells, and formation of capillary vessel lumens. Angiogenesis is also believed to play a role in tumour cell migration and metastasis by allowing tumour cells to enter into systemic circulation (Gershenwald and Fidler, 2002; Satchi-Fainaro *et al.*, 2004).

Under normal physiological conditions, angiogenesis is a highly ordered process that is under tight regulation by an intricate network of opposing signals (Carmeliet and Jain, 2000). Angiogenesis is essential for tumour growth beyond 1-2 mm in diameter due to the tumour's requirement for a network of blood vessels to deliver oxygen and nutrients, and remove metabolic waste products (Parangi *et al.*, 1996; Folkman, 1992). During tumour-associated angiogenesis, the angiogenic switch occurs when the balance between angiogenic stimulators and inhibitors is shifted in favor of angiogenesis by

multiple factors including genetic mutations (Carmeliet, 1999), and low pO_2 resulting in hypoxia-inducible factor-1 gene expression (Maxwell *et al.*, 1997). As a result, tumour cells, as well as host cells that are recruited to the tumour site (e.g., macrophages), produce a microenvironment that is rich in proangiogenic factors (Folkman, 1992). Following stimulatory signals, activated endothelial cells release proteases, which lead to degradation of the ECM surrounding the vessel, followed by endothelial cell proliferation and migration. These proangiogenic factors, in combination with basement membrane degradation by proteolytic enzymes, trigger endothelial cell proliferation, tube formation, and migration toward the tumour site.

Proangiogenic Factors

bFGF and VEGF

Endogenous proangiogenic factors include bFGF and VEGF, which are two of the principle soluble stimulators of angiogenesis (Javerzat *et al.*, 2002; Breier and Risau, 1996; Ribatti *et al.*, 2005). bFGF is a ubiquitously expressed polypeptide growth factor that, under normal conditions, is sequestered in the ECM of healthy tissues (Vlodavsky *et al.*, 1991). In addition, bFGF is also expressed by many human cancer cells, including breast and prostate carcinomas, and is believed to be important for the development of tumour vasculature (Bos *et al.*, 2005; Cronauer *et al.*, 1997) through stimulation of endothelial cell proliferation, migration (Gospodarowicz *et al.*, 1989), as well as endothelial cell production of proteolytic enzymes (Millauer *et al.*, 1993). VEGF is a vascular endothelial cell-specific mitogen that is produced by a variety of cell types, including tumour cells and activated macrophages (Ferrara, 1993; Birck *et al.*, 1999).

Alternate mRNA splicing of a single gene generates six different VEGF isoforms (Robinson and Stringer, 2001), including the most commonly expressed isoform, $VEGF_{165}$, which binds heparan sulfate (Ashikari-Hada *et al.*, 2005). Both bFGF and $VEGF_{165}$ initially interact with the cell through binding heparan sulfate proteoglycans (HSPG). HSPG are a class of complex macromolecules comprised of a protein core with attached heparan sulfate chains, and are widely distributed in nearly all human tissues (Folkman *et al.*, 1988). This binding step is required for the growth factors to subsequently interact with and signal through their respective cell surface receptors (Rapraeger *et al.*, 1991; Gitay-Goran *et al.*, 1992).

Therapeutic use of monoclonal antibodies (mAb) raised against VEGF or VEGF receptors, such as the anti-VEGF mAb bevacizumab that has been assessed as a treatment against renal carcinomas (Yang *et al.*, 2002), have shown promise in the prevention of tumour angiogenesis (Kim *et al.*, 1993; Yang *et al.*, 2002). Anti-VEGF antibodies cause very few side effects, but are not cost-effective for widespread use in a clinical setting (Fraser *et al.*, 2000). Therefore, the development of other compounds that block VEGF signaling, such as low-molecular-weight inhibitors of VEGF receptor kinases, is currently underway. One group found that the orally active and fully synthetic low-molecular-weight glycine ester, CEP-7055, inhibits the activity of all three VEGF receptor kinases, resulting in antitumour and antiangiogenic effects (Ruggeri *et al.*, 2003). Additionally, administration of soluble VEGF decoy-receptors that block VEGF signaling pathways have been under investigation (Ferrara *et al.*, 1998; Gerber *et al.*, 1999). One group engineered a very potent high-affinity VEGF decoy-receptor, VEGF-Trap, with a prolonged *in vivo* half-life resulting in the suppression of tumour growth and

vascularization in a number of cancer types, including mouse B16F10 melanoma, human A673 rhabdomyosarcoma, and rat C6 glioma, with no notable non-specific toxicities (Holash *et al.*, 2002).

Angiopoietins

Angiopoietins, and their endothelium-specific Tie receptors, have multiple roles in angiogenesis. Angiopoietin (Ang)-1 signaling through Tie-2 receptors on endothelial cells (EC) results in the remodeling and stabilization of newly formed blood vessels. In contrast, Ang-2 signaling through Tie-2 receptors antagonizes the effects of Ang-1, leading to blood vessel destabilization, and the promotion of angiogenesis (Thurston *et al.*, 1999; Maisonpierre *et al.*, 1997). The Ang-1 antagonizing effect of Ang-2 contributes to the leakiness and instability of tumour vessels in the presence of VEGF, but results in blood vessel regression when VEGF is not present (Tse *et al.*, 2003). Ang-2 is overexpressed in many human tumour types, including hepatocellular carcinoma and malignant astrocytoma, and therefore is believed to be involved in tumour-induced angiogenesis (Sugimachi *et al.*, 2003; Niu *et al.*, 2004).

Malignant human astrocytoma growth can be inhibited by a kinase-deficient Tie2 construct, which blocks Ang-2 signaling through the Tie-2 receptor. Interruption of Tie-2 signaling in ECs results in the loss of EC viability, which is linked to a block in Akt signaling and increased TSP-1 expression. (Niu *et al.*, 2004; Zadeh *et al.*, 2004).

CXCL8

Elevated CXCL8 mRNA expression has been reported in various cancer tissues where it is believed to act as a growth factor involved in tumour angiogenesis (Yuan *et al.*, 2000). An angiogenic role for CXCL8 was first suggested by studies which showed that macrophages produce CXCL8 and mediate angiogenesis in inflammatory disease (Koch *et al.*, 1986; Koch *et al.*, 1992). CXCL8 activity results in the induction of matrix metalloproteinase (MMP)-2 expression by ECs independent of VEGF and bFGF activity, and therefore CXCL8 levels are believed to directly correlate with the extent of tumour angiogenesis (Bar-Eli, 1999). A recent report showed that an antibody directed against human CXCL8 (ABX-CXCL8) caused a decrease in melanoma cell growth, most likely by preventing CXCL8-dependent MMP-2 production, resulting in impaired cellular invasion (Huang *et al.*, 2002).

Matrix Metalloproteinases

MMPs are a family of proteins that, collectively, can degrade every component of the ECM. These lytic enzymes are expressed by proliferating ECs, and are capable of digesting specific matrix components, thus promoting cellular invasion. MMPs can also release proangiogenic molecules from the basement membrane through proteolytic degradation of ECM components, which further promotes angiogenesis (Hidalgo and Eckhardt, 2001). Expression of MMPs by malignant tumours and their surrounding stroma appears to increase as tumours dedifferentiate, increasing their metastatic potential (Westermarck and Kahari, 1999). MMP expression is believed to be essential in the early stages of angiogenesis, tumour cell invasion, and metastasis (Hidalgo and

Eckhardt, 2001), and it is therefore often associated with a poor prognosis in many cancers (Kodate *et al.*, 1997; Karameris *et al.*, 1997).

Endogenous Antiangiogenic Factors

Angiostatin and Endostatin

A number of endogenous antiangiogenic factors are proteolytic fragments of larger, naturally occurring proteins. Angiostatin is generated by proteolytic cleavage of circulating plasminogen (O'Reilly *et al.*, 1994), while endostatin is a fragment of type XVIII collagen (O'Reilly *et al.*, 1997). Angiostatin and endostatin both inhibit EC migration and proliferation (Griscelli *et al.*, 1998). Angiostatin can inhibit EC proliferation, and induce apoptosis through the induction of p53 and Bax expression, and the activation of Bid, which leads to the release of cytochrome *c* from the mitochondria, resulting in caspase activation and apoptosis (Chen *et al.*, 2003). Angiostatin can also activate the Fas-mediated apoptotic pathway by up-regulating FasL mRNA, and down-regulating c-Flip (Chen *et al.*, 2003). Another group determined that angiostatin can directly inhibit neutrophil and monocyte recruitment, and therefore decrease the level of angiogenic chemokine production at the tumour site (Bennelli *et al.*, 2002).

Endostatin is believed to inhibit angiogenesis by blocking multiple signaling pathways involved in EC migration and proliferation (Sudhakar A *et al.*, 2003; Kim *et al.*, 2002). This is accomplished through binding to $\alpha_v\beta_1$-integrin and VEGFR-2 on the EC membrane, thus inhibiting signals obtained through these receptors. Members of the integrin receptor family of adhesion molecules can bind an array of ECM ligands, including fibronectin and laminin, and not only mediate physical interactions between

ECs and the ECM, but also initiate signaling events involving MAPK and nuclear factor-κB, which are necessary for EC migration (Eliceiri and Cheresh, 1999). Additionally, endostatin can downregulate many proangiogenic signaling pathways, while upregulating many antiangiogenic genes in ECs (Abdollahi *et al.*, 2002). For example, endostatin can downregulate all the genes upregulated by VEGF or bFGF, and can upregulate EC thrombospondin (TSP)-1, which is a potent angiogenesis inhibitor (Folkman, 2006).

Thrombospondin and Class I Interferons

TSP-1 belongs to a family of ECM proteins and is a potent inhibitor of angiogenesis *in vivo* and of endothelial cell responses to angiogenic factors *in vitro* (Sid *et al.*, 2004). TSP-1 appears to mediate its antiangiogenic effect, at least in part, through a specific receptor, CD36, which initiates the antiangiogenic signal, and often results in EC apoptosis (Jimenez *et al.*, 2000; Volpert and Alani, 2003). In addition, TSP-1 can up-regulate Fas ligand expression on ECs, rendering them susceptible to Fas-induced cell death (Volpert *et al.*, 2002). One report showed that administration of exogenous TSP-1 following radiation therapy not only reduced the growth rate of human melanoma xenografts, but also prevented the formation of pulmonary micrometastases (Rofstad *et al.*, 2003). However, the ability to use TSP-1 as a reproducible recombinant protein for clinical application have been limited by its large size (450 kDa), and susceptibility to proteolytic breakdown (Vailhe and Feige, 2003).

The interferons (INFs) are a group of regulatory cytokines that are involved in the control of cell replication and function, and also have immune-modulatory properties (Gutterman, 1994). Class I interferons (IFNα and IFNβ) can directly inhibit cell

proliferation in tumours of different histological origins (Marler *et al.*, 2002; Kubo *et al.*, 2000). Additionally, IFN-α can downregulate the expression of proangiogenic growth factors, including bFGF (Singh *et al.*, 1995) and VEGF (Wang *et al.*, 2003) by tumour cells. Similarly, IFN-β has been shown to inhibit MMP-2 and bFGF production, resulting in decreased migration and invasion of ECs (Gohji *et al.*, 1994).

Antiangiogenic Potential of CAPs

Vascular endothelial cells are an important target for therapeutic intervention in cancer because of the central role of angiogenesis in tumour growth and metastases, and the direct accessibility of EC to drugs via vascular circulation. A number of CAPs have putative antiangiogenic activity (Yoo *et al.*, 1997a; Chavakis *et al.*, 2004). For example, Chavakis *et al.* (2004) have shown that human neutrophil peptide α-defensins (HNPs) can inhibit angiogenesis by interfering with endothelial cell adhesion and migration stimulated by VEGF. It was observed that HNPs can accumulate in the blood vessel wall by binding to ECM-associated fibronectin. HNPs specifically blocked the migration of human umbilical vein endothelial cells (HUVEC) by inhibiting $\alpha_5\beta_1$-integrin-dependent endothelial cell adhesion to fibronectin. HNPs also inhibited VEGF-induced HUVEC proliferation, while HNP-3 induced HUVEC apoptosis. Finally, HNPs inhibited *in vivo* bFGF-induced angiogenesis in the chicken chorioallantoic membrane assay. Interestingly, LfcinB treatment of tumour-bearing mice leads to a reduction in the number of tumour-induced blood vessels (Yoo *et al.*, 1997a), suggesting a possible antiangiogenic role for LfcinB. However, whether this effect is a consequence of LfcinB-

induced apoptosis of endothelial cells or LfcinB-mediated inhibition of tumour blood vessel development remains to be determined.

Hypotheses, Overall Objective and Rational

Hypothesis 1: LfcinB displays selective anticancer activity against a wide variety of human tumour cell lines.

Bovine lactoferrin and LfcinB are known to have *in vitro* and *in vivo* anticancer activity. Bovine lactoferrin treatment resulted in a reduction in methylcholanthrene-induced murine fibrosarcoma growth, as well as experimental lung metastasis by B16-F10 murine melanoma cells in syngeneic mice through a mechanism that is independent of its iron-binding activity (Bezault *et al.*, 1994). The cationic peptide LfcinB is cytotoxic for a diverse range of murine and human tumour cells, exhibiting potent *in vitro* and *in vivo* anticancer activity (Yoo *et al.*, 1997a and b; Iigo *et al.*, 1999; Roy *et al.*, 2002; Eliassen *et al.*, 2002; Mader *et al.*, 2005). However, previous studies did not determine the effect of LfcinB on a wide range of human cancer cell lines, nor did they elucidate the mechanism(s) by which LfcinB kills human cancer cells. For possible future clinical application, I sought to determine whether the apoptosis-inducing activity of LfcinB extended to human cancer cells of epithelial, as well as hematopoietic origin, without causing harm to normal human cells. In addition, I wished to gain a clear understanding of the mechanism of LfcinB-induced cell death in human cancer cell lines. Finally, I sought to determine why the peptide is unable to kill normal human cells.

Hypothesis 2: Upon entry into the cytosol of human tumour cells, LfcinB interacts with, and enters mitochondria leading to mitochondrial membrane destabilization.

I wished to determine whether LfcinB can enter cancer cells to reside in the cytoplasmic compartment. The cationic, amphipathic structure of LfcinB may allow the peptide to target intracellular components, including mitochondria and nucleic acids (Britigan *et al.*, 2001). The common ancestry of prokaryotic cells and the mitochondria of eukaryotic cells, and the knowledge that they both have large transmembrane potentials and are composed of a high content of anionic phospholipids suggests that intracellular LfcinB may interact with mitochondrial membranes (Gray *et al.*, 1999; Risso *et al.*, 2002; Dekroon *et al.*, 1997; Matsuzaki, 1995).

Hypothesis 3: LfcinB displays antiangiogenic activity by binding to heparin-like molecules on the endothelial cell surface, thereby competing with heparin-binding growth factors for surface binding.

A potential antiangiogenic role for LfcinB was suggested by the finding that systemic administration of LfcinB to tumour-bearing mice caused a reduction in the number of tumour-induced blood vessels (Yoo *et al.*, 1997a). I therefore wished to determine whether LfcinB inhibited growth factor-induced angiogenesis *in vivo*, and EC proliferation and migration *in vitro*. Since bovine lactoferrin exhibits heparin-binding activity (Pejler, 1996; Wu and Church, 2003), LfcinB might be inhibiting angiogenesis by preventing heparin-binding growth factor interaction with ECs. I therefore wanted to examine the heparin binding activity of LfcinB, and its ability to compete with heparin-binding and non-heparin-binding growth factors for binding sites on the EC membrane.

CHAPTER 2

Materials and Methods

Animals

Adult (6-8-week-old) C57BL/6 mice and adult (6-8-week-old) SCID/beige mice were purchased from Charles River Canada (Lasalle, Quebec, Canada) and housed in the Carleton Animal Care Facility of Dalhousie University. Mice were maintained on a diet of standard rodent chow and water supplied *ad libitum*. Animal use was in accordance with protocols consistent with the Canadian Council on Animal Care guidelines, and was approved by the Dalhousie University Committee on Laboratory Animals.

Cell Culture

CCRF-CEM cells were kindly provided by Dr. W. Gati (University of Alberta), while MDA-MB-435 cells were a generous gift from Dr. J. Mackey (University of Alberta). Bcl-2 overexpressing Jurkat T leukemia cells and vector-only transfectants (Heibein *et al.*, 2000) were generously contributed by Dr. C. Bleackley (University of Alberta). All other tumor cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained at 37°C in a 5% or 10% CO_2 humidified atmosphere in RPMI 1640 or DMEM medium (Sigma-Aldrich Canada, Oakville, Ont.), respectively, supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES buffer (pH 7.4), and 5% or 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Burlington, Ont.), as appropriate for each cell line. Hereafter, these will be referred to as complete RPMI and complete DMEM. Stock flasks were passaged twice weekly, or as required to maintain optimal cell growth. Normal human T cells, HUVECs, and human fibroblast cells were generous gifts from Drs. J. Marshall, A. Issekutz, and T. Peterson (Dalhousie University), respectively. HUVECs, human dermal fibroblasts, and mammary epithelial cells were purchased from

Cambrex (Rockland, USA). Normal human T cells and human fibroblast cells were maintained at 37°C in a 10% CO_2 humidified atmosphere. HUVECs were maintained at 37°C in a 5% CO_2 humidified atmosphere in complete RPMI medium supplemented with 10% heat-inactivated FCS, 25 μg/mL endothelial cell growth supplement (ECGS), and 45 μg/mL heparin. Human mammary epithelial cells were maintained at 37°C in a 5% CO_2 humidified atmosphere in MEGM medium (Cambrex, Rockland, USA).

Reagents

LfcinB (amino acid sequence: FKCRRWQWRMKKLGAPSITCVRRAF) and its 10-mer (amino acid sequence: FKCRRWQWRM) and 6-mer (amino acid sequence: RRWQWR) derivatives were synthesized in linear form by Sigma Genosys (The Woodlands, TX) with a purity of >95%. For some preliminary experiments, we used LfcinB (generated by pepsin hydrolysis of bovine lactoferrin) that was generously provided by Morinaga Milk Industry Co. (Zama, Japan). Lyophilized peptides were dissolved in serum-free RPMI 1640 medium and aliquots were stored at -70°C. All experiments with LfcinB and its derivatives were performed with medium containing 0.5% FCS because these peptides exhibit optimal cytotoxic activity at lower serum concentrations (Yoo et al., 1997b). Inhibitors of c-Jun N-terminal kinase (JNK; SP 600125), extracellular signal-regulated kinase 1/2 (ERK1/2; PD 98059), and p38 MAPK (SB 203580) were purchased from EMD Biosciences Inc. (San Diego, CA). *N*-acetyl-*L*-cysteine, concanavalin A, bovine serum albumin (BSA), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Canada. Cytochalasin D and dansylcadaverine were purchased from A.G. Scientific, Inc. (San Diego, CA) and Sigma (St. Louis, MO), respectively. Glutathione, caspase

inhibitors (Z-VAD-FMK, Z-VDVAD-FMK, Z-LEHD-FMK, Z-IETD-FMK, and Z-DEVD-FMK), and chromagenic caspase substrates (Ac-IETD-pNA, Ac-VDVAD-pNA, Ac-LEHD-pNA, and Ac-DEVD-pNA) were from EMD Biosciences Inc. (San Diego, CA). Dihydroethidium and 3, 3'- dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes (Eugene, OR). Mouse anti-human Fas and mouse anti-human Fas ligand monoclonal antibodies (mAb) were from BD Pharmingen (Mississauga, Ontario). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) was purchased from Cedarlane Laboratories (Hornby, Ont.). Phycoerythrin (PE)-conjugated mouse antihuman TRAIL (RIK-2) mAb, PE-conjugated mouse anti-human DR4 mAb, PE-conjugated mouse anti-human DR5 mAb, and mouse IgG were from eBioscience (San Diego, CA). Rabbit anti-human caspase-9 antibody, rabbit anti-human caspase-3 antibody, and mouse anti-human caspase-8 mAb were from Cell Signaling Technology (Beverly, MA). Rabbit anti-human caspase-2 antibody was from StressGen Biotechnologies Corp. (Victoria, Brit. Col.) and mouse anti-cytochrome c mAb was from Upstate Biotechnology (Charlottesville, VA). Goat anti-mouse IgG-horse radish peroxidase (HRP) and goat anti-rabbit IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA).

Calcium flux reagents include cell permeate Fluo-4-AM (Molecular Probes, Inc., Eugene, OR), Triton-X 100 and EGTA (Sigma-Aldrich Canada), Hanks Buffered Salt Solution (HBSS, 20 mL 10X HBSS, 0.94 mL 7.5% $NaHCO_3$, 2 mL HEPES, 177 mL dH_2O), Probenecid (Sigma-Aldrich Canada) and HEPES tyrodes buffer (138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 0.5 mM NaH_2PO_4, 20 mM HEPES, pH 7.4). Mitochondria isolation buffer contained 0.2 mM EDTA, 0.25 M sucrose, and 10 mM

Tris-HCl pH 7.8. Streptavidin 10 nm colloidal gold was purchased from Sigma (St. Louis, MO), and streptavidin-TexasRed was from Jackson ImmunoResearch (Mississauga, ON, Canada).

Heparin, hematoxylin, 1, 2-phenylenediamine substrate, Hoechst 33342 trihydrochloride dye, heparinase III, N-hydroxysuccinimido-biotin, N, N-dimethyl formamide, and ECGS were purchased from Sigma-Aldrich Canada. Vascular endothelial growth factor$_{165}$ (VEGF$_{165}$), basic fibroblast growth factor (bFGF), and non-heparin-binding epidermal growth factor (EGF) were obtained from Peprotech Inc. (Rocky Hill, NJ). Growth factor-reduced Matrigel^{TM} was purchased from BD Biosciences (Bedford, MA). Streptavidin-HRP was from Jackson ImmunoResearch (West Grove, PA). Cross-reactive rabbit IgG antibody against human Factor VIII-associated antigen, von Willebrand factor, was purchased from Dako Corp. (Copenhagen, Denmark). Rabbit IgG was from Cedarlane Laboratories (Hornby, Ontario, Canada). Biotinylated goat anti-rabbit IgG was from Invitrogen Corp., (Burlington, Ontario, Canada).

Cell Viability Assays

Hoechst staining

Cells (5×10^4 cells/treatment) were cultured at 37°C in a 5% CO_2 humidified atmosphere in the absence or presence of LfcinB (200 μg/mL) for various time points. Cells were then washed with phosphate buffered saline (PBS), resuspended in 50 μL of 4% paraformaldehyde in PBS, and then placed on silinated microscope slides and allowed to dry overnight, after which they were stained for 10 min at room temperature with Hoechst 33342 trihydrochloride dye (10 μg/mL). Slides were then rinsed with

distilled water and allowed to air dry in the dark. Chromatin condensation and nuclear fragmentation was then assessed at 200× magnification by ultraviolet microscopy.

DNA Fragmentation Assays

The JAM assay was used to measure DNA fragmentation in dying cells (Matzinger 1999). Briefly, cells were labeled with tritiated thymidine (ICN Biomedicals, Irvine, CA; 5 μCi/mL of cells) for 4 h at 37°C, washed extensively with complete medium, and resuspended in fresh medium containing 0.5% FCS. Radiolabeled cells (25 × 10^5 cells/mL) were added in quadruplicate to 96-well flat-bottom tissue culture plates (Sarstedt Inc.). Following the desired treatment, cells were incubated for 18 h at 37°C in a 5% CO_2 humidified atmosphere. DNA was then harvested onto glass fibre filter mats using a multiple sample harvester (Skatron Instruments, Sterling, VA). Radioactivity was measured by liquid scintillation counting. Percentage of DNA fragmentation was calculated as follows: % DNA fragmentation = $(S_{cpm} - E_{cpm}) / S_{cpm} \times 100$, where S is DNA retained from untreated control cells and E is DNA retained from treated cells. Each experiment was performed in triplicate. Alternatively, DNA was isolated from control or LfcinB-treated cells using a kit purchased from Qiagen Inc. (Mississauga, Ont.) and DNA fragmentation was visualized by gel electrophoresis, as previously described (Makrigiannis *et al.*, 1994).

MTT Assay

As an alternative to the JAM assay, changes in cell viability were also measured by MTT assay (Mosmann, 1983). Cells were seeded in quadruplicate into 96-well flat-bottom tissue culture plates at a density of 25 × 10^5 cells/mL in fresh medium containing

0.5% FCS. After 15-h culture in the absence or presence of the desired concentrations of LfcinB, MTT was added to each well at a final concentration of 500 μg/mL and the plates were incubated for an additional 3 h. The medium was then removed and dimethyl sulfoxide (DMSO) (0.1 mL) was added to each well to solubilize the cells. Spectrometric absorbance was measured at 492 nm.

Chromium Release Assay

Cell viability was assessed by ^{51}Cr release from the intracellular compartment (Zawydiwski and Duncan, 1978). Cells were labeled for 1 h with 100 μCi $Na_2{}^{51}CrO_4$ (ICN Canada Ltd., Montreal, PQ). At this time, they were washed extensively with complete medium, and resuspended in 0.5% FCS RPMI-1640 medium. Radiolabeled cells (25×10^5 cells/mL) were added in quadruplicate to 96-well V-bottomed tissue culture plates (Sarstedt, St. Leonard, PQ) in the absence or presence of the desired concentrations of LfcinB. Following incubation at 37°C in a 5% CO_2 humidified atmosphere, radioactivity was determined using gamma counting. All treatments were analyzed versus 10% SDS (positive control). All experiments were performed in triplicate.

Death Receptor/Ligand Expression

Cells (1×10^6 cells/treatment) were incubated for 45 min at 4°C with 10 μg/mL primary mAb (anti-Fas, anti-Fas ligand, PE-anti-TRAIL, PE-anti-DR4, or PE-anti-DR5) in immunofluorescence (IF) buffer (1% bovine serum albumin, 0.2% sodium azide in PBS). Cells were then washed three times with IF buffer and, if appropriate, incubated for an additional 45 min at 4°C with FITC-anti-mouse IgG to detect cell-bound anti-Fas

or anti-Fas ligand mAb. After 3 additional washes with IF buffer, cells were resuspended in 1% paraformaldehyde in PBS and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Determination of Mitochondrial Transmembrane Potential

Flow cytometric analysis of cells stained with $DiOC_6$ were used to measure changes in mitochondrial transmembrane potential (Mosmann, 1983). $DiOC_6$ was stored at 4°C as a 1 mM stock in DMSO. Cells (1×10^6 cells/treatment) were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for 1, 2, and 4 h in wells of a 24-well flat-bottom tissue culture plate (Sarstedt) at 37°C in a 5% CO_2 humidified atmosphere. $DiOC_6$ was then added to untreated or LfcinB-treated cells at a final concentration of 40 nM and 30 min later cells were analyzed by flow cytometry.

Annexin-V Labeling of Apoptotic Cells

Annexin-V staining was used to measure phosphatidylserine headgroup externalization on cells undergoing apoptosis (Koopman *et al.*,1994), according to the instructions provided by the manufacturer of the annexin-V staining kit (Roche Diagnostics, Laval, Que.). Briefly, 1×10^6 cells were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for the desired length of time at 37°C in a 5% CO_2 humidified atmosphere, washed, resuspended in 0.1 mL staining solution (2 % Annexin-V-FITC and 2% propidium iodide-PE by volume in HEPES buffer), and incubated for an additional 10-15 min. Cells were then analyzed by flow cytometry.

Measurement of ROS

Flow cytometry and dihydroethidium was used to measure the production of ROS

during apoptosis (Perticarari *et al.*, 1991). Briefly, cells (1×10^6 cells/treatment) were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for the desired length of time in wells of a 24-well flat-bottom tissue culture plate at 37°C in a 5% CO_2 humidified atmosphere. Dihydroethidium was then added to cells at a final concentration of 2.5 μM and 20 min later cells were analyzed by flow cytometry.

Immunoblotting

Cells (5×10^6) were exposed to LfcinB (200 μg/mL) for 2 h at 37°C in a 5% CO_2 humidified atmosphere and then washed in PBS. For caspase detection, the cell pellet was resuspended in 0.1 mL ice-cold CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) lysis buffer (50 mM PIPES/NaOH at pH 6.5, 2 mM EDTA, 5 mM dithiothreitol, 20 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 0.1% CHAPS) and placed on ice for 1 h with vortexing every 10 min. Samples were then clarified by centrifugation at 10,000 *g* for 10 min at 4°C and the supernatant was collected. For cytochrome c detection, the cell pellet was resuspended in 0.1 mL ice-cold digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 2 mM sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 190 μg/mL digitonin) and incubated on ice for 15 min to allow digitonin to selectively permeabilize the plasma membrane. Cells were then pelleted by centrifugation at 15,000 *g* for 5 min at 4°C and the supernatant was collected. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories Ltd., Mississauga, Ont.). Samples were boiled in SDS sample buffer and 20 μg total protein was loaded into each well of a 12% (for caspase detection) or 15% (for

cytochrome c detection) SDS-polyacrylamide gel for separation by electrophoresis. Protein bands were transferred onto nitrocellulose membranes. The resulting blots were blocked overnight with PBS-Tween (0.25 M Tris at pH 7.5, 150 mM NaCl, and 0.2% Tween 20 in PBS) containing 5% powdered skim milk, and then probed overnight with the desired primary antibody at a 1/500 dilution. Blots were then washed with PBS-Tween and probed for 1 h with HRP-conjugated anti-mouse or anti-rabbit IgG (1/1000 dilution), as appropriate. Following additional washes with PBS-Tween, the protein bands were visualized using an ECL detection system (Bio-Rad).

Caspase Activity Assay

Caspase activity in cell lysates was measured using a chromagenic assay developed from a protocol described by Talanian *et al.* (1997). Briefly, 1×10^6 cells were resuspended in medium containing 0.5% FCS and incubated with or without LfcinB (200 μg/mL) for 2 h at 37°C in a 5% CO_2 humidified atmosphere. Cells were then pelleted and resuspended in ice-cold lysis buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.25% Triton-X-100), followed by intermittent vortexing for 5 min at 4°C. Lysates were clearified by centrifugation at 4°C and aliquots were added in quadruplicate to wells of a 96-well flat-bottom plate (Sarstedt) with or without the appropriate chromagenic caspase substrate (Ac-IETD-pNA, Ac-VDVAD-pNA, Ac-LEHD-pNA, or Ac-DEVD-pNA) at a final concentration of 200 μM. Plates were incubated at 37°C and caspase activity in individual samples was determined by measuring absorbance at 405 nm every 15 min for 2 h with a microplate autoreader (Bio-tek Instruments, Winooski, VT). Relative caspase activity was determined by subtracting background readings

obtained in the absence of cell lysates. An absorbance of 0.001 was arbitrarily defined as 1 unit of caspase activity.

Fluorescent Microscopy

Non-adherent human tumour cell lines or normal human T cells (50×10^4 cells/treatment) were exposed to biotin-LfcinB (200 μg/mL) dissolved in 0.5 % FBS-RPMI for various time points at 37°C in a 5% CO_2 humidified atmosphere. Following treatment, cells were washed two times with PBS, fixed with 4% paraformaldehyde, and pipetted onto silinated microscope slides. Slides were air dried overnight at RT, then incubated with periodate-lysine-paraformaldehyde (PLP) for 5 minutes, washed extensively with PBS, and blocked for 45 min with 2% BSA (w/v in PBS). At this time, slides were washed extensively with PBS, and incubated with Streptavidin-TexasRed (1:1000 in PBS, 1 h, RT) in the dark. Slides were washed with PBS, mounted, and analyzed for fluorescence at 200× magnification.

Variations on this experiment were also performed. Adherent human tumour cell lines and untransformed human primary fibroblasts and HUVECs were placed on coverslips (5×10^4 cells/coverslip) and cultured for 4 h (tumour lines) and overnight (primary cultures) at 37°C in a 5% CO_2 humidified atmosphere to allow adherence to occur. Medium was removed and replaced with RPMI 1640 medium containing 0.5% FCS without or with biotin-LfcinB (200 μg/mL). Following incubation at 37°C in a 5% CO_2 humidified atmosphere, coverslips were washed with PBS, and fixed with 4% paraformaldehyde.

Electron Microscopy

Electron microscopy was done in the Electron Microscope Facility of the Faculty of Medicine, Dalhousie University, using standard protocols (Faulkner and Garduno, 2002). A JEOL JEM 1230 transmission electron microscope operating at 80kV was used to visualize Jurkat T leukemia cells that had been cultured in the absence or presence of 200 μg/mL biotin-LfcinB for 2 hours. Jurkat T leukemia cells were immobilized on 300 mesh nickel grids, and stained with Streptavidin 10 nm colloidal gold (1:20 in PBS, 1 h, RT). Grids were washed three times with PBS, fixed with 4% paraformaldehyde/0.5% gluteraldehyde in 0.1 M sodium cacodylate buffer, washed three times with dH_2O, and then stained with 2% Uranyl Acetate and Lead Citrate. Grids were then analyzed for intracellular LfcinB (60,000× magnification). To investigate possible non-specific binding of Streptavidin colloidal gold during the labeling process, control cells that were not exposed to biotin-LfcinB were also immunolabeled.

Mitochondrial Isolation

Jurkat T leukemia cells (30×10^6 cells/treatment) were washed two times in PBS and resuspended in ice-cold mitochondria isolation buffer (0.5mL, 10 min). The cell membrane of Jurkat T leukemia cells was destabilized by two freeze-thaw cycles (-80°C, then 37°C), and homogenization with 15 strokes of a pre-cooled 10 mL Potter-Elvehjem glass-teflon homogenizer (Fisher Scientific, Nepean, ON, Canada). Jurkat cells were centrifuged for 10 min (1000 *g*, 4°C), and supernatants were removed and centrifuged for a subsequent 15 min (12000 *g*, 4°C). The supernatant was removed and discarded and the cell pellet was resuspended in RPMI-1640 medium containing 0.5% FCS and biotin-

LfcinB (200 μg/mL, 30 min), or medium alone as a control. Electron microscopy was performed.

Real-Time Calcium Flux Analysis

Jurkat T leukemia cells were washed and resuspended at 5×10^6 cells/mL in Hanks buffered salt solution (HBSS) containing 20 mM $CaCl_2$, 20 mM $MgCl_2$, and 4 mM probenecid. Cells were then loaded with 4 μM Fluo-4-AM by incubation at 37°C for 30 min. Following two washes with HBSS containing 5% FCS, Jurkat T leukemia cells were resuspended at 2×10^5 cells/mL in ice-cold HBSS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Aliquots of 4×10^5 Jurkat cells were then warmed to 37°C for 5 min and placed in a 37 °C thermostated quartz cuvette with magnetic stirring in a RF-1501 spectrofluorimetre (Shimadzu, Tokyo, Japan). Jurkat cells were treated with medium alone or with LfcinB (200 μg/mL), and subsequently exposed to 10% Triton-X 100 (positive control), and 0.5 M EGTA (negative control) in a sequential fashion. Fluorescence was measured at 520 nm after excitation at 485 nm. Variations on this experiment were also performed, in which Jurkat T leukemia cells were suspended in calcium-free HEPES tyrodes buffer prior to LfcinB addition.

Matrigel Plug Assay

Mice were injected at 4 different sites along the dorsal midline with growth factor-reduced Matrigel™ (0.3 mL/site) plus sterile distilled water (vehicle for LfcinB), LfcinB alone (200 μg/mL), bFGF (1 μg/mL),$VEGF_{165}$ (5 μg/mL), or non-heparin-binding EGF (2 μg/mL) alone, or LfcinB (200 μg/mL) in combination with bFGF (1 μg/mL), $VEGF_{165}$ (5 μg/mL), or non-heparin-binding EGF (2 μg/mL). After 6 days, mice were

sacrificed and Matrigel plugs were surgically excised, fixed in Carnoy's fixative, and sectioned. Sections were blocked for endogenous peroxidase activity and nonspecific antibody-binding, then stained using rabbit IgG (negative control) or cross-reactive rabbit IgG antibody (1:100 dilution in 1% BSA solution) against human Factor VIII-associated antigen, von Willebrand factor, which is a selective stain for endothelial cells (Travis *et al.*, 1988), followed by sequential treatments with biotinylated goat anti-rabbit IgG (1:3000 dilution in 1% BSA solution) and streptavidin-HRP. von Willebrand factor-specific staining in Matrigel sections was developed with aminoethylcarbazole and visualized by light microscopy (20× magnification). The area of individual Matrigel plugs sections that stained positive for von Willebrand factor relative to the unstained area was determined by computer analysis. As an alternative to von Willebrand factor staining, blood vessel density in hematoxylin and eosin stained Matrigel plug sections was determined on the basis of the number of mature lumens per field of view (n = 10, 200× magnification). Mature lumens were defined by the presence of erythrocytes surrounded by an identifiable endothelial cell layer.

HUVEC Proliferation

HUVECs were plated in quadruplicate in flat-bottomed 96-well microtitre plates (4×10^3 cells/well) that were previously coated with 2% gelatin, and allowed to adhere overnight at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was then replaced with RPMI 1640 medium containing 0.5% FCS without or with LfcinB (200 μg/mL). Plates were then incubated for an additional 15 min. bFGF (10 ng/mL), $VEGF_{165}$ (100 ng/mL), or non-heparin-binding EGF (20 ng/mL) was then added and the plates were incubated for 18 h at 37°C in a 5% CO_2 humidified atmosphere. HUVEC cultures

were then pulsed with 1 μCi/mL tritiated thymidine ([^{3}H]TdR; specific activity 60 Ci/mmol, MP Biomedicals) and 6 h later DNA was harvested onto glass fibre filter mats using a multiple sample harvester (Skatron Instruments, Sterling, VA). [^{3}H]TdR incorporation into DNA was determined by liquid scintillation counting.

HUVEC Migration

Modified Boyden chambers and Costar 12 μM pore transwell inserts (Corning, Acton, MA) pre-coated with growth factor-reduced Matrigel™ were used to assess the effect of LfcinB on HUVEC migration. HUVECs were suspended in RPMI 1640 medium containing 0.1% BSA and 5×10^5 cells were added to the upper chamber of triplicate wells. bFGF (10 ng/mL) or $VEGF_{165}$ (100 ng/mL) in the presence or absence of LfcinB (200 μg/mL) in RPMI 1640 medium containing 0.1% BSA was added to the bottom chamber. Following incubation for 2 and 4 h at 37°C in a 5% CO_2 humidified atmosphere, filters were fixed in ethanol and stained for 10 min with hematoxylin. HUVECs were scraped from the upper chamber and cells that had migrated through the filter were then counted at 400× magnification in 3 non-overlapping fields by light microscopy.

Biotinylation of bFGF and $VEGF_{165}$

$VEGF_{165}$ (40 μg) or bFGF (50 μg) were resuspended in 0.1 ml PBS and combined with 0.1 ml borate buffer. N-hydroxysuccinimido-biotin was resuspended in N, N-Dimethyl Formamide (1 mg/mL), and the resulting solution was slowly added at 4°C to bFGF and $VEGF_{165}$ such that a 40-fold molar excess of biotin to bFGF or $VEGF_{165}$ was achieved. Growth factors were then incubated at 4°C for 8 h. At this time, bFGF and

$VEGF_{165}$ were added to the upper chamber of a 10K Nanosep Centrifugal Device (Pall Life Sciences, Ann Arbor, Maine) and centrifuged for 10 min (10,000 *g*, 4°C). Biotinylated bFGF and $VEGF_{165}$ were then eluted with 0.02 mL PBS from the upper chamber, which retained 90% of the protein. Aliquots of biotinylated bFGF and $VEGF_{165}$ were stored at -20°C.

Solid Phase Heparin-binding Assay

LfcinB binding to plastic-immobilized heparin was determined using a modification of the method described by Silvestri and Sundqvist (2001). Briefly, 10 μg/mL heparin in 15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$ (pH 9.2), and 3 mmol/L NaN_3 was added 96-well flat-bottom microtitre plates that were then incubated for 18 h at 4°C to allow heparin to bind to the plastic. Plates were then washed and 1% (w/v) BSA in blocking buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $CaCl_2$) was added to wells to block nonspecific binding sites. Following incubation at room temperature for 2 h, plates were washed repeatedly with 0.04% Tween 20 in PBS. Then biotinylated LfcinB (50 μg/ml) was added to replicate wells alone or in combination with increasing concentrations of bFGF (5, 10, 20 ng/mL), $VEGF_{165}$ (50, 100, 200 ng/mL), or non-heparin-binding EGF (10, 20, 40 ng/mL as a negative control) in blocking buffer. After incubation for 2 h at 4°C, plates were washed repeatedly with 0.04% Tween 20 in PBS and streptavidin-HRP (1:1000) was added to wells. Following an additional 2 h incubation at 4°C, plates were again washed repeatedly with 0.04% Tween 20 in PBS and 1, 2-phenylenediamine substrate (0.4 mg/mL) was added to the wells. Absorbance was measured at 492 nm using a Microplate Autoreader (Bio-Tek Instruments, Winooski, VT).

Competition Assays

HUVECs were plated in quadruplicate in flat-bottomed 96-well (4×10^3 cells/well) microtitre plates that were previously coated with 2% gelatin, and allowed to adhere overnight at 37°C in a 5% CO_2 humidified atmosphere. Some cultures were then treated with heparinase III (1.5×10^{-2} U/mL) for 2 h at 37°C in a 5% CO_2 humidified atmosphere to remove cell-surface heparin-like molecules. Then culture medium was replaced with RPMI 1640 medium containing 0.5% FCS without or with biotin-LfcinB (50 μg/mL) plus increasing concentrations of bFGF (5, 10, 20 ng/mL), $VEGF_{165}$ (50, 100, 200 ng/mL), or non-heparin-binding EGF (10, 20, 40 ng/mL). Alternatively, RPMI 1640 medium containing 0.5% FCS without or with biotin-bFGF (10 ng/mL) or biotin-$VEGF_{165}$ (100 ng/mL) plus increasing concentrations of LfcinB (10, 25, 50 μg/mL) was added to HUVEC cultures. Following incubation for 2 h at 37°C in a 5% CO_2 humidified atmosphere, wells were washed repeatedly with 0.04% Tween 20 in PBS and streptavidin-HRP (1:1000 dilution) was added to each well. After an additional 2 h incubation at 4°C followed by extensive washing, 1, 2-phenylenediamine substrate (0.4 mg/mL) was added to the wells. Absorbance at 492 nm was determined using a Microplate Autoreader.

Model of Disseminated Ramos B lymphoma

Three groups (n = 5/group) of adult (6-8-week-old) SCID/beige mice were injected via the tail vein with 2.5×10^6 Ramos B lymphoma cells on day 0. Group 1 received intraperitoneal (i.p.) injections of saline on days 1 and 3, Group 2 received i.p. injections of LfcinB on day 1 (1 mg/mouse), and Group 3 received i.p. injections of

LfcinB on days 1 and 3 (1 mg/mouse/injection). Mice were examined daily for signs of distress, and weighed every 2 days beginning at day 0. Paralysis of the hind legs was used as the survival end point.

Statistical Analysis

One way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test were performed for dose response analyses using the Instat statistics program (Graphpad Software, CA). When appropriate, the two-tailed student's *t*-test was used to compare differences between test and control groups. $P < 0.05$ was considered to be statistically significant.

CHAPTER 3

Bovine Lactoferricin Selectively Induces Apoptosis in Human Leukemia and Carcinoma Cell Lines: Role of Reactive Oxygen Species, Mitochondrial Damage, and Caspases Associated with the Intrinsic Pathway of Apoptosis

Portions of this chapter appeared in the following publications:

Mader JS, Salsman J, Conrad DM, Hoskin DW. (2005) Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Molecular Cancer Therapeutics 4: 612-624.

Furlong SJ, Mader JS, and Hoskin DW. (2006) Lactoferricin-induced apoptosis in estrogen-nonresponsive MDA-MB-435 breast cancer cells is enhanced by C_6 ceramide or tamoxifen. Oncology Reports 15: 1385-1390.

Preamble

LfcinB is a cationic, amphipathic peptide that is cytotoxic for human and rodent cancer cells. However, the mechanism(s) by which LfcinB causes the death of cancer cells is not well understood. The objectives of the current study are 5-fold. First, it is important for potential future clinical applications to determine whether the apoptosis-inducing activity of LfcinB extends to human cancer cells of epithelial, as well as hematopoietic origin, without causing harm to normal human cells. In this regard, toxicity by conventional chemotherapeutic agents remains a major challenge in the treatment of human malignancies (Savarese *et al.*, 2003). The second goal is to determine the role of death receptor(s) and mitochondria, as well as caspases, in LfcinB-induced apoptosis of human cancer cells. A detailed understanding of the mechanism of LfcinB-induced killing of human neoplasms may suggest possible synergic interactions with other anticancer agents, as has already been established for recombinant TRAIL and different chemotherapeutic drugs in several tumour cell lines (Morgan *et al.*, 2002; Ohtsuka *et al.*, 2003). The third goal is to confirm the contribution of ROS to LfcinB-induced apoptosis in cultures of human cancer cells, and determine whether ROS production is involved in the initiation or execution phase of apoptosis. I also wanted to examine shorter derivatives of LfcinB for apoptosis-inducing activity since smaller peptide fragments would be more easily synthesized in large quantities for therapeutic use. Finally, I determined the *in vivo* activity of LfcinB in a mouse model of disseminated B cell lymphoma.

Results

LfcinB Caused Apoptosis in Human Leukemia and Carcinoma Cells without Affecting the Viability of Untransformed Cells.

Figure 3.1A shows that synthetic LfcinB (200 μg/mL, 18 h) was cytotoxic for different human leukemia and carcinoma cell lines, as determined using the JAM assay. A similar cytotoxic effect was obtained when Jurkat T leukemia cells or MCF-7 breast carcinoma cells were exposed to 200 μg/mL pepsin-generated LfcinB (86 ± 2% cell death and 43 ± 3% cell death, respectively). Since the JAM assay measures DNA fragmentation (Matzinger, 1991), these data strongly suggest that LfcinB caused tumour cells to die by apoptosis. In contrast, LfcinB was not cytotoxic to primary cultures of normal human T lymphocytes (resting and mitogen-activated), fibroblasts, or endothelial cells. The slow rate of primary cell proliferation did not allow for the high level of tritiated thymidine incorporation that is required to measure cell death by JAM assay, therefore, the MTT assay (Mosmann, 1983) was used to determine the effect of LfcinB on the viability of untransformed cells. In addition, normal human breast epithelial cells were not harmed by a 24 h exposure to increasing concentrations of LfcinB as determined by JAM assay (Figure 3.2A), as well as by Hoechst staining (Figure 3.2B, 24 h). LfcinB treatment triggered DNA fragmentation in T leukemia cells (Jurkat), breast cancer cells (MCF-7), and colon cancer cells (Colo-35) in a dose-dependent fashion with a cytotoxic effect being evident at LfcinB concentrations as low as 25 μg/mL (Figure 3.1B). A time course analysis revealed that LfcinB-induced DNA fragmentation in Jurkat T leukemia cells was detectable within 2 h of treatment with 200 μg/mL LfcinB and became maximal by the 8 h time-point (Figure 3.1C). LfcinB-induced DNA fragmentation in MDA-MB-435 breast

Figure 3.1 *LfcinB was cytotoxic to human cancer cells, but not untransformed cells, and mediated its effects in a dose- and time-dependent fashion.* **A.** The indicated human cancer cell lines of hematopoeitic and epithelial (breast, colon, and ovary) origin, as well as normal human T cells (resting and stimulated with 5 μg/mL concanavalin A), Primary human endothelial and fibroblast cells were treated with 200 μg/mL LfcinB for 18 h. Cytotoxicity against cancer cells was determined by JAM assay while cytotoxicity against normal cells was determined by MTT assay. **B.** Jurkat T leukemia cells, MCF-7 breast cancer cells, or Colo-35 colon cancer cells were treated with the indicated concentrations of LfcinB for 18 h. Cytotoxicity was then determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (ns and *** denote not significant and $p<0.001$, respectively, when compared to treatment with medium control). **C.** Jurkat T leukemia cells or MDA-MB-435 breast cancer cells were exposed to 200 μg/mL LfcinB for the indicated periods of time, after which cytotoxicity was determined by JAM assay. Data are shown as mean % cell death ± SD of quadruplicate determinations and are representative of 3 independent experiments. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (*** denotes $p<0.001$ when compared to treatment with medium control).

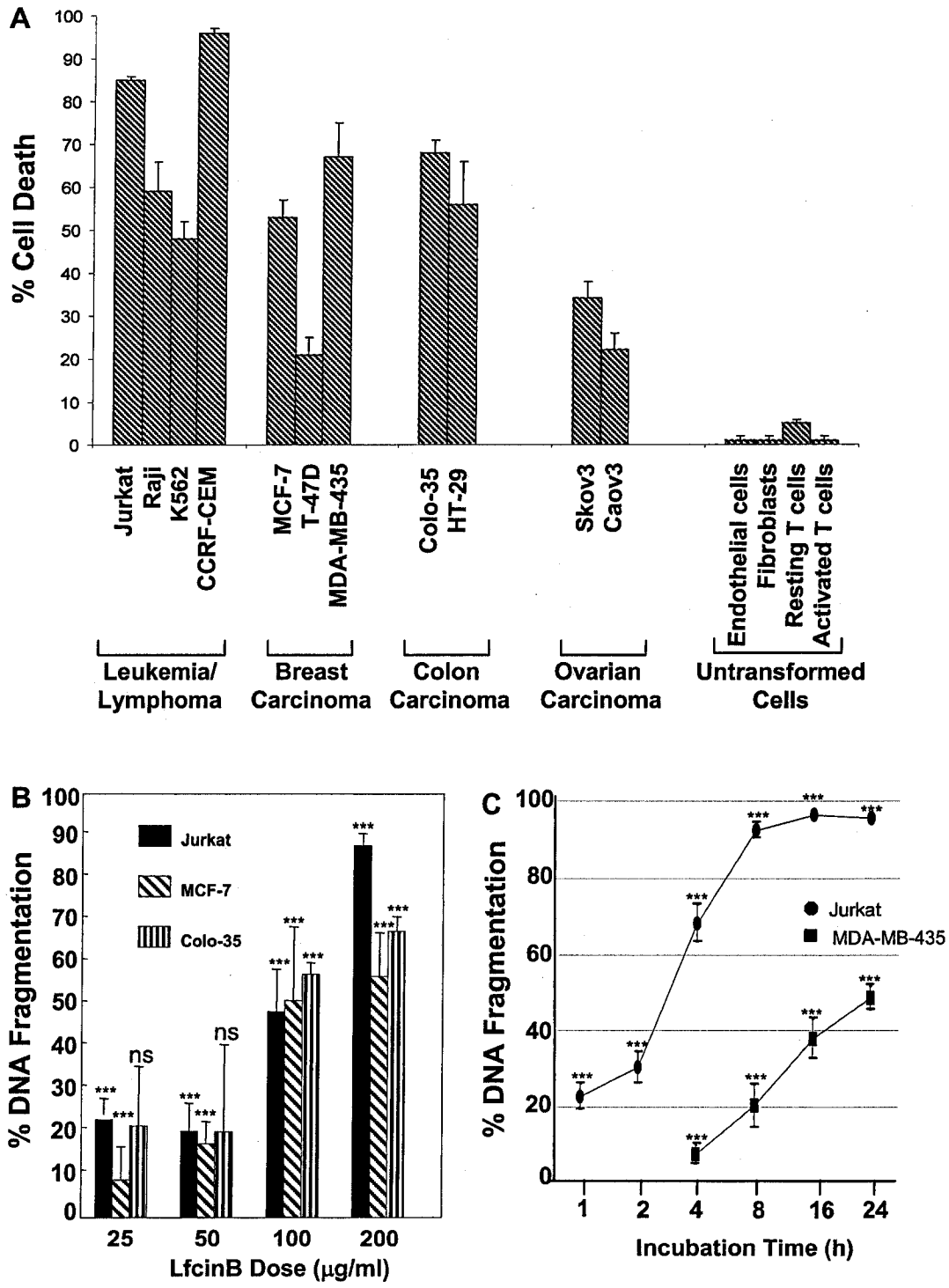

A
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
Jurkat
Raji
K562
CCRF-CEM
MCF-7
T-47D
MDA-MB-435
Colo-35
HT-29
Skov3
Caov3
Endothelial cells
Fibroblasts
Resting T cells
Activated T cells
Leukemia/ Lymphoma
Breast Carcinoma
Colon Carcinoma
Ovarian Carcinoma
Untransformed Cells
B
% DNA Fragmentation
Jurkat
MCF-7
Colo-35
ns
ns
***
25
50
100
200
LfcinB Dose (µg/ml)
C
% DNA Fragmentation
Jurkat
MDA-MB-435
1
2
4
8
16
24
Incubation Time (h)

Figure 3.1

Figure 3.2 *LfcinB did not affect the viability of normal human epithelial cells.* **A.** Normal human breast epithelial cells were treated with the indicated concentrations of LfcinB for 24 h. Cytotoxicity was then determined by JAM assay. Differences were not significant by ANOVA ($p>0.05$) or Tukey-Kramer multiple comparison tests (ns denotes not significant when compared to treatment with medium control). **B.** Hoechst staining analyzed chromatin condensation and nuclear fragmentation in normal human breast epithelial cells exposed to LfcinB (200 μg/mL, 24 h).

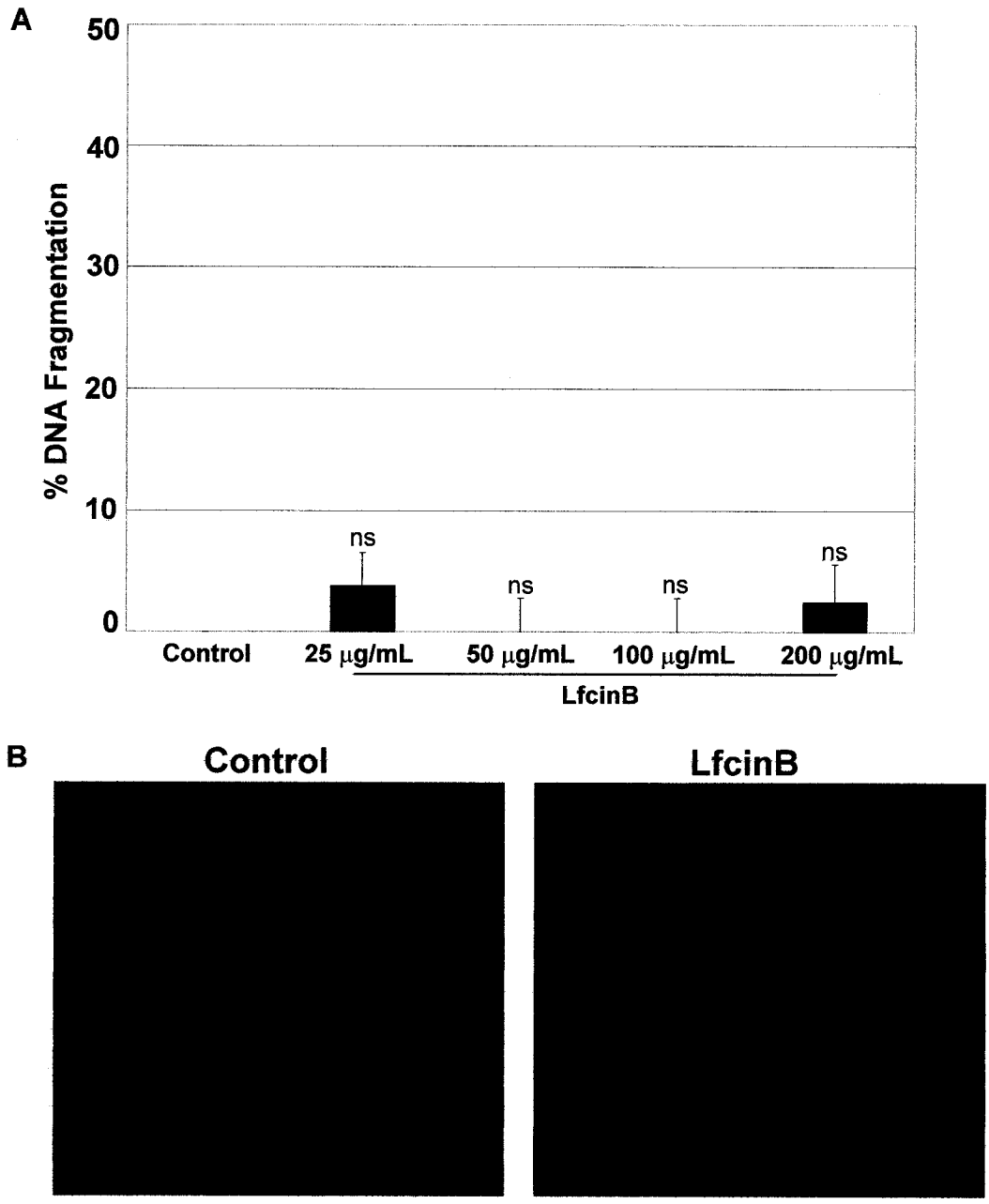

A
50
40
30
20
10
0
% DNA Fragmentation
ns
ns
ns
ns
Control
25 μg/mL
50 μg/mL
100 μg/mL
200 μg/mL
LfcinB
B
Control
LfcinB

Figure 3.2

carcinoma cells was slightly delayed in comparison to Jurkat T leukemia cells, although substantial cell death was evident by the 16 h time-point. Jurkat T leukemia cells were used in most of the subsequent experiments because of the sensitivity that these transformed T cells exhibited to the cytotoxic activity of LfcinB.

Of interest, cyclic structure was not required for LfcinB-induced apoptosis in human cancer cell lines, since pepsin-generated LfcinB and synthetic LfcinB lacking the disulfide bond displayed equivalent anticancer activity in Jurkat T leukemia and MCF-7 breast carcinoma cultures. This is consistent with a report that cyclic and linear forms of LfcinB are equally effective in killing various microbes (Hoek *et al.*, 1997). I also confirmed the ability of linear LfcinB to kill Jurkat T leukemia cells by adding the reducing agent, 2-ME to synthetic LfcinB in Jurkat cell cultures and then measuring DNA fragmentation by JAM assay. LfcinB had equivalent cytotoxic activity against Jurkat T leukemia cells under reducing conditions in comparison to non-reducing conditions (Figure 3.3). This confirmed that linear LfcinB has equivalent anticancer activity to that of the cyclic form of the peptide.

To confirm that LfcinB caused apoptosis in human cancer cells, I treated Jurkat T leukemia cells with 200 μg/mL LfcinB for 4 h and isolated DNA, which was subsequently electrophoresed in an agarose gel and visualized by ethidium bromide staining. Figure 3.4A shows that only intact high-molecular weight DNA was present in the lane containing DNA from untreated control cells (lane 2), whereas nucleosomal-sized DNA fragments were present in the lane containing DNA from LfcinB-treated cells (lane 3). Moreover, the DNA laddering effect induced by LfcinB was comparable to that

Figure 3.3 *LfcinB-induced DNA fragmentation in Jurkat T leukemia cells was not dependent on the disulfide bond between cysteine residues.* Jurkat T leukemia cells were treated with increasing concentrations of the reducing agent, 2-ME, alone or in combination with 200 μg/mL of LfcinB for 18 h. Cytotoxicity was determined by JAM assay.

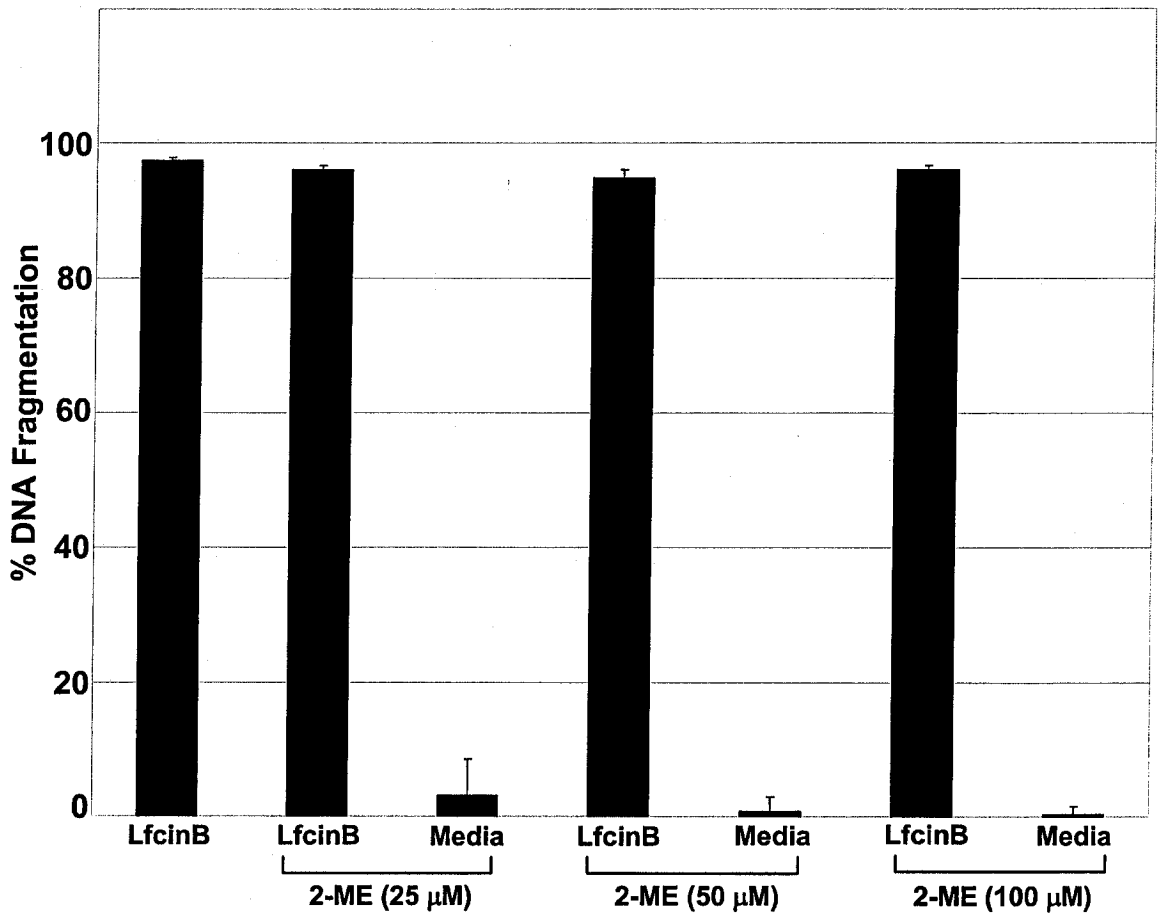
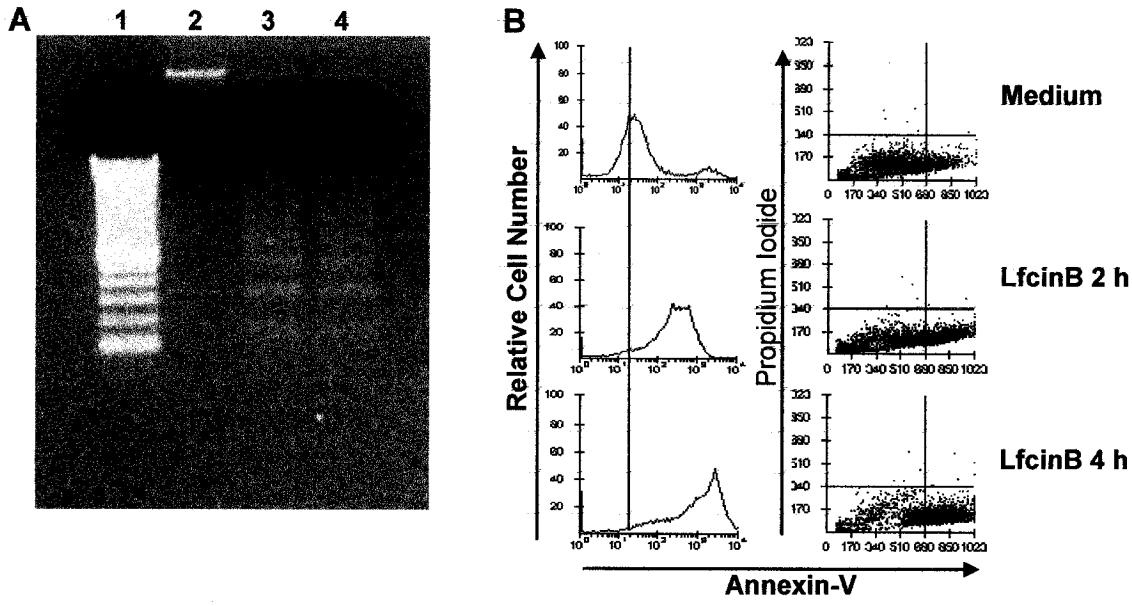

% DNA Fragmentation
100
80
60
40
20
0
LfcinB
LfcinB
Media
LfcinB
Media
LfcinB
Media
2-ME (25 μM)
2-ME (50 μM)
2-ME (100 μM)

Figure 3.3

Figure 3.4 *LfcinB induced apoptosis in Jurkat T leukemia cells.* **A.** Gel electrophoresis analysis of DNA fragmentation in Jurkat T leukemia cells following 4 h treatment with medium alone (lane 2) or 200 μg/mL Lfcin B (lane 3) or 18 h treatment with 50 μM etoposide (lane 4). Lane 1 contained a standard 200 kilobase DNA ladder. **B.** Flow cytometric analysis of Annexin V-FITC stained Jurkat T leukemia cells prior to treatment and after 2 or 4 h treatment with 200 μg/mL Lfcin. Lower left quadrant, living cells: 88.28% (untreated), 64.17% (2 h LfcinB), 38.02% (4 h LfcinB); lower right quadrant, Annexin-V-positive cells: 11.57% (untreated), 35.65% (2 h LfcinB), 61.67% (4 h LfcinB); upper left quadrant, propidium iodide-positive cells: 0.13% (untreated), 0.10% (2 h LfcinB), 0.17% (4 h LfcinB); upper right quadrant, Annexin-V-positive and propidium iodide-positive late apoptotic cells: 0.02% (untreated), 0.05% (2 h LfcinB), 0.14% (4 h LfcinB). **C.** Jurkat T leukemia cells were cultured in the absence (panel i) or presence (panel ii) of 200 μg/mL Lfcin for 18 h and then photographed using a digital camera and a phase contrast light microscope.


A
1
2
3
4
B
Relative Cell Number
Propidium Iodide
Annexin-V
Medium
LfcinB 2 h
LfcinB 4 h

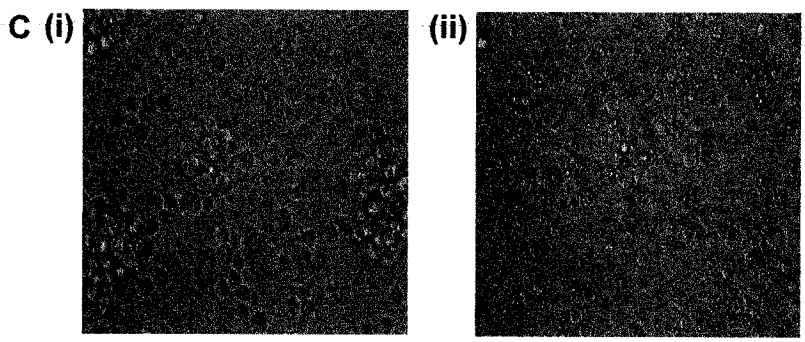

C (i)
(ii)

Figure 3.4

induced by etoposide (lane 4), which is a topoisomerase II inhibitor that is known to cause the apoptotic death of leukemia cells (Barry *et al.*, 1993). I further confirmed that LfcinB was triggering apoptosis in human cancer cells by staining Jurkat T leukemia cells with FITC-conjugated Annexin-V and phycoerythrin-conjugated propidium iodide following LfcinB treatment. Annexin-V binds specifically to phosphatidylserine headgroups that translocate to cell surface during the earliest stages of apoptosis, while propidium iodide binds nuclear components within leaky necrotic cells (Koopman *et al.*, 1994). A time-dependent increase in the percentage of Annexin-V positive cells was observed after 2 and 4 h of exposure to LfcinB (Figure 3.4B). After 4 h exposure to LfcinB, greater than 60% of cells stained positive for Annexin-V and negative for propidium iodide. The lack of propidium iodide staining of LfcinB-treated Jurkat T leukemia cells confirmed that these cells were not dying by necrosis. In addition, light microscopy showed that, in comparison to untreated control cells, LfcinB-treated Jurkat T leukemia cells exhibited the typical apoptotic characteristics of cell shrinkage and membrane blebbing (Figure 3.4C).

Caspase-2, -3, and -9 were Required for LfcinB-Induced Apoptosis.

I next determined the role of individual caspases in LfcinB-induced apoptosis. Figure 3.5A shows that treatment of Jurkat T leukemia cells with selective inhibitors of caspase-2 (Z-VDVAD-FMK), caspase-9 (Z-LEHD-FMK), or caspase-3 (Z-DEVD-FMK) prior to exposure to LfcinB had a significant inhibitory effect on subsequent induction of apoptosis. In contrast, inhibition of caspase-8 activity with Z-IETD-FMK did not significantly impair LfcinB-induced apoptosis. Similar results were obtained when MDA-MB-435 breast carcinoma cells were treated with LfcinB in the presence of

Figure 3.5 *LfcinB-induced apoptosis was mediated by caspase-2, -3, and -9.* **A.** Jurkat T leukemia cells or **B.** MDA-MB-435 breast cancer cells were treated with 100 μg/mL and 200 μg/mL LfcinB, respectively, in the absence or presence of an inhibitor of caspase-2 (Z-VDVAD-FMK), caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), or caspase-9 (Z-LEHD-FMK). After 18 h cytotoxicity was determined by JAM assay. Data are shown as mean % cell death ± SD of quadruplicate determinations and are representative of 3 independent experiments. Differences were extremely significant by Tukey-Kramer multiple comparison test (ns and *** denote not significant and $p<0.001$, respectively, when compared to LfcinB treatment alone).

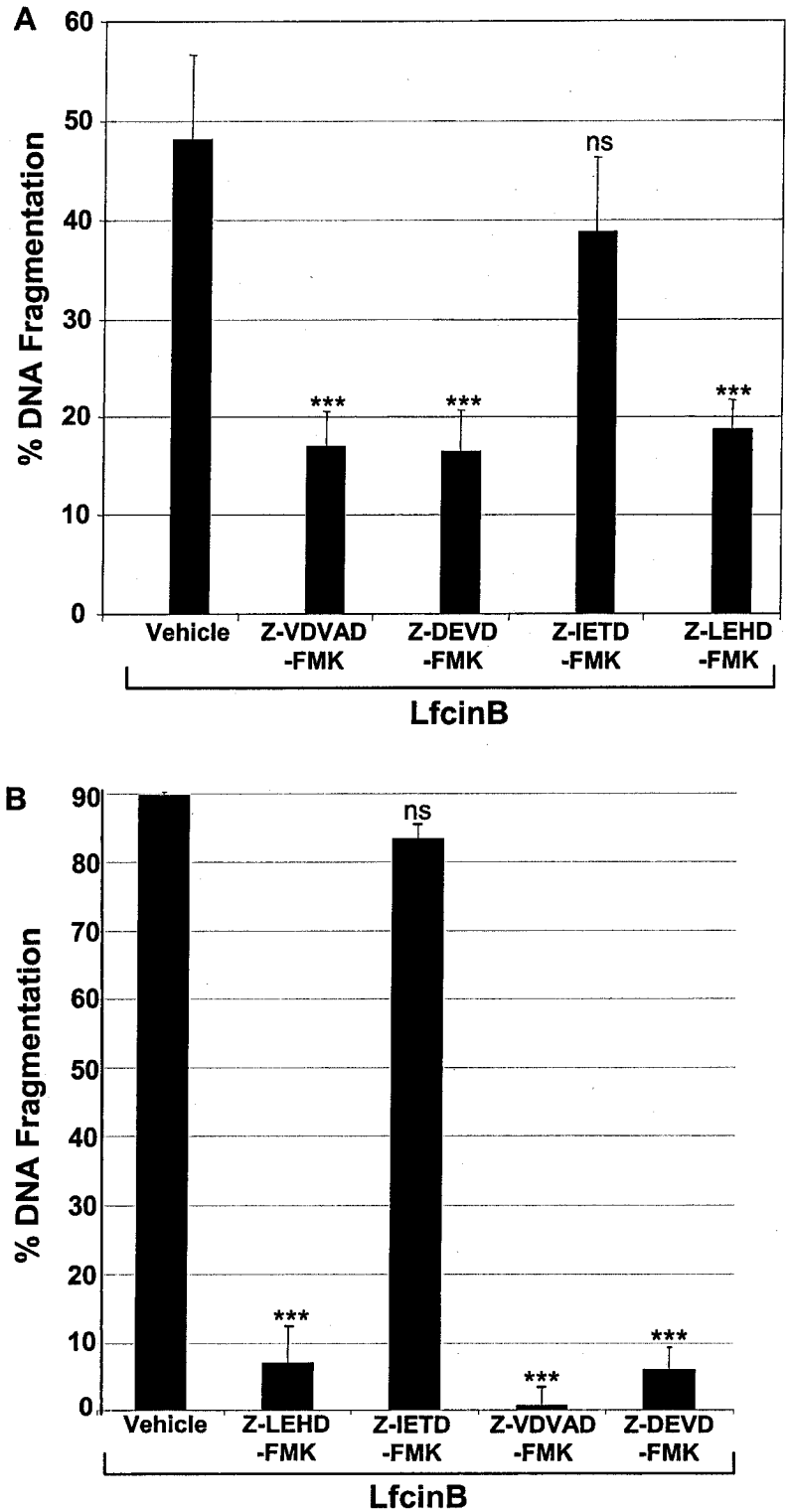

A
% DNA Fragmentation
60
50
40
30
20
10
0
ns
***
***
***
Vehicle
Z-VDVAD -FMK
Z-DEVD -FMK
Z-IETD -FMK
Z-LEHD -FMK
LfcinB
B
% DNA Fragmentation
90
80
70
60
50
40
30
20
10
0
ns
***
***
***
Vehicle
Z-LEHD -FMK
Z-IETD -FMK
Z-VDVAD -FMK
Z-DEVD -FMK
LfcinB

Figure 3.5

selective caspase inhibitors (Figure 3.5B). These findings suggest that LfcinB killed leukemia and carcinoma cells by activating caspase-2, -3, and -9, but not -8, which was consistent with induction of the intrinsic pathway of apoptosis. Immunoblot detection of cleavage products of specific procaspases was used to confirm the activation of caspase-2, -3, and -9 by LfcinB. Figure 3.6A shows the presence of cleavage products of procaspase-2 (34 and 27 kDa protein bands), procaspase-3 (17 kDa protein band), and procaspase-9 (35 and 17 kDa protein bands) in Jurkat T leukemia cells that were exposed to 200 μg/mL LfcinB for 2 h. In contrast, there was no apparent increase in cleavage products of procaspase-8 (41 and 18 kDa protein bands) following LfcinB treatment. A time course analysis of caspase activity in cellular lysates extracted from LfcinB-treated Jurkat T leukemia cells revealed that caspase-2 was activated within 2 h of LfcinB treatment (Figure 3.6B). Substantial activation of caspase-3 was also observed at the 2 h time-point. Following 4 h exposure to LfcinB, caspase-2 and caspase-3 activity in Jurkat T leukemia cells had increased dramatically. Low level caspase-8 and caspase-9 activation was detected at the 4 h time-point. Failure to detect caspase-8 activity prior to caspase-3 activity, as well as the failure of Z-IETD-FMK to prevent LfcinB-induced apoptosis argued against an important role for death receptors, even though death receptors have been implicated in apoptosis induction by other anticancer agents (Morgan *et al.*, 2002; Friesen *et al.*, 1996; Williams *et al.*, 1997).

To confirm that LfcinB-induced apoptosis in cancer cells was independent of Fas, TRAIL DR4, and TRAIL DR5 death receptors, I used flow cytometry to measure Fas, Fas ligand, DR4, DR5, and TRAIL expression by Jurkat T leukemia cells following a 4 h

Figure 3.6 *LfcinB treatment resulted in the cleavage of procaspase-2, -3, and -9.* **A.** Jurkat T leukemia cells were treated with 200 μg/mL LfcinB for 2 h, washed, and lysed with CHAPS buffer. Cell lysates were clarified by centrifugation prior to immunoblot analysis using anti-caspase-2, -3, -8, and -9 antibodies. The solid arrowhead indicates the procaspase form while the open arrowheads indicate the major cleavage products. The blot was stripped between each antibody use. Actin expression was determined to confirm equal loading and transfer of protein samples. **B.** Cell lysates were prepared from Jurkat T leukemia cells following 2 h (open bars) and 4 h (filled bars) treatment with 200 μg/mL LfcinB, and combined with chromagenic substrates that are selective for caspase-2, -3, -8, and -9. Caspase activity was determined by absorbance at 405 nm. Data shown are representative of 3 independent experiments.

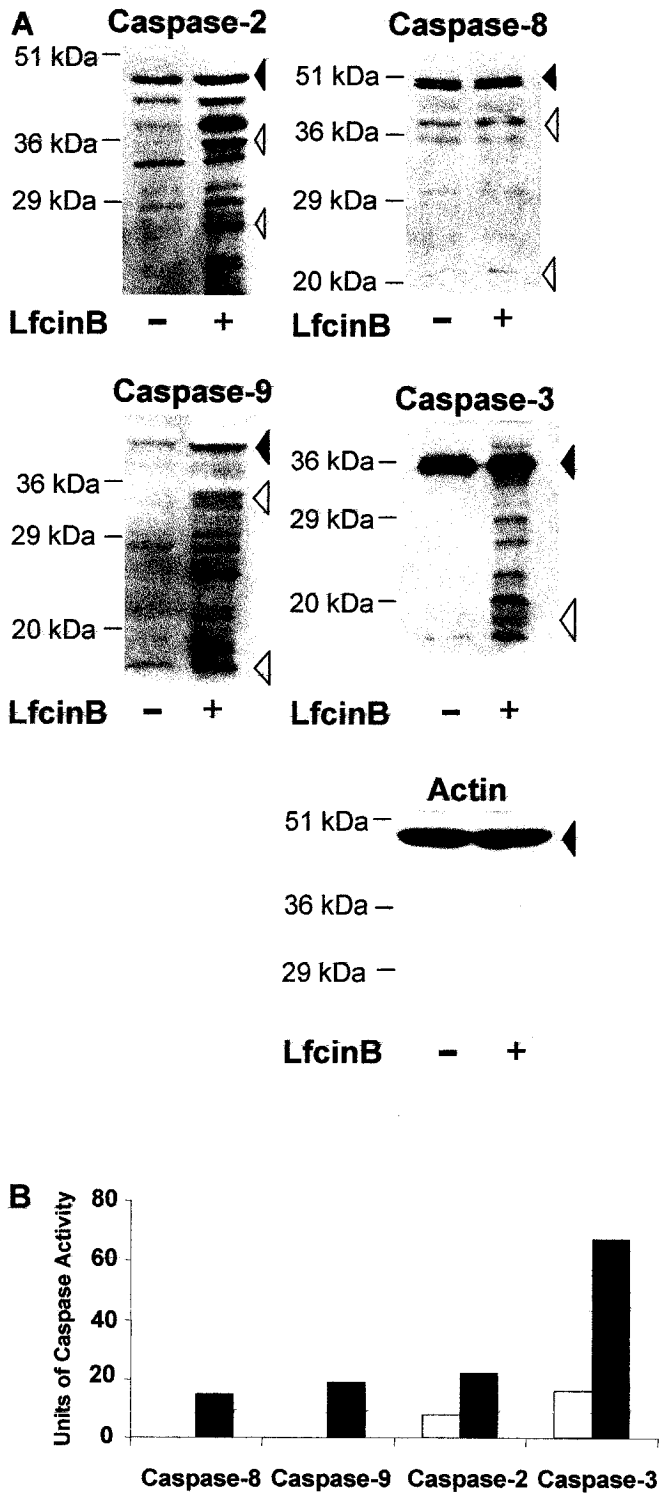

A
Caspase-2
51 kDa
36 kDa
29 kDa
LfcinB - +
Caspase-8
51 kDa
36 kDa
29 kDa
20 kDa
LfcinB - +
Caspase-9
36 kDa
29 kDa
20 kDa
LfcinB - +
Caspase-3
36 kDa
29 kDa
20 kDa
LfcinB - +
Actin
51 kDa
36 kDa
29 kDa
LfcinB - +
B
Units of Caspase Activity
80
60
40
20
0
Caspase-8 Caspase-9 Caspase-2 Caspase-3

Figure 3.6

exposure to LfcinB. There was no change in Fas, Fas ligand, DR4, or DR5 expression when cells were exposed to 200 μg/mL LfcinB, although a slight decrease in TRAIL expression was noted (3.7A). In addition, I treated Jurkat T leukemia cells with 100 μg/mL LfcinB in the absence or presence of neutralizing mAbs to Fas ligand or TRAIL at a concentration (10 μg/mL) that effectively inhibited Fas ligand- or TRAIL-induced apoptosis, respectively. Neither neutralizing mAb affected the ability of LfcinB to trigger apoptosis (3.7B), indicating that Fas/Fas ligand and/or TRAIL/DR4/DR5 interactions were not important for LfcinB-induced apoptosis.

Inhibitors of MAPKs had no effect on LfcinB-induced cytotoxicity in Jurkat T leukemia cells.

MAPKs are a group of serine-threonine kinases that are involved in the regulation of cell survival and death (Dong *et al.*, 2002; Hommes *et al.*, 2003). I therefore wanted to understand whether LfcinB-induced apoptosis in human tumour cells required MAPK activity. Jurkat T leukemia cells were pre-treated with inhibitors of JNK (10 μM SP600125), ERK1/2 (10 μM PD98059), and p38 MAPK (10 μM SB203580) prior to LfcinB exposure (100 μg/mL). Then cytotoxicity was determined by JAM assay. The MAPK inhibitors had no effect on LfcinB-induced DNA fragmentation (Figure 3.8), which suggests that MAPKs are not involved in LfcinB-induced cytotoxicity of human tumour cells.

LfcinB Caused Dissipation of the Mitochondrial Transmembrane Potential, Mitochondrial Swelling, and Cytochrome c Release.

The involvement of caspase-2 and caspase-9 in the LfcinB-induced apoptotic

Figure 3.7 *LfcinB-induced apoptosis was independent of death receptors, Fas, and TRAIL DR4 and DR5.* **A.** Jurkat T leukemia cells were treated with 200 μg/mL LfcinB for 4 h, and flow cytometric analysis was performed to measure Fas, Fas ligand, DR4, DR5, and TRAIL expression was performed. **B.** Jurkat T leukemia cells were treated for 18 hours with 100 μg/mL LfcinB in the absence or presence of neutralizing mAbs to Fas ligand or TRAIL (10 μg/ml). Differences were significant by Tukey-Kramer multiple comparison test (ns, *, and ** denote not significant, $p<0.05$, and $p<0.01$, respectively, when compared to LfcinB treatment alone).

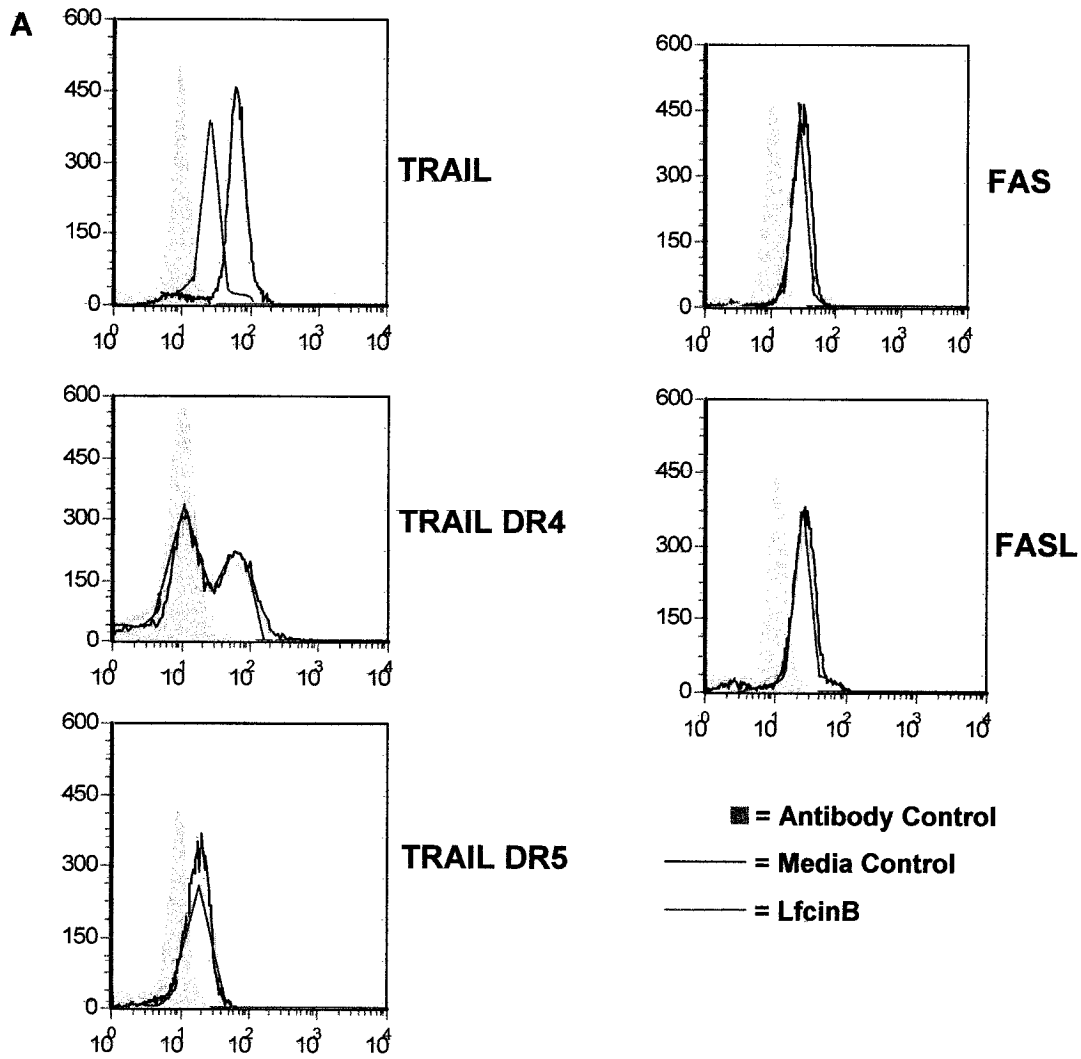

A
600
450
300
150
0
TRAIL
FAS
TRAIL DR4
FASL
TRAIL DR5
= Antibody Control
= Media Control
= LfcinB

Figure 3.7

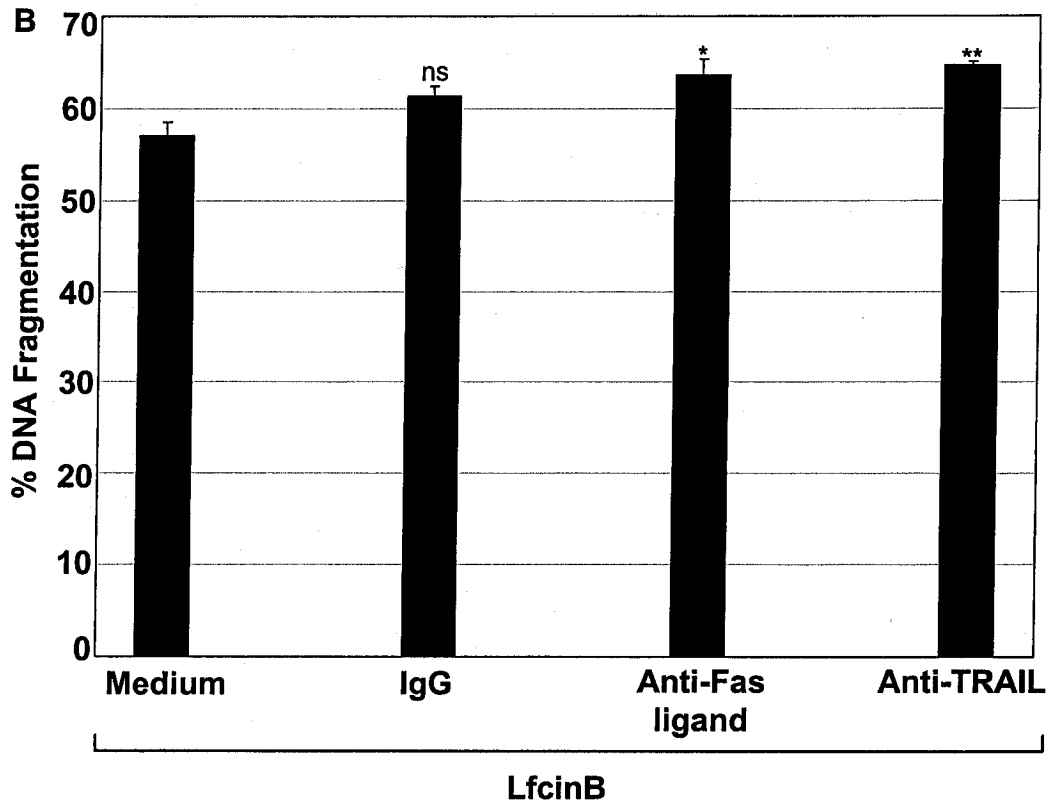

B
70
60
50
40
30
20
10
0
% DNA Fragmentation
ns
*
**
Medium
IgG
Anti-Fas ligand
Anti-TRAIL
LfcinB

Figure 3.7

Figure 3.8 *Inhibitors of MAPKs had no effect on LfcinB-induced cytotoxicity in Jurkat T leukemia cells.* Jurkat T leukemia cells were pre-treated with inhibitors of c-Jun N-terminal kinase (JNK; SP 600125, 10 μM), extracellular signal-regulated kinase ½ (ERK1/2; PD 98059, 10 μM), and p38 MAPK (SB 203580, 10 μM) for 30 min, followed by treatment with 100 μg/mL of LfcinB for 18 h. Cytotoxicity was determined by JAM assay. Differences were extremely significant by Tukey-Kramer multiple comparison test (ns, and *** denote not significant and $p<0.001$, respectively, when compared to LfcinB treatment alone).

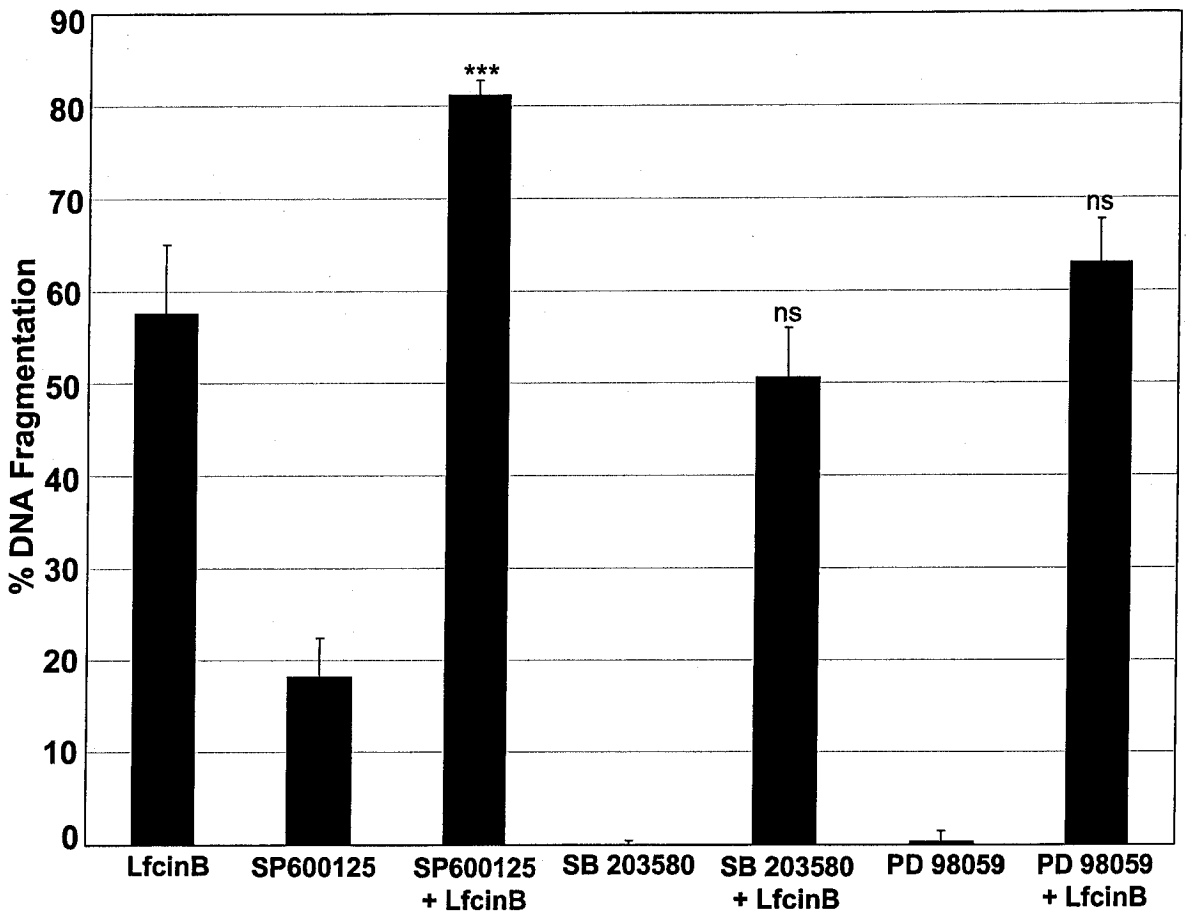

90
80
70
60
50
40
30
20
10
0
% DNA Fragmentation
***
ns
ns
LfcinB
SP600125
SP600125 + LfcinB
SB 203580
SB 203580 + LfcinB
PD 98059
PD 98059 + LfcinB

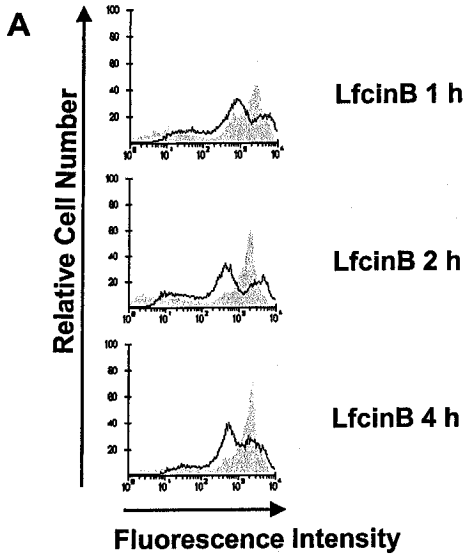
Figure 3.8

death of Jurkat T leukemia cells suggested that LfcinB treatment might disrupt the membrane integrity of cancer cell mitochondria. I therefore employed $DiOC_6$, a fluorescent dye that localizes to intact mitochondria (Zamzami *et al.*, 1995), to determine the effect of LfcinB on mitochondrial membrane integrity. Figure 3.9A shows a time-dependent leftward shift in $DiOC_6$ fluorescence of Jurkat T leukemia cells following exposure to LfcinB, indicating that LfcinB treatment resulted in the loss of mitochondrial membrane integrity and dissipation of mitochondrial transmembrane potential. In addition, Jurkat T leukemia cells that were engineered to overexpress the mitochondria-associated anti-apoptotic protein Bcl-2 were rendered less sensitive to killing by LfcinB (Figure 3.9B). The mitochondria of LfcinB-treated Jurkat T leukemia cells were also swollen in comparison to mitochondria of control cells (Figure 3.10A, panel ii vs. panel i). Examination of 200 untreated and 200 LfcinB-treated Jurkat T leukemia cells revealed that over 60% of mitochondria in LfcinB-treated cells were swollen (Figure 3.10B). Some swollen mitochondria were also observed in untreated cells, which was most likely a reflection of background apoptosis that normally occurs in Jurkat T leukemia cell cultures. Importantly, LfcinB treatment resulted in the release of cytochrome c from mitochondria to the cytosol of Jurkat T leukemia cells (Figure 3.10C). In contrast, no cytosolic cytochrome c was detected in control cells. Taken together, these data indicate that LfcinB induced the mitochondrial pathway of apoptosis in human cancer cells.

Generation of ROS and Caspase-2 Activity was Required for LfcinB-Induced Damage to Mitochondria.

To determine whether exposure of cancer cells to LfcinB resulted in the generation of ROS, Jurkat T leukemia cells were loaded with dihydroethidium, which is

Figure 3.9 *LfcinB-induced apoptosis was a mitochondria-dependent process.* **A.** Jurkat T leukemia cells were cultured for the indicated periods of time in the absence or presence or 200 μg/mL LfcinB. Cells were loaded with $DiOC_6$ (40 nM) 30 min prior to the end of the assay. Loss of mitochondrial transmembrane potential is seen as a leftward shift in $DiOC_6$ fluorescence by LfcinB-treated cells (open peak) in comparison to untreated cells (filled peak), as determined by flow cytometry. **B.** Unmodified Jurkat T leukemia cells, vector-only-transfected cells, and Bcl-2-overexpressing cells were treated with 200 μg/mL LfcinB for18 h. Cytotoxicity was determined by JAM assay. Data are shown as mean % cell death ± SD of quadruplicate determinations and are representative of 3 independent experiments. Differences were extremely significant by Tukey-Kramer multiple comparison test (ns and *** denote not significant and $p<0.001$, respectively, when compared to LfcinB treatment alone).


A
Relative Cell Number
LfcinB 1 h
LfcinB 2 h
LfcinB 4 h
Fluorescence Intensity

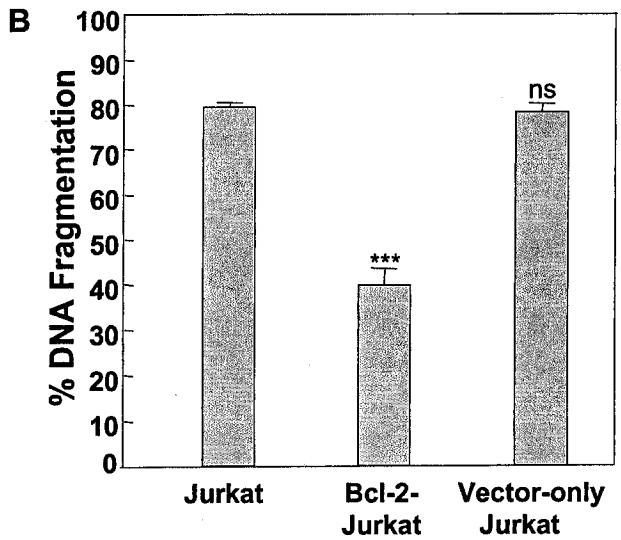

B
% DNA Fragmentation
100
90
80
70
60
50
40
30
20
10
0
***
ns
Jurkat
Bcl-2-Jurkat
Vector-only Jurkat

Figure 3.9

Figure 3.10 *LfcinB treatment resulted in swelling of mitochondria and cytochrome c release.* **A.** Jurkat T leukemia cells were cultured for 2 h in the absence (panel i) or presence (panel ii) of 200 μg/mL LfcinB, fixed with glutaraldehyde, sectioned, and mitochondria were visualized by transmission electron microscopy (30,000× magnification). **B.** The % swollen mitochondria in untreated versus LfcinB-treated Jurkat T leukemia cells was determined by examining 200 untreated cells and 200 LfcinB-treated cells. **C.** Jurkat T leukemia cells were cultured in the absence or presence of 200 μg/mL LfcinB for 2 h, washed, and lysed with digitonin buffer. Cell lysates were clarified by centrifugation prior to immunoblot analysis using anti-cytochrome c antibody (15 kDa). The blot was then stripped and actin expression was determined to confirm equal loading and transfer of protein samples.

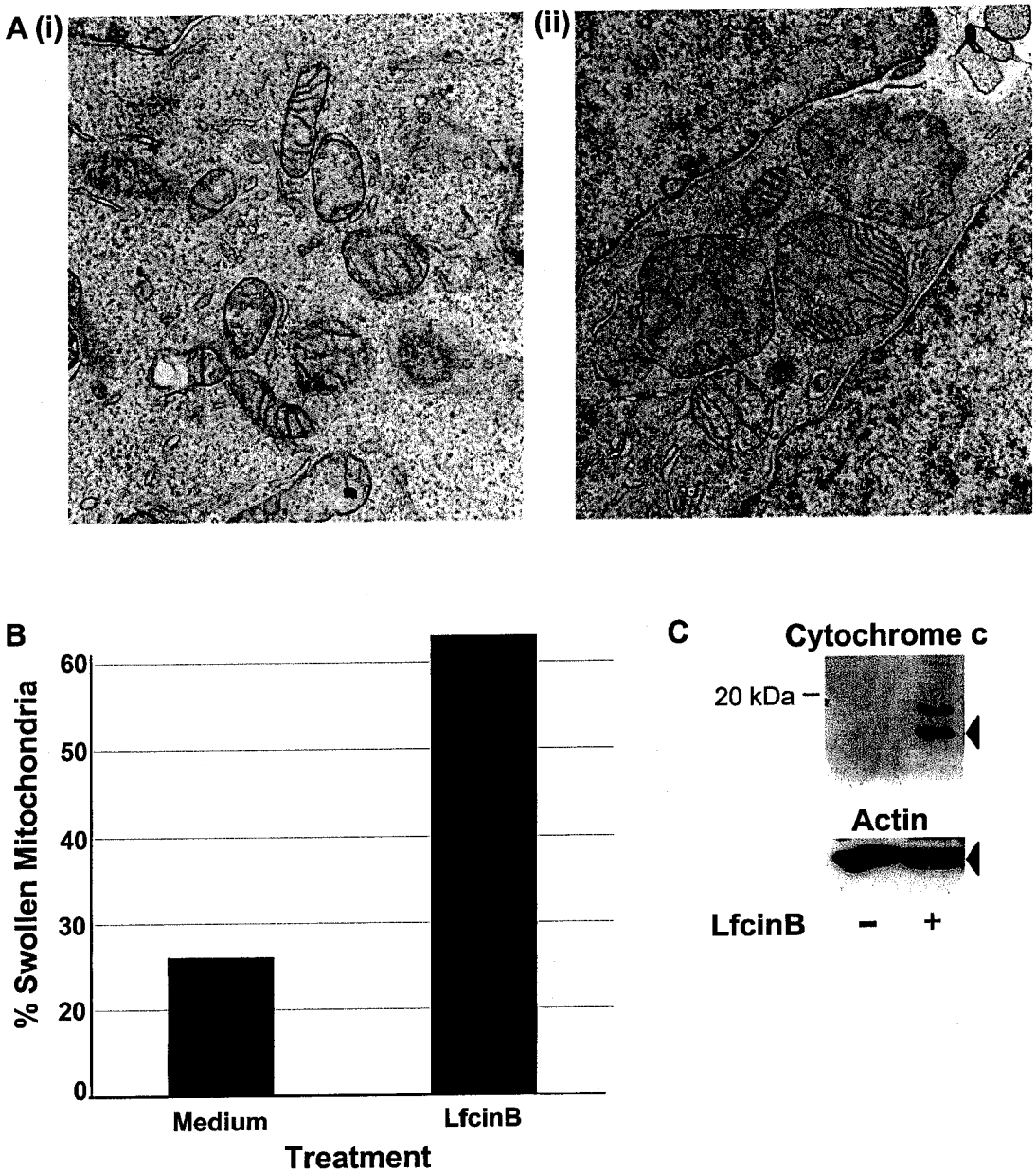
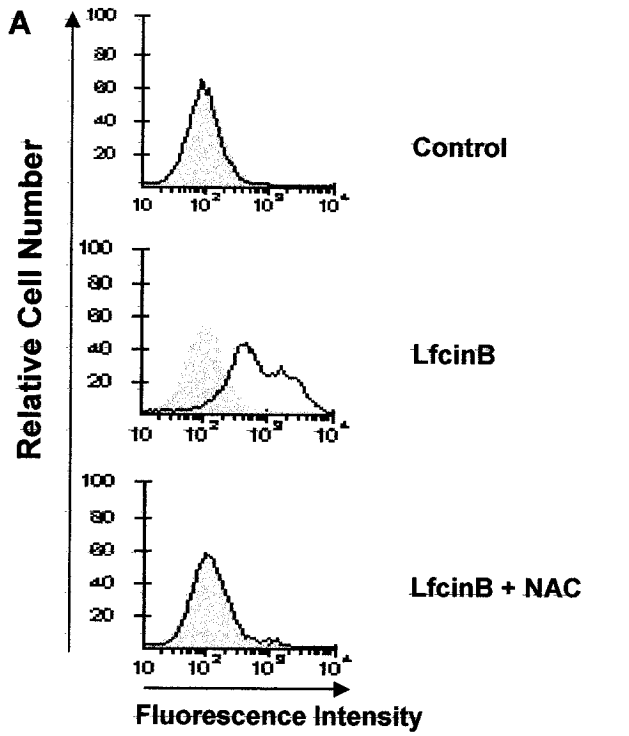

A (i)
(ii)
B
% Swollen Mitochondria
60
50
40
30
20
0
Medium
LfcinB
Treatment
C
Cytochrome c
20 kDa
Actin
LfcinB
-
+

Figure 3.10

oxidized to red-fluorescent ethidium that accumulates in the nucleus (Perticarari *et al.*, 1991), and then treated with LfcinB. Flow cytometric analysis revealed a dramatic increase in fluorescence 1 h after cell exposure to LfcinB, indicating the early generation of ROS (Figure 3.11A). The antioxidant *N*-acetyl-*L*-cysteine ablated the LfcinB-induced accumulation of ROS in Jurkat T leukemia cells. Furthermore, LfcinB-induced DNA fragmentation was reduced in a dose-dependent fashion when Jurkat T leukemia cells were exposed to LfcinB in the presence of increasing concentrations of the antioxidants glutathione or *N*-acetyl-*L*-cysteine (Figure 3.11B). I next showed that the LfcinB-induced generation of ROS in Jurkat T leukemia cells did not occur as a consequence of caspase activation since pretreatment with the broad spectrum caspase inhibitor Z-VAD-FMK did not prevent intracellular accumulation of ROS in response to LfcinB treatment (Figure 3.12A). However, activation of caspase-2 was a necessary condition for the LfcinB-induced reduction in mitochondrial membrane integrity since pretreatment of Jurkat T leukemia cells with the caspase-2 inhibitor Z-VDVAD-FMK prevented dissipation of mitochondrial transmembrane potential in response to LfcinB (Figure 3.12B). In contrast, inhibition of caspase-3 or caspase-9 activity by pretreatment with Z-DEVD-FMK or Z-LEHD-FMK, respectively, did not prevent LfcinB-induced loss of mitochondrial transmembrane potential, implying that these particular caspases are activated downstream of caspase-2 as a consequence of diminished mitochondrial membrane integrity. The loss of mitochondrial transmembrane potential by Jurkat T leukemia cells in response to LfcinB treatment was partially reversed in the presence of the antioxidant *N*-acetyl-*L*-cysteine (Figure 3.12C), suggesting that ROS are involved in the initiation of LfcinB-induced apoptosis. Moreover, preliminary experiments suggested that caspase-2

Figure 3.11 *LfcinB-induced apoptosis involved the production of ROS.* **A.** Jurkat T leukemia cells were cultured in the absence or presence of 200 μg/mL LfcinB without or with 10 mM *N*-acetyl-*L*-cysteine (NAC) for 1 h and then loaded with dihydroethidium. The presence of ROS was determined by flow cytometry (open peak) after 20 min. Background fluorescence is indicated by the filled peaks. **B.** Jurkat T leukemia cells were treated with 200 μg/mL LfcinB in the absence or presence of the indicated concentrations of glutathione (GSH) or NAC. After 18 h, cytotoxicity was determined by JAM assay. Data are shown as mean % cell death ± SD of quadruplicate determinations and are representative of 3 independent experiments. Differences were extremely significant by ANOVA ($p>0.0001$) and Tukey-Kramer multiple comparison tests (*** denotes $p<0.001$ when compared to LfcinB treatment alone).


A
Relative Cell Number
Control
LfcinB
LfcinB + NAC
Fluorescence Intensity

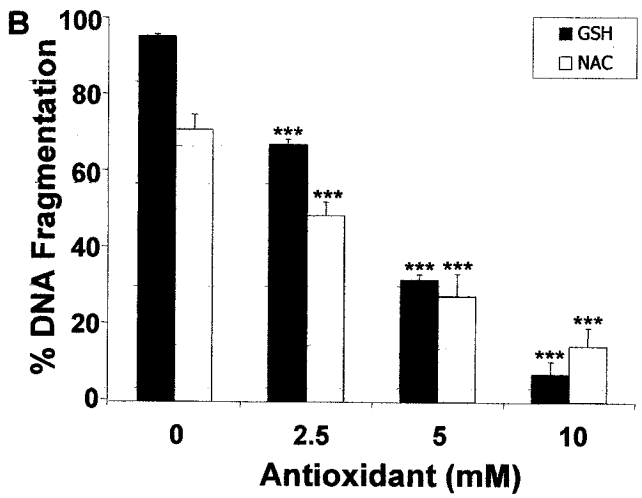

B
% DNA Fragmentation
GSH
NAC
Antioxidant (mM)

Figure 3.11

Figure 3.12 *Sequential contribution of ROS and caspase-2 activation to LfcinB-induced changes in mitochondrial membrane integrity.* **A.** Jurkat T leukemia cells were pretreated with the drug vehicle (DMSO) or 50 μM Z-VAD-FMK and then cultured in the absence or presence of 200 μg/mL LfcinB plus the drug vehicle or Z-VAD-FMK for 1 h, followed by loading with dihydroethidium. LfcinB-induced production of ROS was determined by flow cytometry (open peak) after 20 min. Background fluorescence by cells treated with the drug vehicle or Z-VAD-FMK alone is indicated by the filled peaks. **B.** Jurkat T leukemia cells were pretreated with the drug vehicle (DMSO) or 50 μM Z-VDVAD-FMK (caspase-2 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), or Z-LEHD-FMK (caspase-9 inhibitor) and then cultured for 2 h in the absence or presence of 200 μg/mL LfcinB plus the drug vehicle or the appropriate caspase inhibitor. Cells were loaded with $DiOC_6$ (40 nM) 30 min prior to the end of the assay. Loss of mitochondrial transmembrane potential is seen as a leftward shift in $DiOC_6$ fluorescence by LfcinB-treated cells (open peak) in comparison to control cells (filled peak), as determined by flow cytometry. **C.** Jurkat T leukemia cells were cultured for 2 h in the absence or presence of 200 μg/mL LfcinB without or with 10 mM *N*-acetyl-*L*-cysteine (NAC). Cells were loaded with $DiOC_6$ (40 nM) 30 min prior to the end of the assay and flow cytometry was used to compare changes in mitochondrial transmembrane potential in LfcinB-treated cells (open peak) with control cells (filled peak). Data in panels A, B, and C are representative of 2 independent experiments.

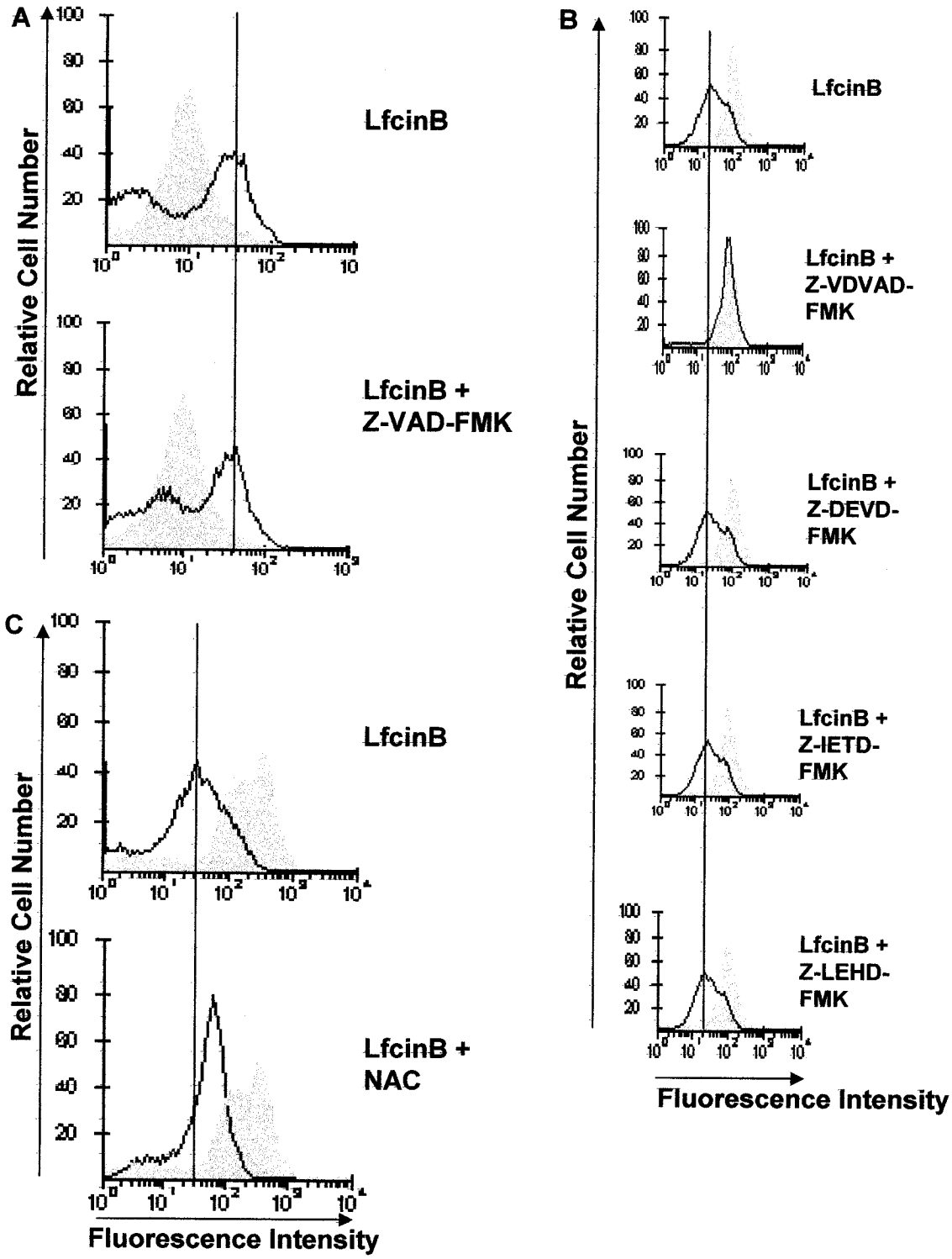

A
Relative Cell Number
LfcinB
LfcinB +
Z-VAD-FMK
B
Relative Cell Number
LfcinB
LfcinB +
Z-VDVAD-
FMK
LfcinB +
Z-DEVD-
FMK
LfcinB +
Z-IETD-
FMK
LfcinB +
Z-LEHD-
FMK
Fluorescence Intensity
C
Relative Cell Number
LfcinB
LfcinB +
NAC
Fluorescence Intensity

Figure 3.12

Figure 3.13 *LfcinB-induced apoptosis in Jurkat T leukemia cells was partially blocked by caspase-2 inhibition or NAC treatment.* **A.** Gel electrophoresis analysis of DNA fragmentation in Jurkat T leukemia cells following 4 h treatment with medium alone (lane 2), DMSO (lane 3), 50 μM caspase-2 inhibitor (Z-VDVAD-FMK, lane 4), 200 μg/ml Lfcin B (lane 5), caspase-2 inhibitor + Lfcin B (lane 6), or 18 h treatment with 50 μM etoposide (lane 7). Lane 1 contained a standard 200 kilobase DNA ladder. (n=1). **B.** Gel electrophoresis analysis of DNA fragmentation in Jurkat T leukemia cells following 4 h treatment with medium alone (lane 2), DMSO (lane 3), NAC (lane 4), 200 μg/ml Lfcin B (lane 5), NAC + Lfcin B (lane 6), or 18 h treatment with 50 μM etoposide (lane 7). Lane 1 contained a standard 200 kilobase DNA ladder. (n=1).

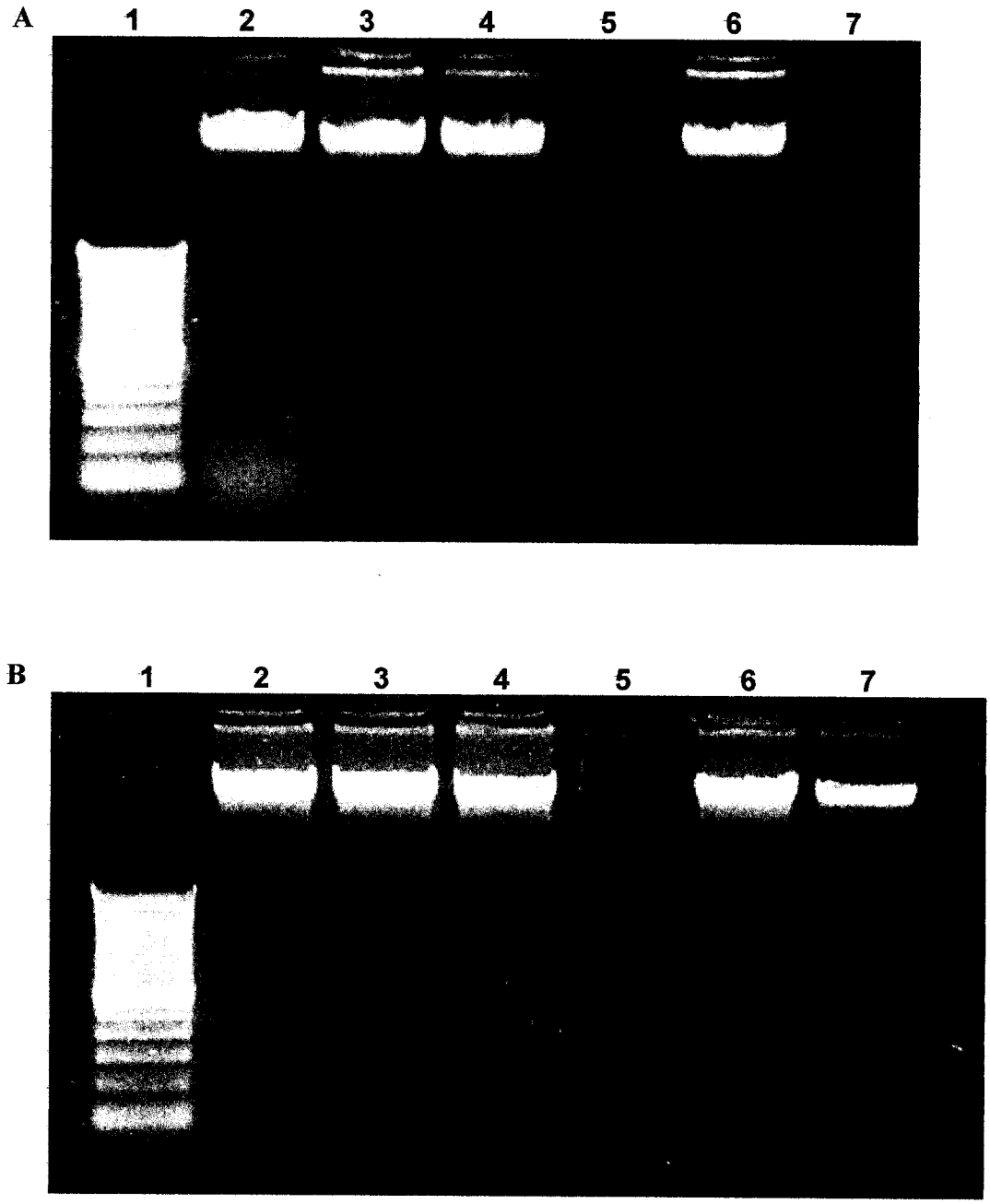


Figure 3.13

inhibition or NAC addition prior to LfcinB treatment of Jurkat T leukemia cells may, at least partially, block DNA fragmentation (Figure 3.13A and B).

Cytotoxic Activity of a 10 Amino Acid Fragment Derived from LfcinB.

I next investigated 10 amino acid and 6 amino acid fragments of LfcinB for cytotoxic activity against Jurkat T leukemia cells. I hypothesized that the cytotoxic activity of LfcinB might reside within the same sequence of 10 amino acid (FKCRRWQWRM) and/or 6 amino acid (RRWQWR) residues that have previously been reported to be responsible for the antimicrobial activity of LfcinB (Hoek *et al.*, 1997; Schibli *et al.*, 1999). A comparison of the apoptosis-inducing activity of the 10-mer and 6-mer peptide fragments of LfcinB with that of intact LfcinB revealed that the 10-mer peptide fragment induced DNA fragmentation in Jurkat T leukemia cells (37 ± 10%), but not to the same extent as LfcinB (93 ± 1%) at equimolar (63μM) concentrations over 18 h of culture (Fig. 3.14). In contrast, the 6-mer peptide fragment was unable to cause any substantial DNA fragmentation. Taken together, these data indicated that the apoptosis-inducing activity of LfcinB may in part be due to the 10 amino acid residues that also have antimicrobial activity (Hoek *et al.*, 1997), but that this 10-mer sequence is not sufficient to induce the same high level of DNA fragmentation that occurs following treatment with full length LfcinB. Of interest, preliminary fluorescent microscopy analysis of Jurkat T leukemia cells exposed to biotin-6-mer showed that the 6-mer peptide bound to the surface of these cells (Figure 3.15A). A chromium release assay was then used to determine whether the 6-mer peptide was cytotoxic to Jurkat T leukemia

Figure 3.14 *FKCRRWQWRM, a 10 amino acid derivative of LfcinB, had apoptogenic activity.* Jurkat T leukemia cells were cultured in the absence or presence of 31 μM or 63 μM intact LfcinB (25-mer), 10 amino acid (10-mer), or 6 amino acid (6-mer) derivatives of LfcinB for 18 h. A 63 μM concentration of the LfcinB 25-mer is equivalent to 200 μg/mL. Cytotoxicity was then determined by JAM assay. Data are shown as mean % cell death ± SD of quadruplicate determinations and are representative of 3 independent experiments. Differences were not significant for the 6-mer by ANOVA (>0.05) or Tukey-Kramer multiple comparison tests (ns denotes not significant when compared to medium control). Differences were extremely significant for the 10-mer and 25-mer by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (*** denotes $p<0.001$ when compared to medium control).

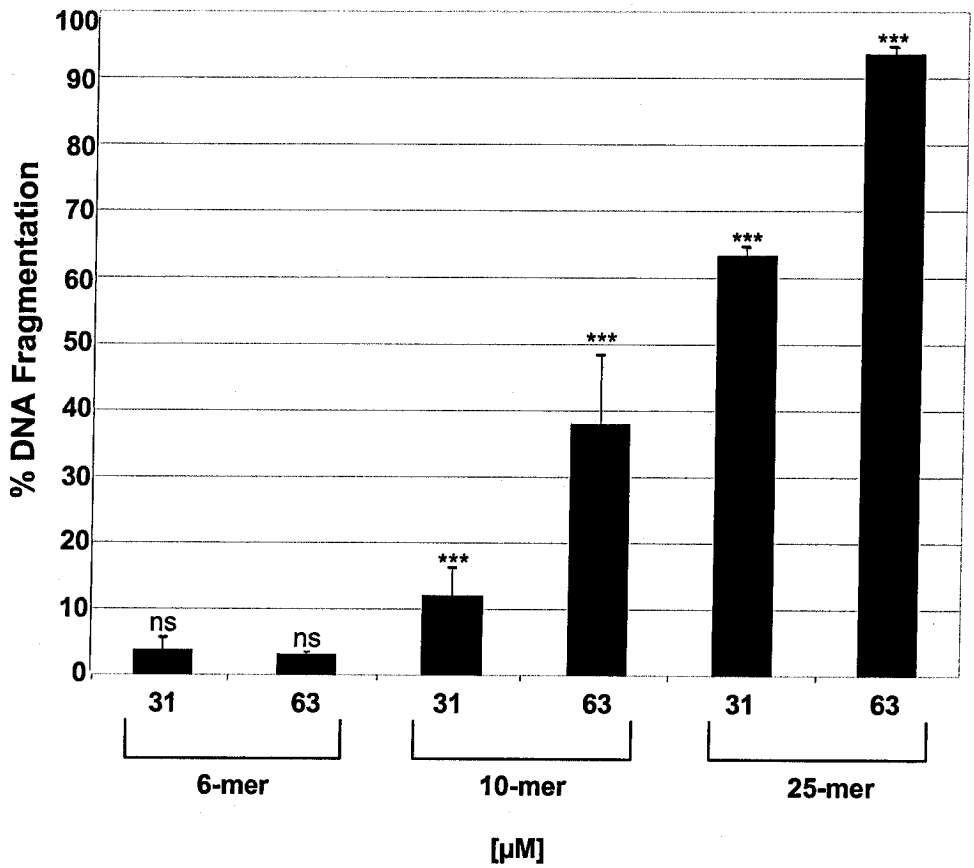

% DNA Fragmentation
100
90
80
70
60
50
40
30
20
10
0
ns
ns
***
***
***
***
31
63
31
63
31
63
6-mer
10-mer
25-mer
[µM]

Figure 3.14

Figure 3.15 *The 6 amino acid LfcinB fragment, RRWQWR, bound to Jurkat T leukemia cells, but did not induce cell death.* **A.** Jurkat T leukemia cells were treated with 63 μM of LfcinB, or 63 μM 6-mer peptide in RPMI-1640 medium containing 0.5% FCS for 8 h. Cytotoxicity was determined by chromium release assay. **B.** Jurkat T leukemia cells were treated with 32 μM biotin-6-mer for 1 h, and fluorescent microscopy was performed (200× magnification).

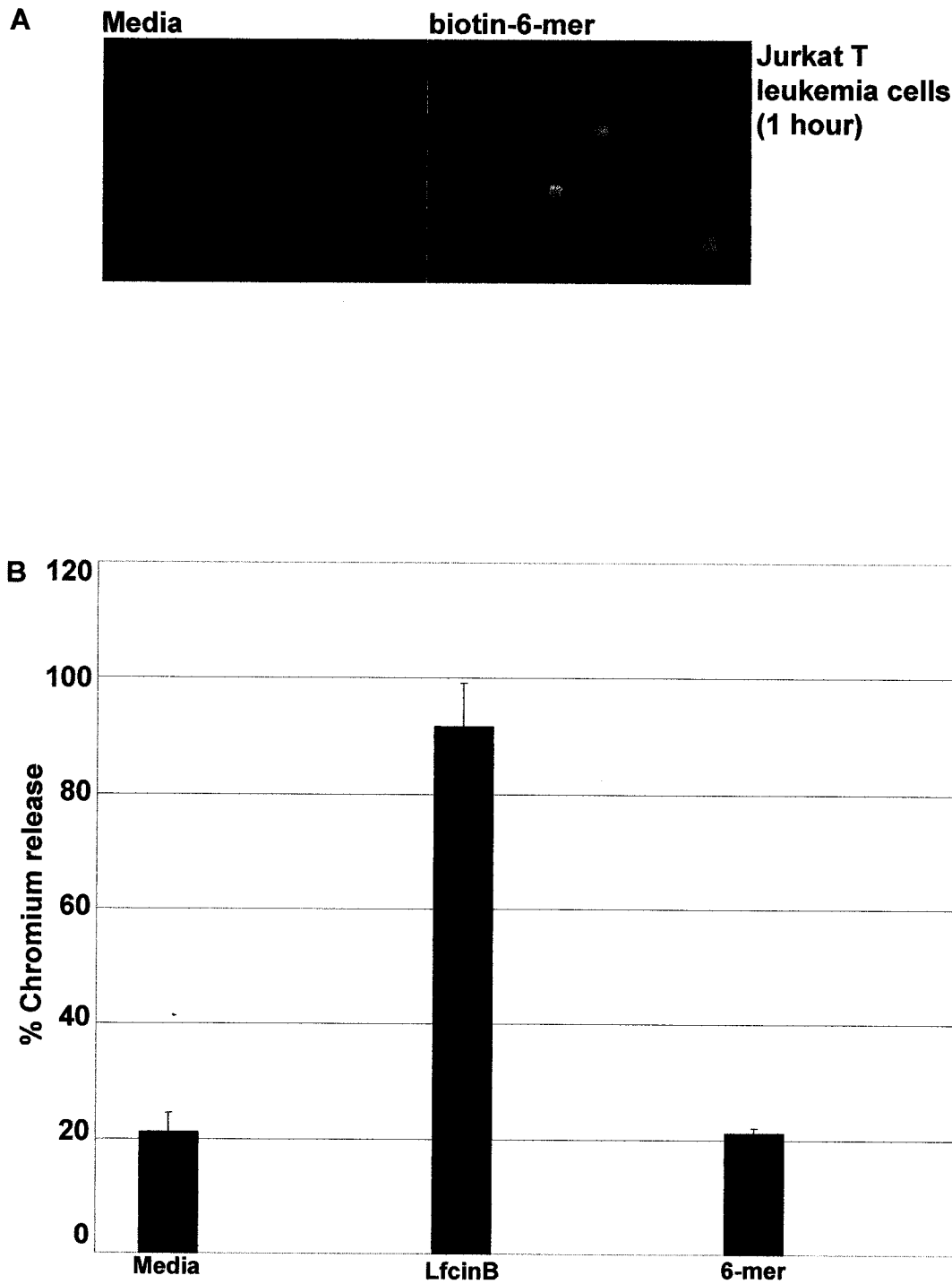

A
Media
biotin-6-mer
Jurkat T leukemia cells (1 hour)
B
120
100
80
60
40
20
0
% Chromium release
Media
LfcinB
6-mer

Figure 3.15

cells, even though there was no DNA fragmentation (Figure 3.15B). This experiment showed that the 6-mer peptide was not cytotoxic to Jurkat T leukemia cells.

LfcinB-Induced Apoptosis in Raji and Ramos B Lymphoma Cell Lines is Inhibited by Increasing Serum Concentration.

JAM assays were performed to determine whether exposure of Raji and Ramos human B lymphoma cells to LfcinB resulted in the cell death. Figure 3.16 shows that LfcinB strongly induced DNA fragmentation at 0.5% and 1% FCS concentrations in both B lymphoma cell lines. However, apoptosis was completely inhibited in Raji cells by 2.5% and 5% FCS (Figure 3.16A). Apoptosis was also inhibited by 2.5% and 5% FCS in Ramos cells (Figure 3.16B), albeit to a lesser extent than in Raji cells (Figure 3.16A). This finding was consistent with a previous report that LfcinB exhibits optimal cytotoxic activity at lower serum concentrations (Yoo *et al.*, 1997b). The significance of this observation will be further addressed in Chapter 4. Analysis of LfcinB-induced apoptosis in Raji and Ramos cells was also carried out by Hoechst staining, which showed that LfcinB treatment resulted in chromatin condensation and apoptotic body formation characteristic of apoptosis (Figure 3.16C).

LfcinB Treatment Increased Mean Survival Time of Immune-Deficient Mice Bearing Disseminated Tumours.

Investigation of LfcinB as a possible therapeutic agent was accomplished using SCID/beige mice bearing disseminated Ramos B lymphoma, which models B cell non-Hodgkin's lymphoma (Yoshida *et al.*, 1997; Griffiths *et al.*, 2003). Mice treated with LfcinB on day 1 after lymphoma cell injection showed a 1.4-fold increase in median

Figure 3.16 *LfcinB killed Raji and Ramos B lymphoma lines by apoptosis. Increasing serum concentrations had inhibitory effects.* The JAM assay was used to measure DNA fragmentation in Raji (**A**) and Ramos (**B**) B lymphoma cells undergoing apoptosis. Briefly, B lymphoma cells were labeled with tritiated thymidine for 4 hours at 37°C. Radiolabeled cells (50×10^5 cells/mL) were added in quadruplicate to 96-well flat-bottomed tissue culture plates in the absence or presence of the desired concentrations of BSA and LfcinB. Following 18-hour incubation, DNA was harvested and percent fragmentation was determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (ns and *** denote not significant and $p<0.001$, respectively, when compared to medium control).
C. Further analysis of cell death was performed by Hoechst 33342 staining. Raji and Ramos B lymphoma cell lines were treated with or without LfcinB (200 μg/mL) for 8 hours. At this time, slides were stained with Hoechst 33342 trihydrochloride dye (10 μg/mL, 10 min) and fluorescence was monitored with an ultraviolet microscopy (200× magnification).

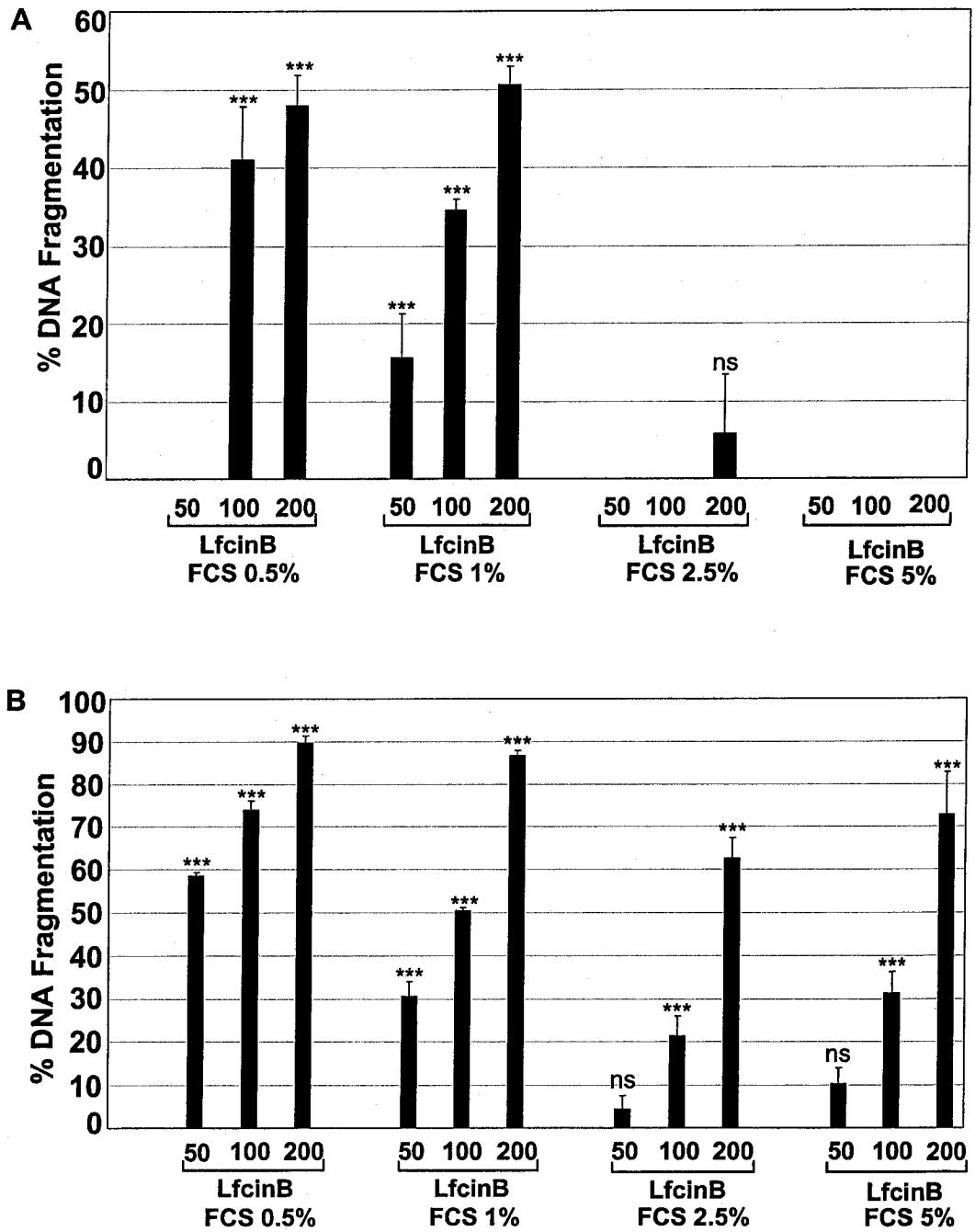

A
% DNA Fragmentation
60
50
40
30
20
10
0
***
ns
50 100 200
LfcinB
FCS 0.5%
FCS 1%
FCS 2.5%
FCS 5%
B
100
90
80
70

Figure 3.16

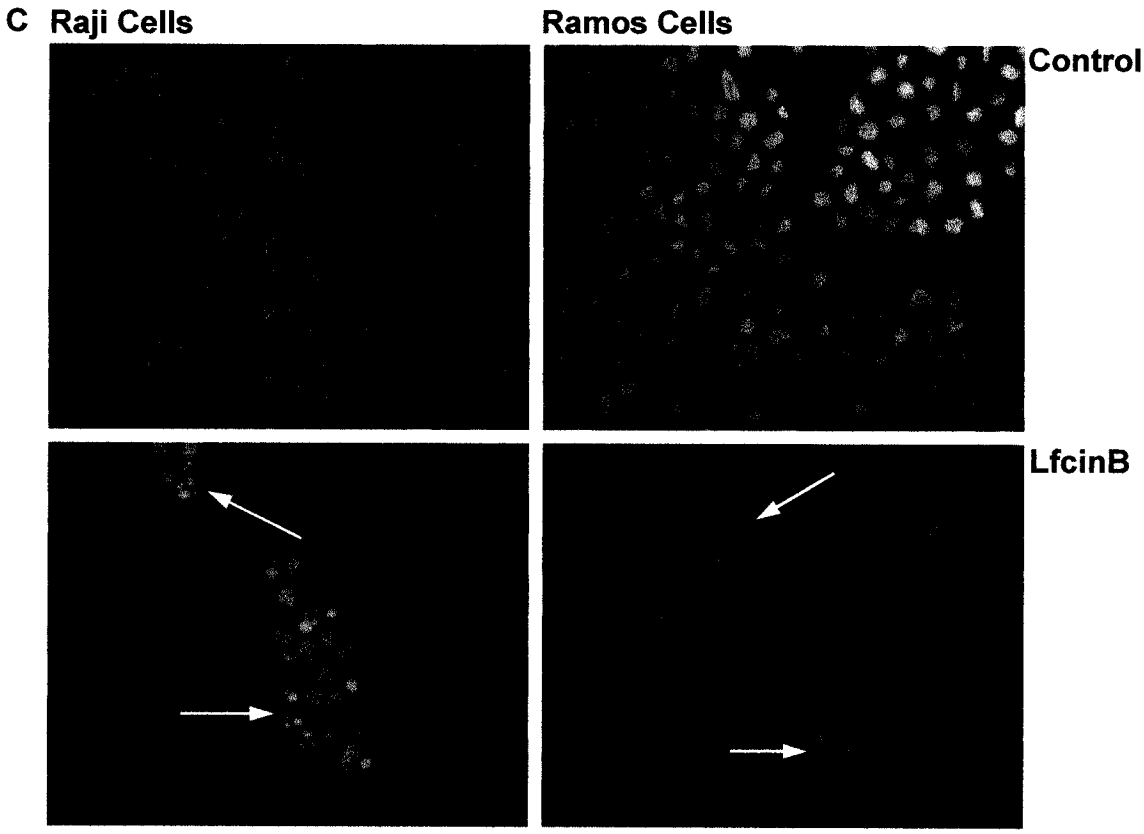


Figure 3.16

survival compared to controls, whereas mice that were given LfcinB on days 1 and 3 after lymphoma cell injection showed a 1.9-fold increase in median survival, as well as an increased proportion of long-term survivors (Figure 3.17A). Additionally, mice treated with LfcinB showed weight gain over the course of the experiments, while control mice showed an average loss in weight (Figure 3.17B). I conclude that LfcinB shows potential as an effective treatment for B cell lymphomas.

Figure 3.17 *LfcinB treatment increased mean survival time of immune-deficient mice bearing disseminated tumours.* **A.** Analysis of LfcinB as a therapeutic agent was accomplished using SCID/beige mice bearing disseminated Ramos B lymphoma. Three groupings of mice were injected i.v. with 2.5×10^6 Ramos cells on day 0 (n = 5/group). Group 1 received i.p. injections of saline day 1 and 3, Group 2 received i.p. injections of LfcinB on day 1 (1 mg/mouse), Group 3 received i.p. injections of LfcinB on day 1 and day 3 (1 mg/mouse/injection). Mice were examined daily for signs of distress, and weighed every 2 days. Paralysis of the hind legs was used as the survival end point. **B.** Analysis of mouse weight in SCID/beige mice injected with disseminated Ramos B lymphoma. Mice were weighed every two days, bars represent mean percent weight change (±SD). Differences were very significant ($p<0.001$, days 6 and 12) and extremely significant ($p<0.0001$, days 12 and 24) by ANOVA and Tukey-Kramer multiple comparison tests (ns, *, **, and *** denote not significant, $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to saline control).

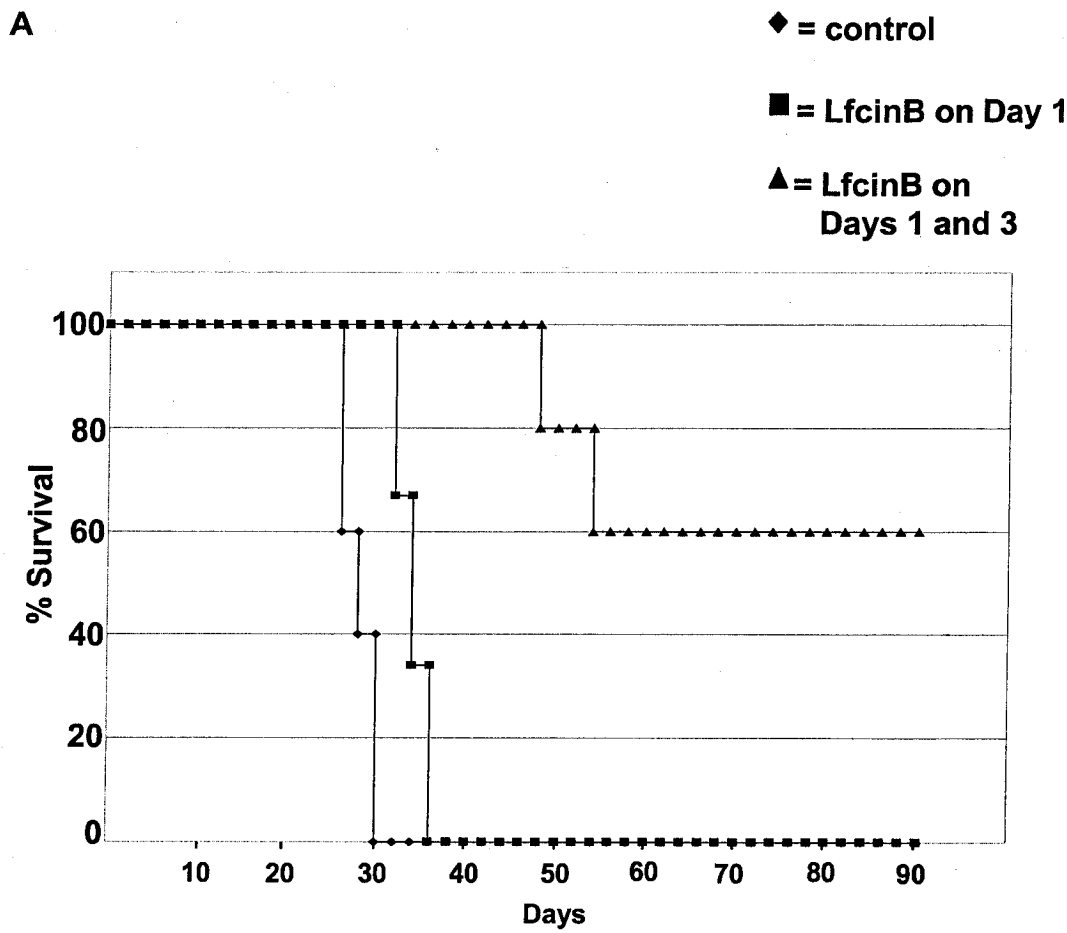

A
◆ = control
■ = LfcinB on Day 1
▲ = LfcinB on Days 1 and 3
100
80
60
40
20
0
% Survival
10
20
30
40
50
60
70
80
90
Days

Figure 3.17

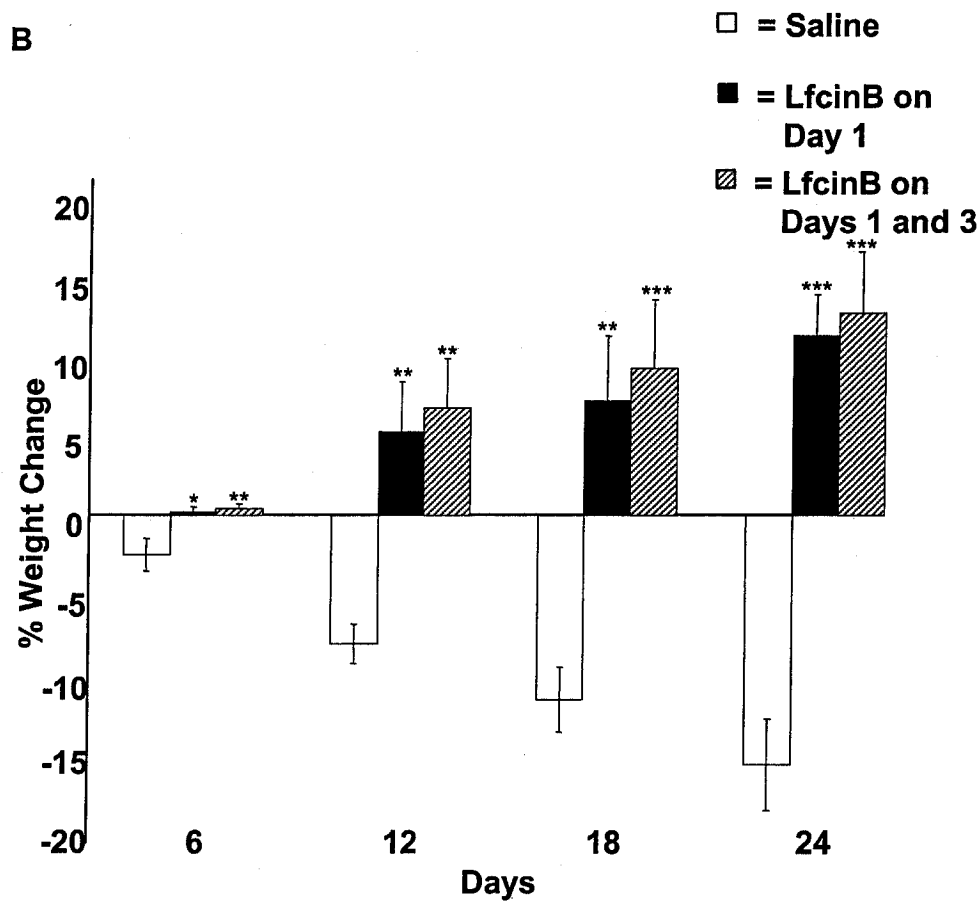

B
□ = Saline
■ = LfcinB on Day 1
▨ = LfcinB on Days 1 and 3
% Weight Change
20
15
10
5
0
-5
-10
-15
-20
6
12
18
24
Days
*
**
**
**
**
***
***
***

Figure 3.17

CHAPTER 4

Bovine Lactoferricin Binds to Human Tumour Cells and Forms Pores in the Tumour Cell Membrane

Portions of this chapter appeared in the following publication:

D Top, R de Antueno, J Salsman, J Corcoran, JS Mader, D Hoskin, A Touhami, MH Jericho, and R Duncan. 2005. Lipid reconstitution of a minimal protein-mediated membrane fusion machine. EMBO J., 24: 2980-2988.

Preamble

LfcinB kills Jurkat T leukemia through the mitochondria-dependent pathway of apoptosis, involving a ROS-dependent, caspase-2-dependent loss in $\Delta\Psi_m$, and sequential caspase-9, and caspase-3 activation (Mader *et al.*, 2005). Peptide fragments of LfcinB, FKCRRWQWRM and RRWQWR, found to display equivalent antimicrobial activity to that of LfcinB (Hoek *et al.*, 1997; Schibli *et al.*, 1999), were shown to have significantly less and no cytotoxic effect, respectively, against Jurkat T leukemia cells (Mader *et al.*, 2005). Additionally, LfcinB is selective in its apoptogenic activity, displaying no cytotoxic effect against normal human primary cells, including primary fibroblasts, human umbilical vein endothelial cells, breast epithelial cells, and resting and activated T cells (Mader *et al.*, 2005; Furlong *et al.*, 2006). However, the basis for this selectivity and the mechanism by which LfcinB interacts with tumour cells has not been elucidated.

In the studies detailed in the present chapter, I compared LfcinB-treated Jurkat T leukemia cells and normal human T cells to determine the specific interaction of LfcinB with the cell membrane. Biotinylated LfcinB (biotin-LfcinB) was employed in fluorescent microscopy experiments to establish whether LfcinB interacted with the membrane of human tumour and normal T cells. Calcium flux experiments in combination with chromium release and DNA fragmentation assays were used to elucidate the potential pore forming ability of LfcinB, while inhibitors of receptor-mediated endocytosis were used to determine whether receptor binding was necessary for the cytotoxic effects of LfcinB, as well as the entry of LfcinB into human tumour cells. Electron microscopy was performed to determine whether LfcinB crossed the tumour cell membrane into the cytosol of Jurkat T leukemia cells. Finally, LfcinB administered in

fusogenic liposomal vehicles was analyzed for its ability to kill tumour cells and LfcinB-resistant normal human primary cells. Liposomes are small spherical vesicles composed of a membrane bilayer made up of phospholipids and cholesterol (Woodle, 1993) that allow for the encapsulation and delivery of drugs, proteins, and a variety of other therapeutic agents via the intravenous route of administration, therefore enhancing drug activity while decreasing toxic side effects (Yuan *et al.*, 1994; Swenson *et al.*, 2004).

Results

LfcinB Binds to Jurkat T Leukemia cells but did not Bind to or Kill Normal Human T Cells.

I have previously shown that LfcinB kills Jurkat T leukemia cells but that human primary T cells and fibroblasts are resistant to LfcinB-induced cell death (Figure 3.1; Mader *et al.*, 2005). I therefore wanted to determine whether this selectivity resulted from a lack of LfcinB binding to the membrane of normal human T cells. Many normal human cells display a neutral net charge on their cell surface, compared to the net negative charge of many cancer cell membranes (Vogel *et al.*, 2002). This knowledge supports the hypothesis that LfcinB might be initially interacting with human tumour cells through electrostatic interactions. Figure 4.1 shows that biotin-LfcinB (200 μg/mL) bound avidly to Jurkat T leukemia cells as early as 5 min following exposure to the peptide. However, biotin-LfcinB (200 μg/mL) did not bind to the surface of normal human T cells or fibroblasts, even after 1 h exposure (Figure 4.2). JAM assays comparing non-biotin- and biotin-LfcinB were then performed to ensure that the biotinylated peptide had equivalent killing activity. Equivalent molar concentrations of LfcinB and biotin-LfcinB had comparable cytotoxicity in Jurkat T leukemia cells (Figure 4.3). This data suggested that electrostatic interaction between the cationic LfcinB peptide and the negatively charged tumour cell membrane might be necessary for LfcinB binding.

Figure 4.1 *LfcinB bound to the surface of Jurkat T leukemia cells prior to cell death.* Jurkat T leukemia cells were treated with biotin-LfcinB (200 μg/mL) for the indicated times. Then fluorescent microscopy was performed (200× magnification).

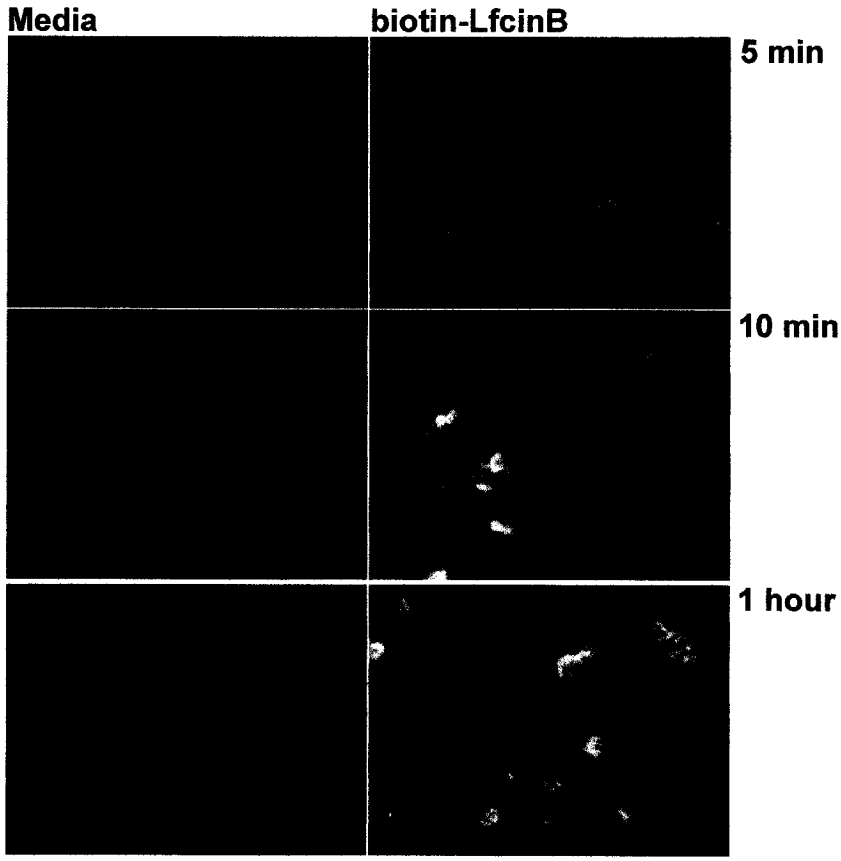


Figure 4.1

Figure 4.2 *LfcinB did not bind to the plasma membrane of normal human T cells or fibroblasts.* Jurkat T leukemia cells, normal human T cells, and normal human fibroblasts were treated with biotin-LfcinB (200 μg/mL, 1 h) and fluorescent microscopy was performed (200× magnification).

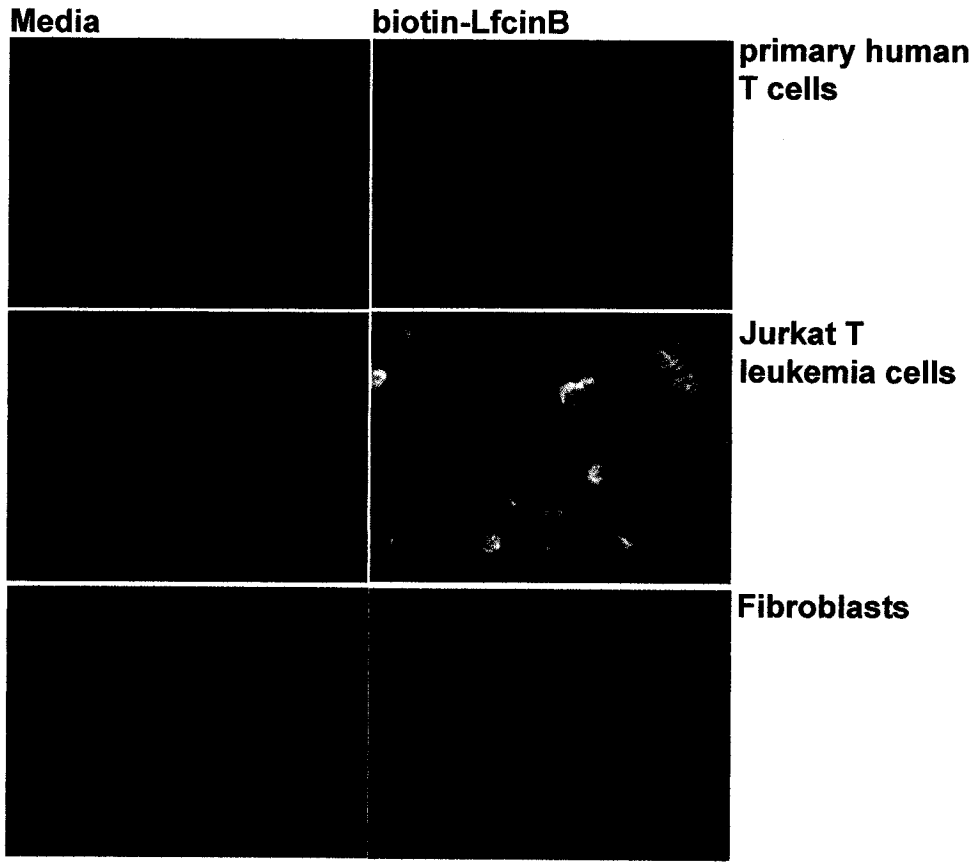

Media
biotin-LfcinB
primary human T cells
Jurkat T leukemia cells
Fibroblasts

Figure 4.2

Figure 4.3 *Biotinylated LfcinB and non-biotinylated LfcinB display similar cytotoxic activity at equimolar concentrations.* Jurkat T leukemia cells were treated with increasing molar concentrations of LfcinB and biotin-LfcinB for 18 h. Cytotoxicity against Jurkat T leukemia cells was determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$).

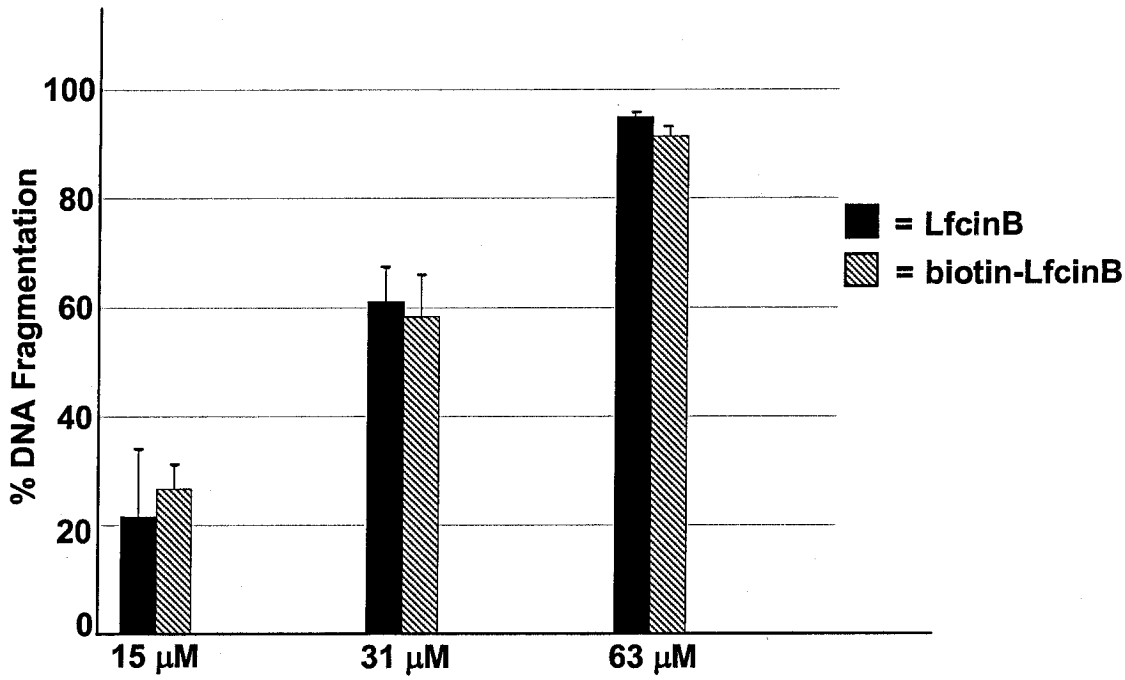

100
80
60
40
20
0
% DNA Fragmentation
15 μM
31 μM
63 μM
= LfcinB
= biotin-LfcinB

Figure 4.3

LfcinB Delivered by Fusogenic Liposomes Killed Untransformed HUVECs, and Human Fibroblast Cells.

Since LfcinB did not kill normal HUVECs or human fibroblasts (Figure 3.1; Mader *et al.*, 2005) or bind to normal T cells or fibroblasts (Figure 4.2). I wanted to determine whether LfcinB would be cytotoxic to resistant cells when the peptide was delivered directly to the cytoplasmic compartment. Liposomes containing the p14 reptilian reovirus (RRV) fusion protein isolated from *Python regius* (Ahne *et al.*, 1987) were used to deliver LfcinB directly into normal HUVECs or fibroblasts. P14 is a complex, type I integral membrane protein that mediates fusion between the viral envelope and the target cell membrane allowing for virus entry into the cell (Corcoran and Duncan, 2004). Figure 4.4 shows that LfcinB (63 μM, equivalent to 200 μg/mL) contained within fusogenic liposomes ($p14^+$) was cytotoxic to LfcinB-resistant HUVECs (Figure 4.4A), and human fibroblasts (Figure 4.4B) following 18 h exposure. Control liposomes had no effect on the viability of either cell type. In addition, HUVECs were killed by 6-mer peptide delivered via fusogenic liposomes (Figure 4.4A), supporting the hypothesis that the 6-mer peptide is unable to destabilize the human cell membrane but is cytotoxic once inside the cytosolic compartment. Together, these data suggest that the cytotoxic activity of free LfcinB, as well as the 6-mer derivative of LfcinB, is determined by the ability of these peptides to penetrate the cell membrane.

High Concentrations of BSA Inhibited LfcinB-Induced Cell Death in Jurkat T Leukemia Cells, but did not Block LfcinB Binding to the Cell Membrane.

It is well known that high serum concentrations inhibit the cytotoxic activity of LfcinB against human tumour cell lines (Yoo *et al.*, 1997b). Electrostatic interactions

Figure 4.4 *Jurkat T leukemia cells, and normal human fibroblasts and endothelial cells were killed by LfcinB delivered in fusogenic liposomal vehicles.* **A.** HUVECs were treated with free LfcinB or 6-mer, or LfcinB or 6-mer contained within fusogenic ($p14^+$) liposomes (63 μM, 18 h). Control cells were treated with $p14^-$ liposome containing LfcinB, or 6-mer (63 μM), or empty liposomes ($p14^+$, or $p14^-$). Cytotoxicity against HUVECs was determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (ns, **, and *** denote not significant, $p<0.01$, and $p<0.001$, respectively, when compared to LfcinB or 6-mer treatment alone). **B.** Normal human fibroblast cells were treated with free LfcinB or LfcinB contained within fusogenic ($p14^+$) liposomes (200 μg/mL, 18 h). Control cells were treated with $p14^-$ liposome containing LfcinB (200 μg/mL), or empty liposomes ($p14^+$, or $p14^-$). Cytotoxicity against normal human fibroblast cells was determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (ns, *, and *** denote not significant, $p<0.05$, and $p<0.001$, respectively, when compared to LfcinB treatment alone).

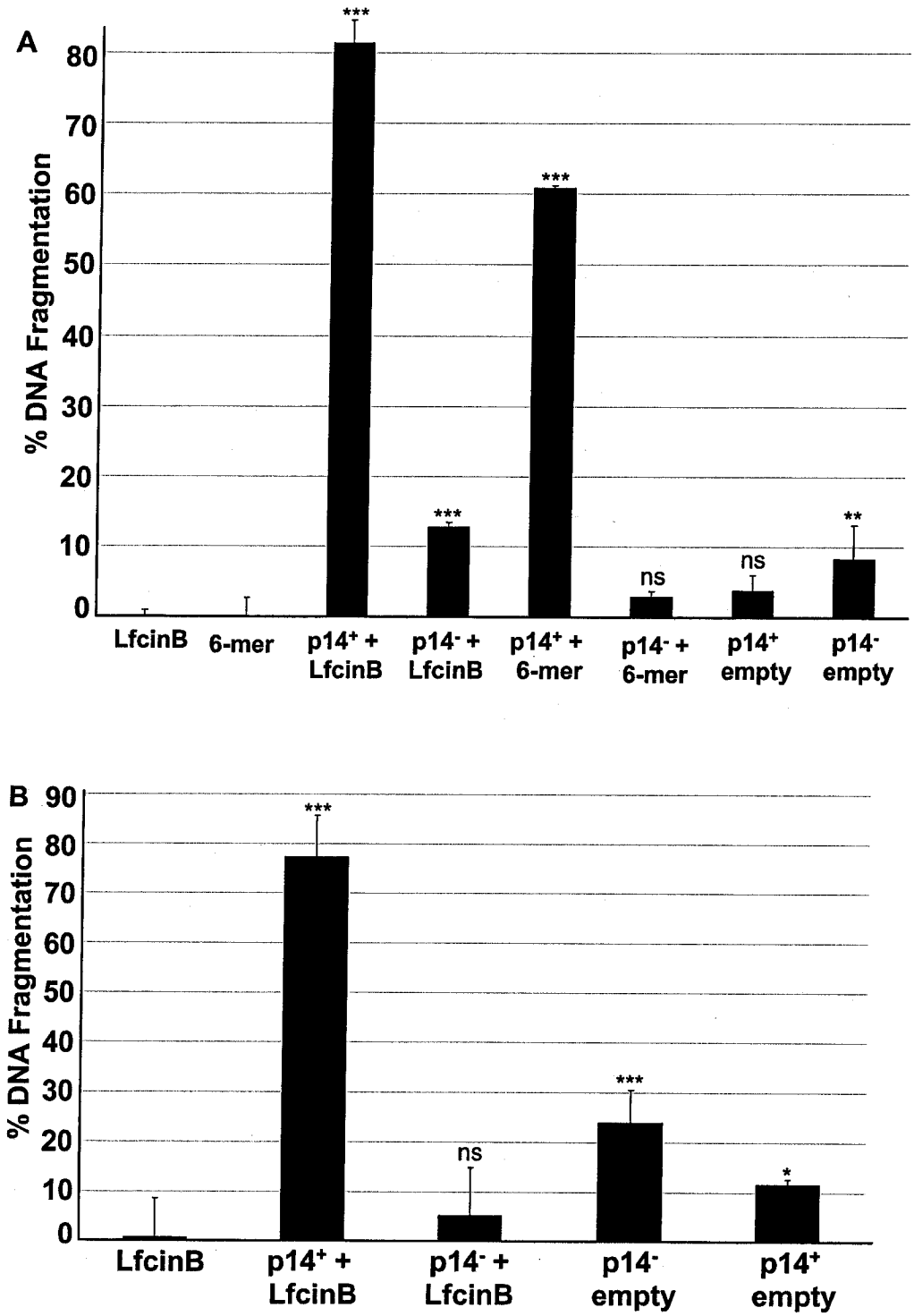

A
% DNA Fragmentation
0
10
20
30
40
50
60
70
80
***
***
***
ns
ns
**
LfcinB
6-mer
p14+ + LfcinB
p14- + LfcinB
p14+ + 6-mer
p14- + 6-mer
p14+ empty
p14- empty
B
% DNA Fragmentation
0
10
20
30
40
50
60
70
80
90
***
ns
***
*
LfcinB
p14+ + LfcinB
p14- + LfcinB
p14- empty
p14+ empty

Figure 4.4

between the cationic LfcinB peptide and anionic membrane components may be required for LfcinB-induced cytotoxicity. These interactions may be blocked by anionic serum components. To test this hypothesis, JAM assays were performed in which Jurkat T leukemia cells were exposed to LfcinB (100 μg/mL) following pre-incubation with and in the presence of increasing concentrations of the anionic serum component, BSA (Figure 4.5A). BSA concentrations of 7.5-12.5% significantly ($p<0.001$) inhibited LfcinB-induced DNA fragmentation in Jurkat T leukemia cells, which suggests that BSA might be inhibiting the interaction between LfcinB and the human tumour cells. Together with the known inhibitory effect of serum components on the LfcinB-induced cytotoxicity, this data supports the belief that LfcinB is initially interacting with human tumour cells through electrostatic interactions. However, fluorescent microscopy analysis of Jurkat T leukemia cells pre-exposed to BSA or FCS (10% w/v) prior to biotin-LfcinB (200 μg/mL) showed LfcinB binding to Jurkat cells in the presence of BSA and FCS (Figure 4.5B). Jurkat cells treated with LfcinB following BSA or FCS exposure exhibited morphological features that might indicate cell death (indicated by arrows) at 30 min (Figure 4.5B). This suggests that BSA and FCS may inhibit DNA fragmentation in Jurkat cells following LfcinB treatment; however, these cells may still be undergoing cell death. To determine whether LfcinB-treated cells were dying in the presence of high BSA and FCS concentrations, chromium release assays were performed in which Jurkat T leukemia cells were exposed to LfcinB (200 μg/mL) following pre-incubation with and in the presence of a high concentration of BSA and FCS (Figure 4.6). Both BSA and FCS inhibited chromium release from Jurkat T leukemia cells exposed to LfcinB,

Figure 4.5 *LfcinB-induced cell death was inhibited by BSA.* **A.** Jurkat T leukemia cells were pre-exposed to increasing concentrations of the anionic bovine serum component, BSA, resuspended in PBS (w/v) for 30 min, followed by treatment with 100 μg/mL LfcinB for 18 h. Cytotoxicity against Jurkat T leukemia cells was determined by JAM assay. Differences were extremely significant by ANOVA ($p<0.0001$) and Tukey-Kramer multiple comparison tests (ns and *** denote not significant and $p<0.001$, respectively, when compared to LfcinB treatment alone). **B.** Jurkat T leukemia cells were pre-exposed to BSA or FCS (10%) and then treated with biotin-LfcinB (200 μg/mL, 30 h) and fluorescent microscopy was performed (200× magnification).

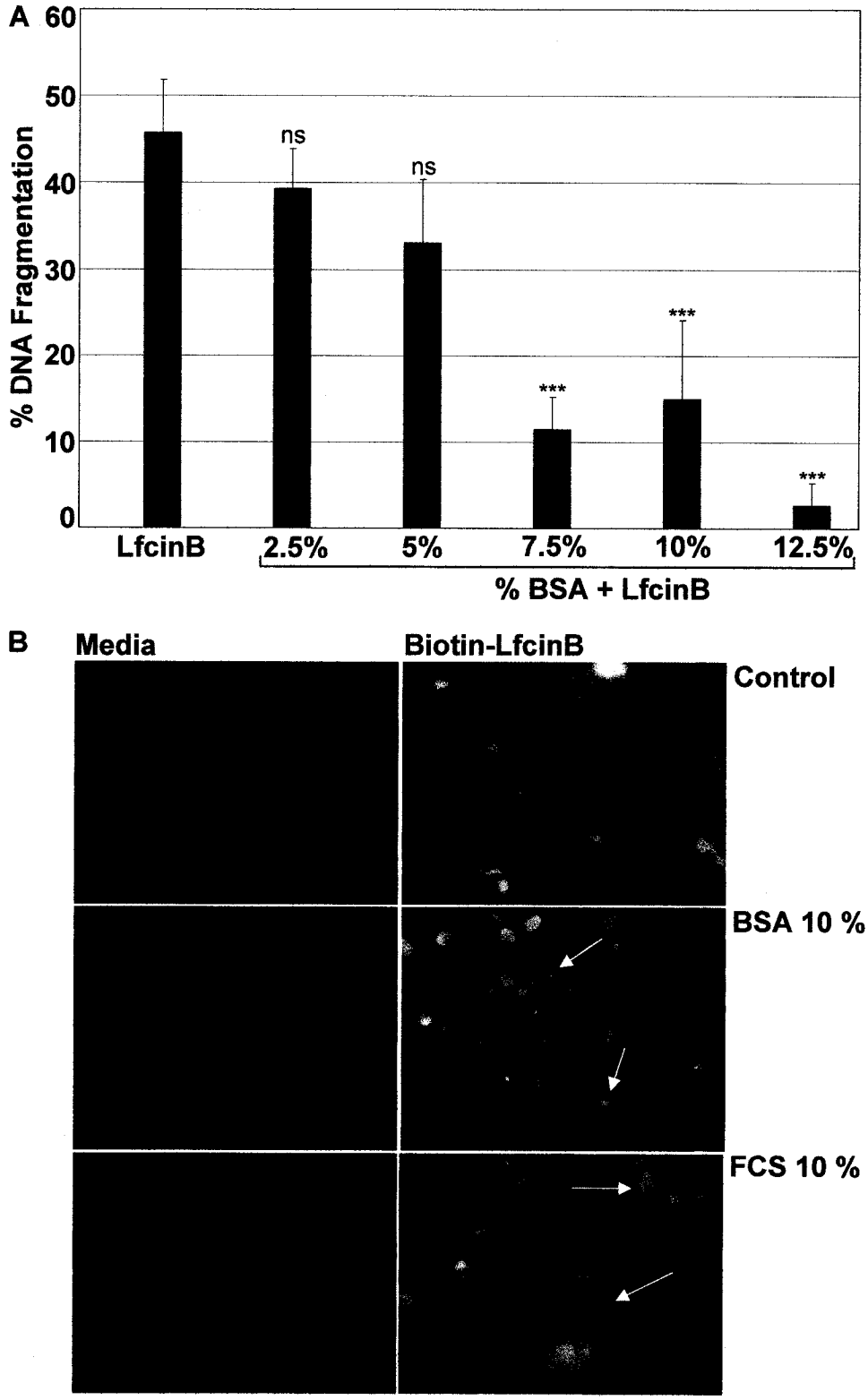

A
60
50
40
30
20
10
0
% DNA Fragmentation
ns
ns
***
***
***
LfcinB
2.5%
5%
7.5%
10%
12.5%
% BSA + LfcinB
B
Media
Biotin-LfcinB
Control
BSA 10 %
FCS 10 %

Figure 4.5

Figure 4.6 *A high concentration of BSA or FCS inhibited LfcinB-induced cell death.* Jurkat T leukemia cells were pre-exposed to a high concentration (10%) of BSA or FCS 30 min, followed by treatment with 200 μg/mL LfcinB for 8 h. Cytotoxicity against Jurkat T leukemia cells was determined by chromium release assay. Differences were extremely significant by Student's t-test (ns and *** denote not significant and $p<0.0001$, respectively).

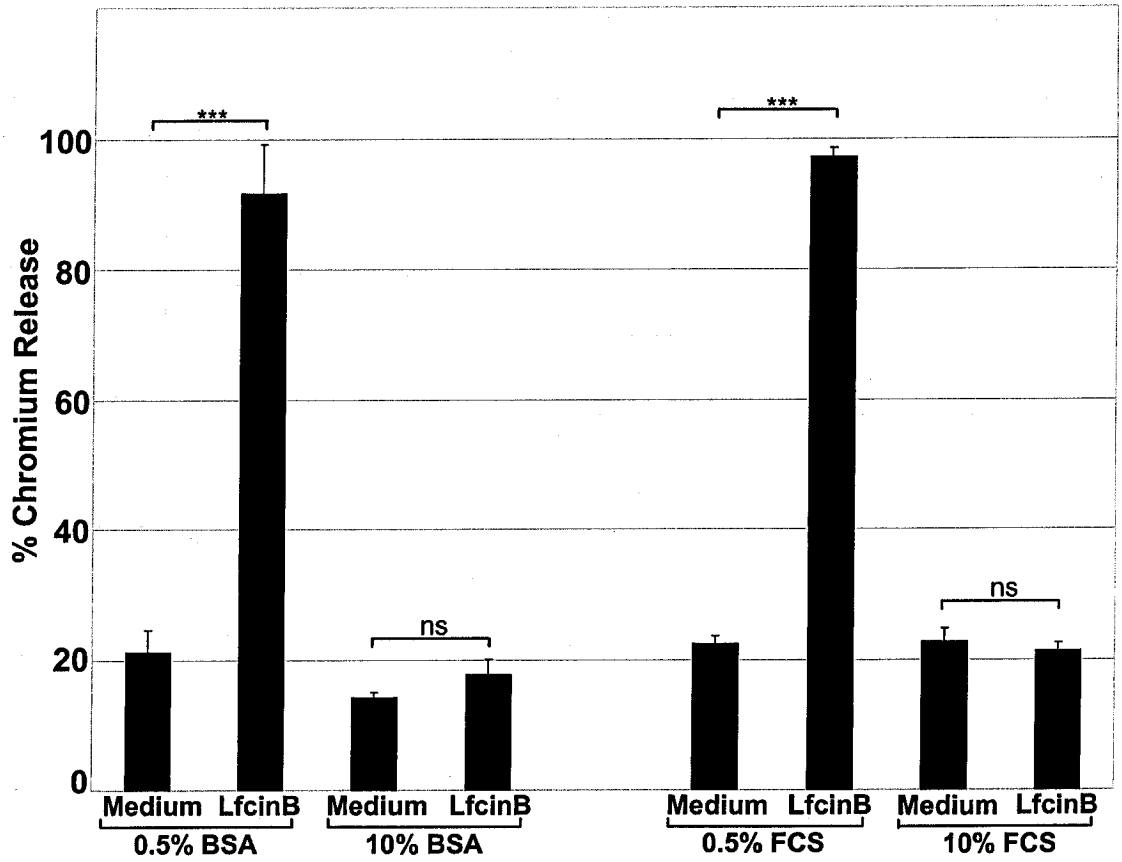

% Chromium Release
100
80
60
40
20
0
***
***
ns
ns
Medium
LfcinB
Medium
LfcinB
Medium
LfcinB
Medium
LfcinB
0.5% BSA
10% BSA
0.5% FCS
10% FCS

Figure 4.6

suggesting that BSA and FCS blocked LfcinB-induced apoptosis in a mechanism that was independent of LfcinB interaction with the cell membrane.

Receptor-Mediated Endocytosis was not Required for LfcinB-Induced Apoptosis.

Internalization of extracellular macromolecules can occur by two main mechanisms in eukaryotic cells; pinocytosis, which is a non-specific process whereby molecules are ingested in combination with other extracellular fluids, and receptor-mediated endocytosis, which involves the binding of ligands to specific receptors at the plasma membrane (de Figueiredo and Soares, 2000; Schwartz, 1995; Mukherjee *et al.*, 1997). I wanted to understand whether receptor-mediated endocytosis was necessary for LfcinB-induced cell death in human tumour cell lines. Experiments were performed using two well-characterized inhibitors of receptor-mediated endocytosis, dansylcadaverine and cytochalasin D. Dansylcadaverine inhibits transglutaminase activity in the cell membrane thus blocking the formation of coated pits (Haigler *et al.*, 1980), while cytochalasin D inhibits fluid phase endocytosis and clathrin- and caveolae-mediated endocytosis (Blok *et al.*, 1982). Figure 4.7 shows that, at concentrations that are known to inhibit receptor-mediated endocytosis (Dawe *et al.*, 2002), neither inhibitor had an effect on LfcinB-induced cell death in Jurkat T leukemia cells, suggesting that receptor-mediated endocytosis was not required for LfcinB-induced cytotoxicity.

LfcinB may have Formed Pores in Jurkat T Leukemia Cell Membranes.

Many CAPs destabilize microbial membranes through formation of oligomerized membrane pores, allowing the peptide to access the cytoplasmic compartment

Figure 4.7 *The cytotoxic effect of LfcinB was not dependant on receptor-mediated endocytosis.* Jurkat T leukemia cells were pre-treated with inhibitors of receptor-mediated endocytosis, cytochalasin D (2.5 μg/mL) and dansylcadaverine (0.1 mM), for 30 min. Following pre-treatment, cells were exposed to 200 μg/mL LfcinB for 18 h. Cytotoxicity against Jurkat T leukemia cells was determined by JAM assay.

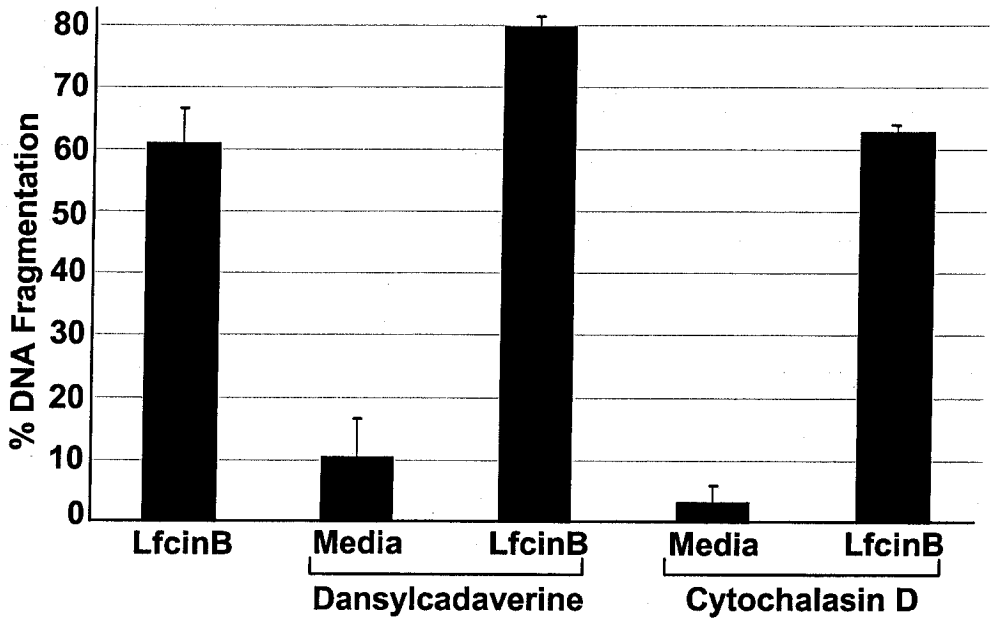

% DNA Fragmentation
80
70
60
50
40
30
20
10
0
LfcinB
Media
LfcinB
Media
LfcinB
Dansylcadaverine
Cytochalasin D

Figure 4.7

(Ehrenstein and Lecar, 1997; Haukland *et al.*, 2001; Ye *et al.*, 2004). I wanted to determine whether LfcinB formed membrane pores in Jurkat T leukemia cells. I therefore performed chromium release assays in conjunction with DNA fragmentation assays to determine whether intracellular proteins were released from LfcinB-treated cells prior to DNA fragmentation. Figure 4.8 shows that chromium release from Jurkat T leukemia cells preceded any substantial DNA fragmentation, occurring 5 to 30 min following LfcinB-exposure (200 μg/mL). This finding suggested that membrane pores were formed by LfcinB prior to the activation of cell death. Further evidence of pore formation in LfcinB-treated (200 μg/mL) Jurkat T leukemia cells was obtained using Ca^{2+} flux analysis. Cells were pre-loaded with 4 μM Fluo-4-AM and placed in a 37 °C thermostated quartz cuvette with magnetic stirring. Jurkat T leukemia cells were then treated with medium alone or with LfcinB (200 μg/mL). I observed a considerable Ca^{2+} flux upon LfcinB addition (Figure 4.9i); however, this flux was not observed when cells were suspended in calcium-free HEPES tyrodes buffer prior to LfcinB exposure (Figure 4.9ii). This data is consistent with LfcinB treatment allowing extracellular Ca^{2+} to enter the tumour cell, potentially due to the formation of membrane pores, rather than triggering the release of intracellular Ca^{2+} stores.

LfcinB Entered Jurkat T leukemia Cells to Reside in the Cytosol where it Interacted with Mitochondria.

Previous reports have determined that LfcinB can cross the cell membrane in various bacteria to reside in the cytoplasmic compartment (Haukland *et al.*, 2001). However, it is not known whether LfcinB enters human tumour cells to reside in the

Figure 4.8 *Exposure to LfcinB caused chromium release prior to DNA fragmentation.* Jurkat T leukemia cells were treated with 200 μg/mL LfcinB for various time points. Leakage of intracellular proteins was analyzed by chromium release assay, while cytotoxicity against Jurkat T leukemia cells was determined by JAM assay. Differences were significant by Student's t-test (*, **, and *** denote $p<0.05$, $p<0.01$ and $p<0.001$, respectively).

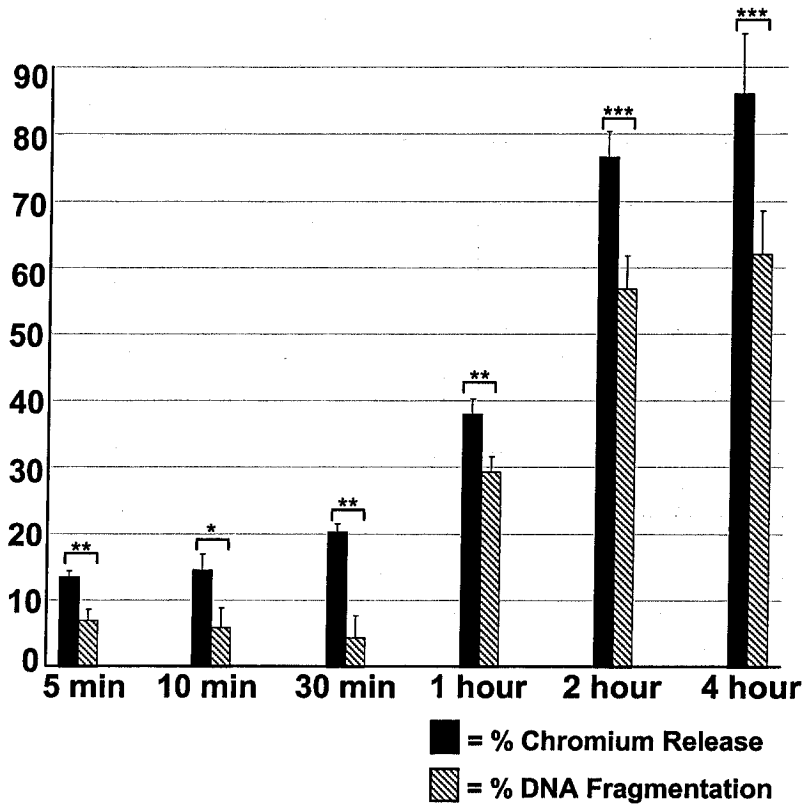

90
80
70
60
50
40
30
20
10
0
5 min
10 min
30 min
1 hour
2 hour
4 hour
**
*
**
**
***
***
= % Chromium Release
= % DNA Fragmentation

Figure 4.8

Figure 4.9 *LfcinB formed pores in the membrane of Jurkat T leukemia cells allowing for extracellular calcium to enter the cell.* **(i)** Jurkat T leukemia cells were pre-loaded with 4 μM Fluo-4-AM and placed in a 37°C thermostated quartz cuvette with magnetic stirring. Following treatment with medium alone, Jurkat T leukemia cells were exposed to LfcinB (200 μg/mL) and analyzed for calcium flux. Cells were subsequently exposed to 10% Triton-X 100, as a positive control, and 0.5 M EGTA, as a negative control, in sequential fashion. Fluorescence was measured at 520 nm after excitation at 485 nm. **(ii)** Jurkat T leukemia cells were loaded with 4 μM Fluo-4-AM and placed in a 37°C thermostated quartz cuvette with magnetic stirring. Cells were suspended in calcium-free HEPES tyrodes buffer and then exposed to LfcinB (200 μg/mL) and analyzed for calcium flux. Cells were subsequently exposed to 10% Triton-X 100, as a positive control, and 0.5 M EGTA, as a negative control, in sequential fashion.

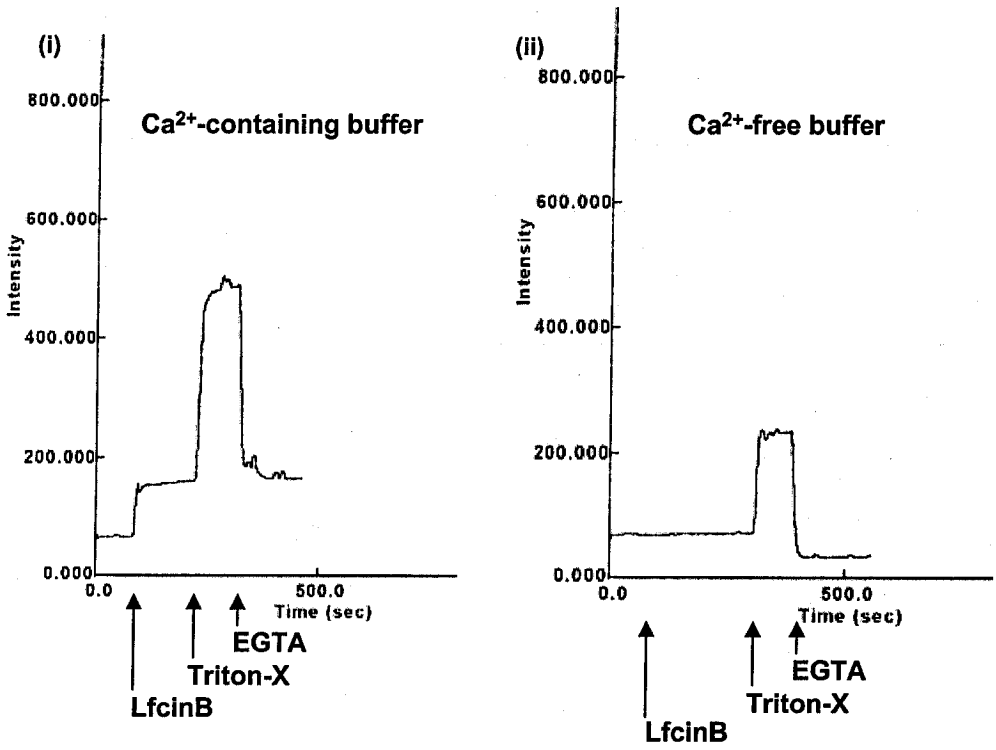

(i)
800.000
600.000
400.000
200.000
0.000
Intensity
Ca2+-containing buffer
0.0
500.0
Time (sec)
EGTA
Triton-X
LfcinB
(ii)
800.000
600.000
400.000
200.000
0.000
Intensity
Ca2+-free buffer
0.0
500.0
Time (sec)
EGTA
Triton-X
LfcinB

Figure 4.9

cytosol. Transmission electron microscopy using Streptavidin colloidal gold (Hainfeld and Powell, 2000) was performed on Jurkat T leukemia cells exposed to biotin-LfcinB (200 μg/mL, 2 h) alone or following pre-treatment with cytochalasin D (2.5 μg/mL) to block receptor-mediated endocytosis. Figure 4.10 shows that biotin-LfcinB crossed the plasma membrane in Jurkat T leukemia cells, and that entry was independent of receptor-mediated endocytosis.

Like human tumour cell membranes, mitochondrial membranes possess a net negative charge (Gray *et al.*, 1999; Risso *et al.*, 2002). I therefore hypothesized that, upon entering the human tumour cell, LfcinB might target and lyse mitochondrial membranes. Transmission electron microscopy analysis of mitochondria isolated from Jurkat T leukemia cells exposed to biotin-LfcinB (200 μg/mL, 30 min) showed that biotin-LfcinB bound avidly to and entered mitochondria (Figure 4.11). This observation suggests that LfcinB may specifically target the mitochondrial membrane once LfcinB is inside the tumour cell.

Figure 4.10 *LfcinB entered Jurkat T leukemia cells in a receptor-independent manner to reside in the cytosol.* Jurkat T leukemia cells were exposed to biotin-LfcinB (200 μg/mL, 2 h) alone or following pre-treatment with cytochalasin D (2.5 μg/mL). Localization of LfcinB was determined using Streptavidin colloidal gold and transmission electron microscopy (panel i and ii, 100, 000× magnification; panel iii and iv, 60, 000× magnification).

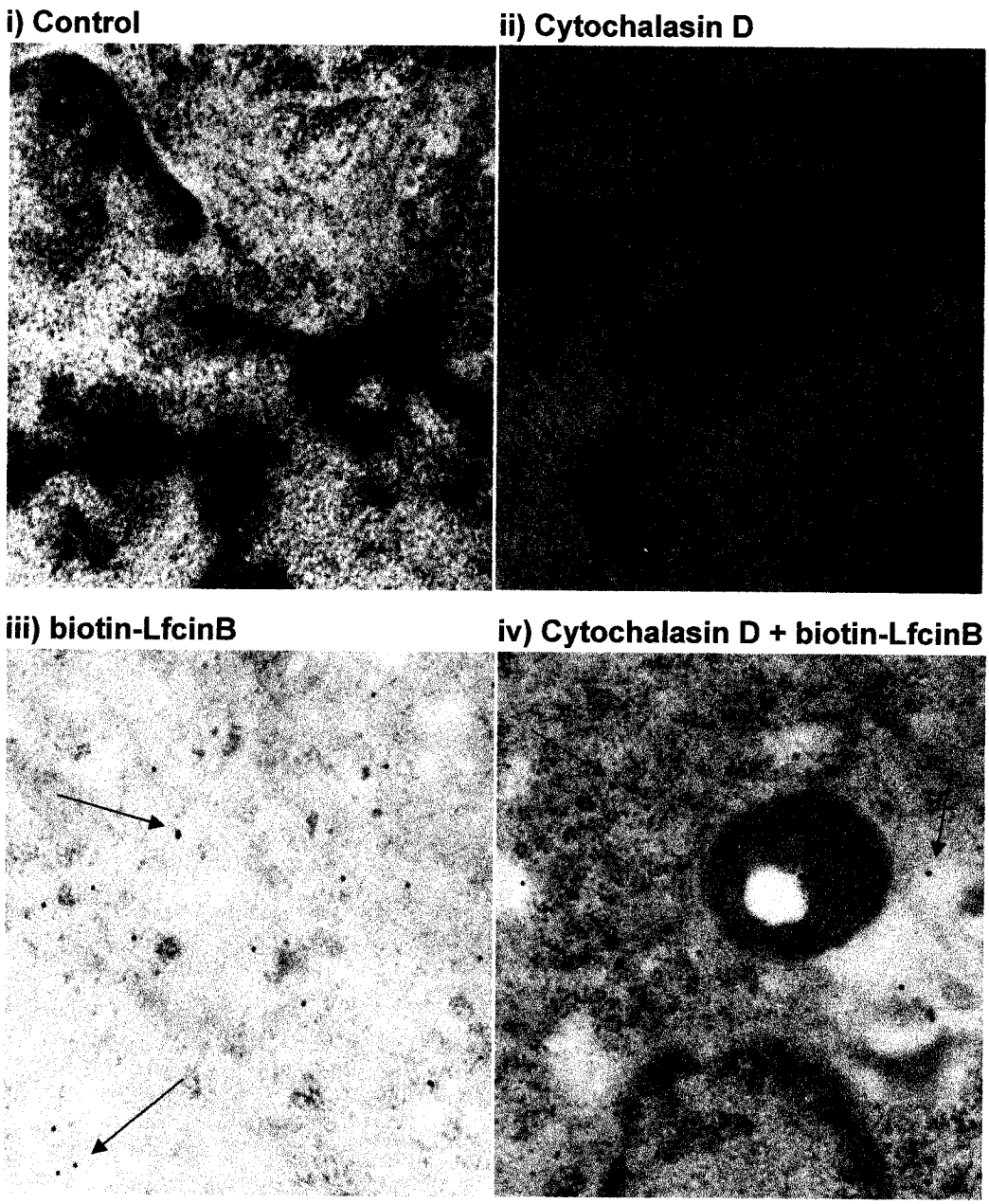
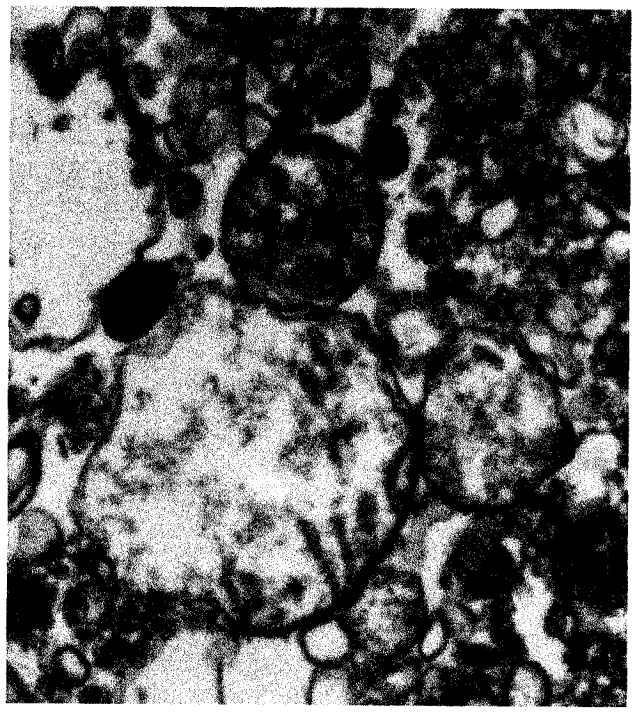

i) Control
ii) Cytochalasin D
iii) biotin-LfcinB
iv) Cytochalasin D + biotin-LfcinB

Figure 4.10

Figure 4.11 *LfcinB interacted with mitochondria isolated from Jurkat T leukemia cells.* Mitochondria were isolated from Jurkat T leukemia cells (30×10^6 cells/treatment), and exposed to 200 μg/mL biotin-LfcinB for 30 min. Binding of LfcinB to mitochondria was determined using Streptavidin colloidal gold and transmission electron microscopy (60, 000× magnification).



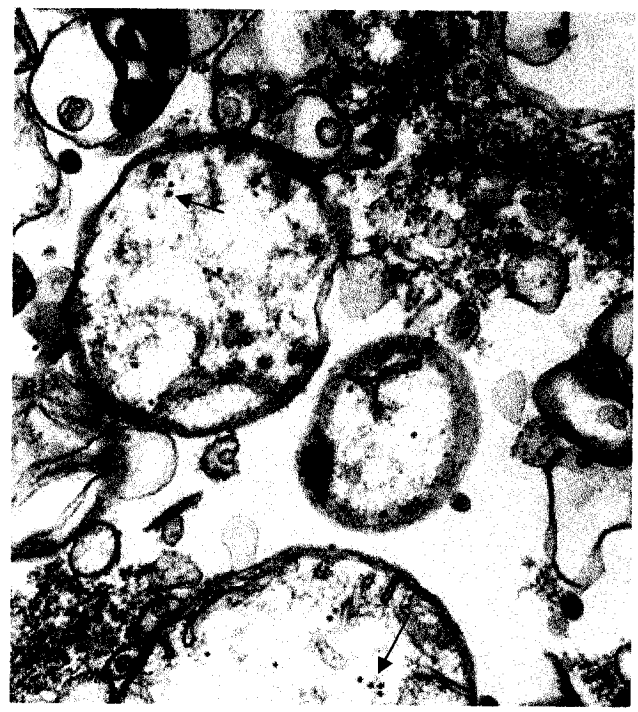


Figure 4.11

CHAPTER 5

Bovine Lactoferricin Inhibits Basic Fibroblast Growth Factor- and Vascular Endothelial Growth Factor$_{165}$-Induced Angiogenesis by Competing for Heparin-Like Binding Sites on Endothelial Cells

Portions of this chapter will appear in the following manuscript:

Mader JS, Smyth D, Marshall J, Hoskin DW. (Submitted) Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor$_{165}$-induced angiogenesis by competing for heparin-like binding sites on endothelial cells.

Preamble

There is an urgent need for innovative forms of cancer treatment that not only avoid the serious problem of chemotherapeutic resistance that results from the inherent genetic instability and heterogeneity of cancer cells, but also limit the level of toxic side effects. In this regard, angiogenesis inhibitors have gained recent attention due to their ability to target genetically stable untransformed endothelial cells comprising the tumour vasculature (Fayette *et al.*, 2005).

In the present study, I used both *in vivo* and *in vitro* approaches to investigate LfcinB for possible antiangiogenic activity. The Matrigel plug assay, which is a well-established method of assessing the *in vivo* activity of antiangiogenic factors (Akhtar *et al.*, 2002), was used to determine the effect of LfcinB on bFGF- and $VEGF_{165}$-induced blood vessel development in mice. I also investigated the effect of LfcinB on the *in vitro* proliferation and migration of HUVECs in response to bFGF and VEGF. Endothelial cell proliferation and migration induced by proangiogenic factors are crucial steps in the development of tumour vasculature (Folkman, 1992). In addition, I determined the ability of LfcinB to bind heparin-like molecules that are involved in bFGF and $VEGF_{165}$ interaction with their respective receptors because LfcinB is derived from lactoferrin, which exhibits heparin-binding activity (Pejler, 1996; Wu and Church, 2003).

Results

LfcinB Inhibited In Vivo Angiogenesis.

Subcutaneous implantation of Matrigel plugs was used to determine the *in vivo* antiangiogenic effect of LfcinB. Matrigel plugs that contained the drug vehicle, LfcinB, bFGF, or $VEGF_{165}$ alone, or LfcinB in combination with bFGF or $VEGF_{165}$ were sectioned and stained for von Willebrand factor. Figure 5.1A shows representative histological images of Matrigel plug sections. Figure 5.1B shows the percent angiogenesis in sections of replicate Matrigel plugs (n = 8). Neither the vehicle nor LfcinB caused substantial angiogenesis, whereas bFGF, $VEGF_{165}$, and non-heparin-binding EGF strongly stimulated endothelial cell migration and tube formation. The difference between the computer analysis of % angiogenesis for the vehicle control (Figure 5.1B) and the representative image (Figure 5.1A) may be explained by artifactual staining detected during computer analysis. The isotype control for the von Willebrand factor-specific antibody gave results that were not significantly different from the vehicle control (data not shown). Importantly, bFGF and $VEGF_{165}$-induced angiogenesis in Matrigel plugs was reduced by 40% and 45% ($p < 0.001$), respectively, in the presence of LfcinB. In contrast, LfcinB did not affect endothelial cell migration and tube formation induced by non-heparin-binding EGF. To rule out the possibility that LfcinB decreased von Willebrand factor expression by endothelial cells, I determined blood vessel density (number of mature lumens/field of view) in hematoxylin and eosin-stained Matrigel plug sections. Table 5.1 shows that the number of vessels that developed in response to bFGF and $VEGF_{165}$ was reduced by 61% ($P < 0.002$) and 67% ($P < 0.001$), respectively, in the presence of LfcinB. In contrast, LfcinB did not significantly affect blood vessel

Figure 5.1 *LfcinB inhibited bFGF- and $VEGF_{165}$-induced angiogenesis in Matrigel plugs.* **A**. Histological analysis of implanted Matrigel plugs. Matrigel containing distilled water (vehicle), bFGF (1 μg/mL), $VEGF_{165}$ (5 μg/mL), or non-heparin-binding EGF (2 μg/mL) in the presence or absence of 200 μg/mL LfcinB were implanted in mice by subcutaneous injection. After 6 days mice were sacrificed, the Matrigel plugs were surgically excised, sectioned, and blood vessel formation was visualized by staining with antibody against von Willebrand factor (20× magnification). **B**. Quantitation of mean capillary area in Matrigel plugs. The area of individual Matrigel plugs sections that stained positive for von Willebrand factor relative to the unstained area was determined by computer analysis. Each bar represents mean percent angiogenesis ± SEM (n = 8). Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$when compared to growth factor treatment alone).

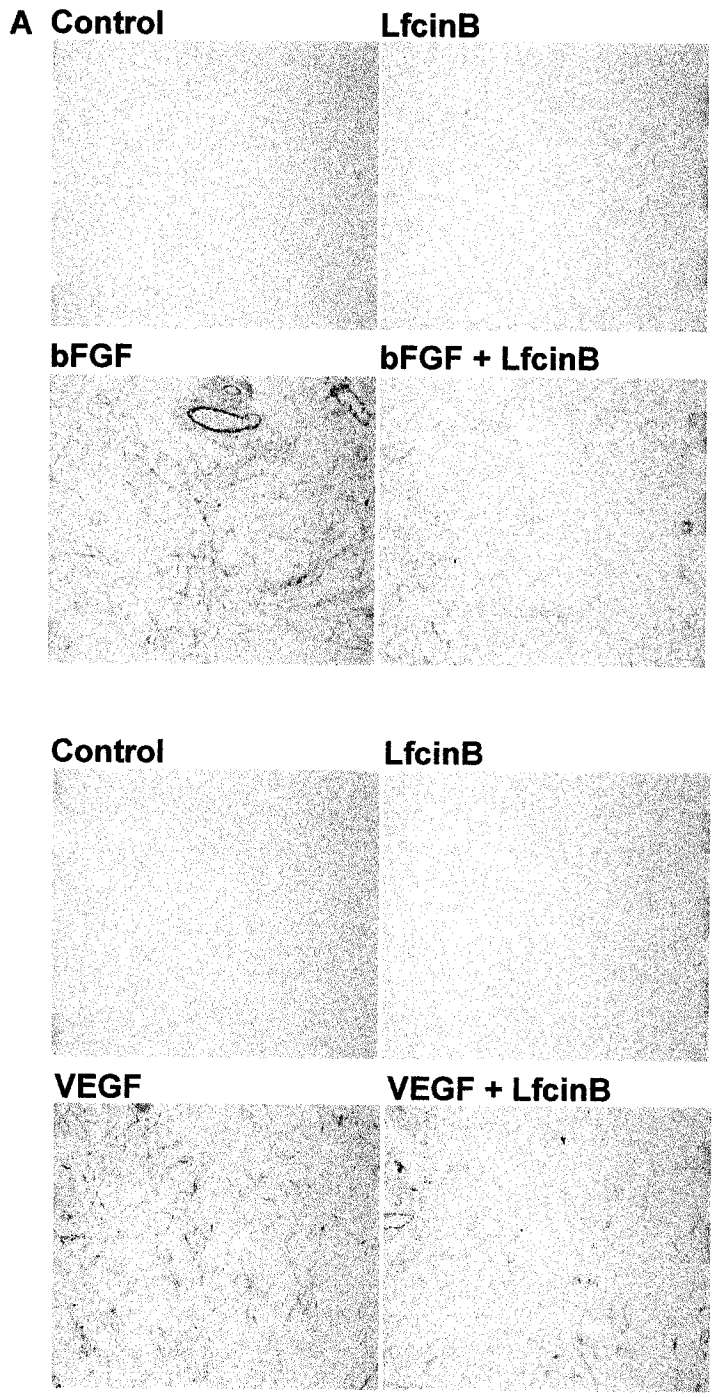

A Control
LfcinB
bFGF
bFGF + LfcinB
Control
LfcinB
VEGF
VEGF + LfcinB

Figure 5.1

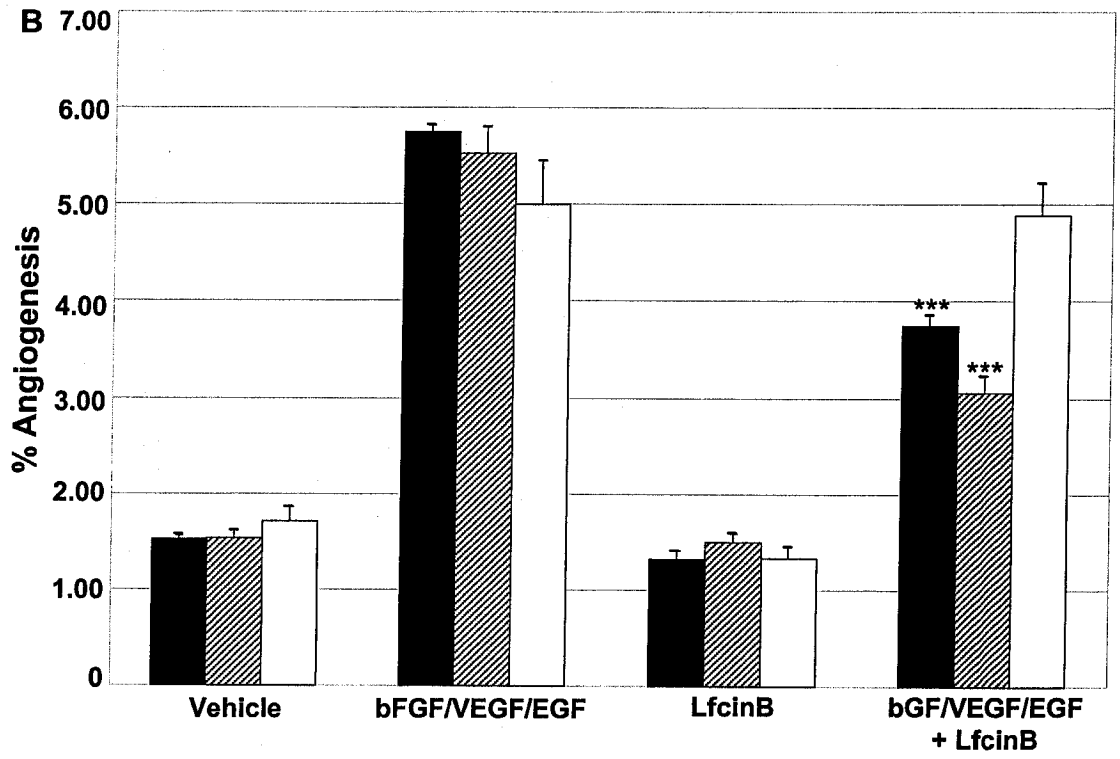

B
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0
% Angiogenesis
***
***
Vehicle
bFGF/VEGF/EGF
LfcinB
bGF/VEGF/EGF
+ LfcinB

Figure 5.1

Table 5.1 *Effect of LfcinB on blood vessel development.*

	Treatment*	Mature lumens/Field of view †	% Decrease
I.	Vehicle	0	
	bFGF	23 ± 8	
	LfcinB	0	
	bFGF + LfcinB	9 ± 7‡	61
II.	Vehicle	0	
	$VEGF_{165}$	24 ± 9	
	LfcinB	0	
	$VEGF_{165}$ + LfcinB	8 ± 6¶	67
III.	Vehicle	0	
	Non-heparin-binding EGF	22 ± 8	
	LfcinB	0	
	Non-heparin-binding EGF + LfcinB	20 ± 9	9

* Matrigel containing distilled water (vehicle), LfcinB alone (200 μg/mL), bFGF alone (1 μg/mL), $VEGF_{165}$ alone (5 μg/mL) or non-heparin-binding EGF alone (2 μg/mL), or LfcinB (200 μg/mL) in combination with bFGF (1 μg/mL), $VEGF_{165}$ (5 μg/mL) or non-heparin-binding EGF alone (2 μg/mL) was implanted in mice by subcutaneous injection. After 6 days, mice were sacrificed, and Matrigel plugs were surgically excised and sectioned.

† Blood vessel density in hematoxylin and eosin-stained Matrigel plug sections was determined on the basis of the number of mature lumens per field of view (n = 10, 200× magnification). Data are mean values ± SD.

‡ Significant reduction relative to bFGF alone ($p < 0.002$), as determined by Tukey-Kramer multiple comparisons test.

¶ Significant reduction relative to $VEGF_{165}$ alone ($p < 0.001$), as determined by Tukey-Kramer multiple comparisons test.

development in response to non-heparin-binding EGF. Taken together, these data suggest that LfcinB inhibited endothelial cell migration and tube formation in response to heparin-binding growth factors.

LfcinB Inhibited bFGF- and $VEGF_{165}$-Induced HUVEC Proliferation and Migration.

It is well known that the proliferation and migration of endothelial cells is an essential component of angiogenesis (Folkman, 1992). In light of the results from my *in vivo* studies, I wanted to determine whether LfcinB inhibition of bFGF- and $VEGF_{165}$-induced angiogenesis was due to an inhibitory effect of LfcinB on the proliferation and/or migration of endothelial cells. Figure 5.2 shows that LfcinB strongly inhibited ($p < 0.001$) the *in vitro* proliferation of HUVECs in response to bFGF or $VEGF_{165}$. In contrast, HUVEC proliferation induced by non-heparin-binding EGF was not affected by LfcinB. Figure 5.3 shows that LfcinB dramatically reduced ($p < 0.001$) the migration of HUVECs in response to bFGF or $VEGF_{165}$ over a 2 and 4 h period. Collectively, these data indicate that LfcinB inhibited the bFGF- and $VEGF_{165}$-induced proliferation and migration of endothelial cells.

LfcinB Does Not Affect HUVEC Viability.

Given that LfcinB induces apoptosis in a variety of human cancer cell lines as early as 1 h post treatment (Yoo *et al.*, 1997b; Mader *et al.*, 2005), I examined the possibility that the antiangiogenic activity of LfcinB resulted from a cytotoxic effect on endothelial cells. Figure 5.4A shows that ^{51}Cr-labeled HUVECs that were exposed to medium, bFGF, $VEGF_{165}$, or LfcinB for 6 h released similar amounts of ^{51}Cr into culture

Figure 5.2 *LfcinB inhibited bFGF- and $VEGF_{165}$ -induced HUVEC proliferation.* HUVECs (4×10^3 cells/well) were cultured for 24 h in the presence of medium, bFGF (10 ng/mL), $VEGF_{165}$ (100 ng/mL), or non-heparin-binding EGF (20 ng/mL) in the presence or absence of LfcinB (200 μg/mL). DNA synthesis was measured as $[H^3]$–TdR incorporation. Data are shown as mean cpm ± SD of quadruplicate determinations. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to growth factor treatment alone), (*, and ** denote $p<0.05$, and $p<0.01$, respectively when compared to LfcinB treatment alone).

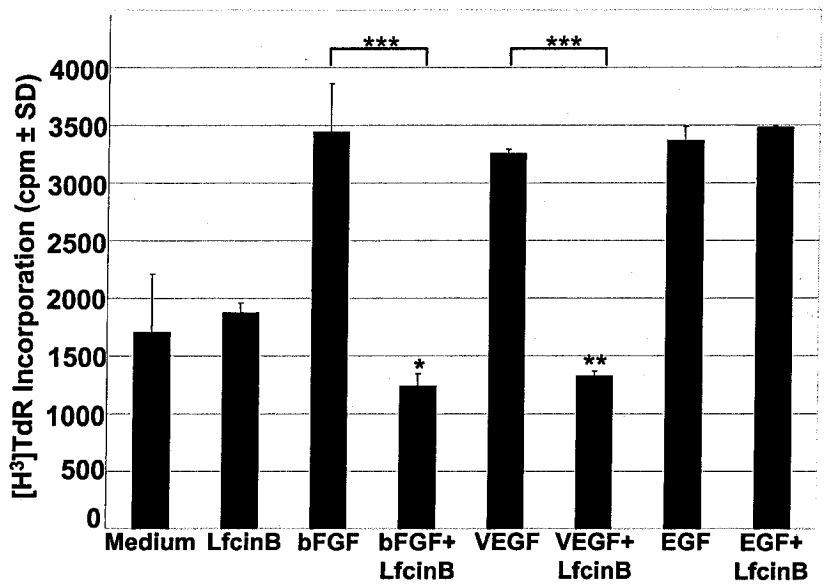

[H3]TdR Incorporation (cpm ± SD)
4000
3500
3000
2500
2000
1500
1000
500
0
***
***
*
**
Medium
LfcinB
bFGF
bFGF+ LfcinB
VEGF
VEGF+ LfcinB
EGF
EGF+ LfcinB

Figure 5.2

Figure 5.3 *LfcinB inhibited bFGF- and $VEGF_{165}$-induced HUVEC migration.* HUVECs (5×10^5 cells) were added to the upper chamber of costar transwell inserts, and medium, bFGF (10 ng/mL), or $VEGF_{165}$ (100 ng/mL) in the presence or absence of LfcinB (200 μg/mL) was added to the bottom chamber. Following incubation (2 h and 4 h) filters were fixed, stained with hematoxylin, and HUVECs that had migrated across the filter were enumerated by light microscopy. Data are shown as mean number of migrated HUVECs ± SD of triplicate determinations. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to growth factor treatment alone).

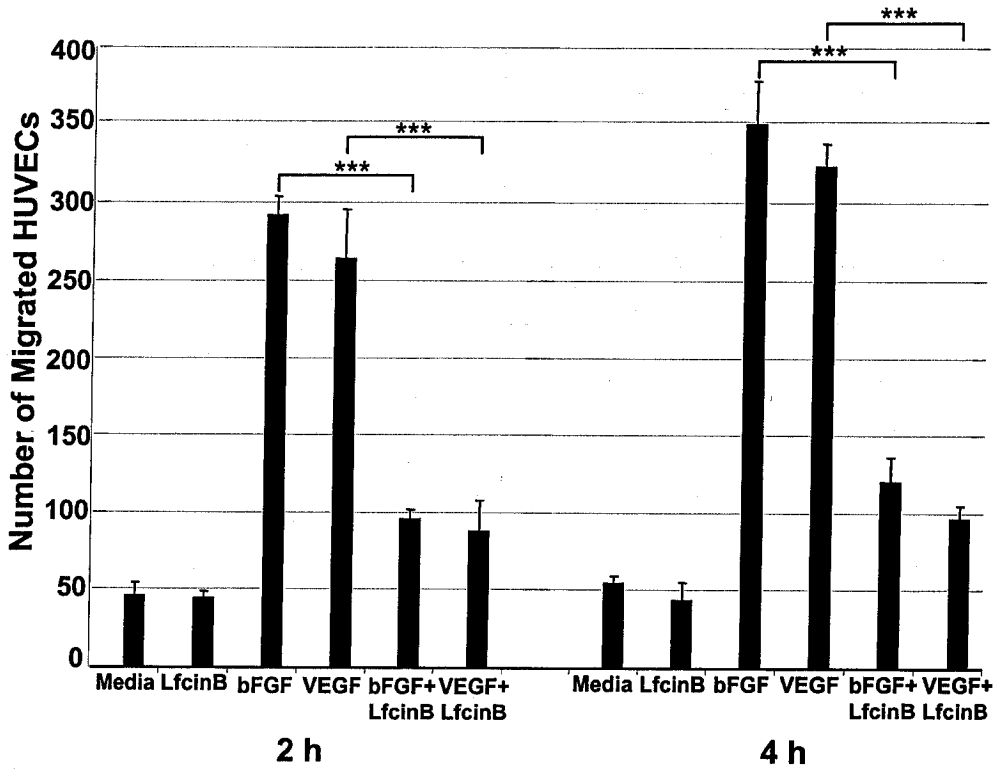

Number of Migrated HUVECs
400
350
300
250
200
150
100
50
0
***
***
***
***
Media
LfcinB
bFGF
VEGF
bFGF+ LfcinB
VEGF+ LfcinB
Media
LfcinB
bFGF
VEGF
bFGF+ LfcinB
VEGF+ LfcinB
2 h
4 h

Figure 5.3

Figure 5.4 *LfcinB did not affect HUVEC viability.* **A.** Effect of LfcinB on cell membrane integrity. HUVECs were labeled with ^{51}Cr, washed extensively, and then exposed to 200 μg/mL LfcinB in the presence or absence of bFGF (10 ng/mL), or $VEGF_{165}$ (100 ng/mL) for 6 h. ^{51}Cr present in cell-free supernatants at the end of the incubation period was determined by gamma counting. Data are expressed as percent ^{51}Cr release ± SD of triplicate determinations. **B.** Identification of apoptotic cells by Hoechst staining. HUVECs were cultured for 24 h in the presence of medium, LfcinB (200 μg/mL), bFGF (10 ng/mL) or $VEGF_{165}$ (100 ng/mL) alone, or bFGF (10 ng/mL) or $VEGF_{165}$ (100 ng/mL) in combination with LfcinB (200 μg/mL). HUVECs were then fixed and stained with Hoechst 33342 trihydrochloride dye to detect chromatin condensation and nuclear fragmentation (200× magnification).

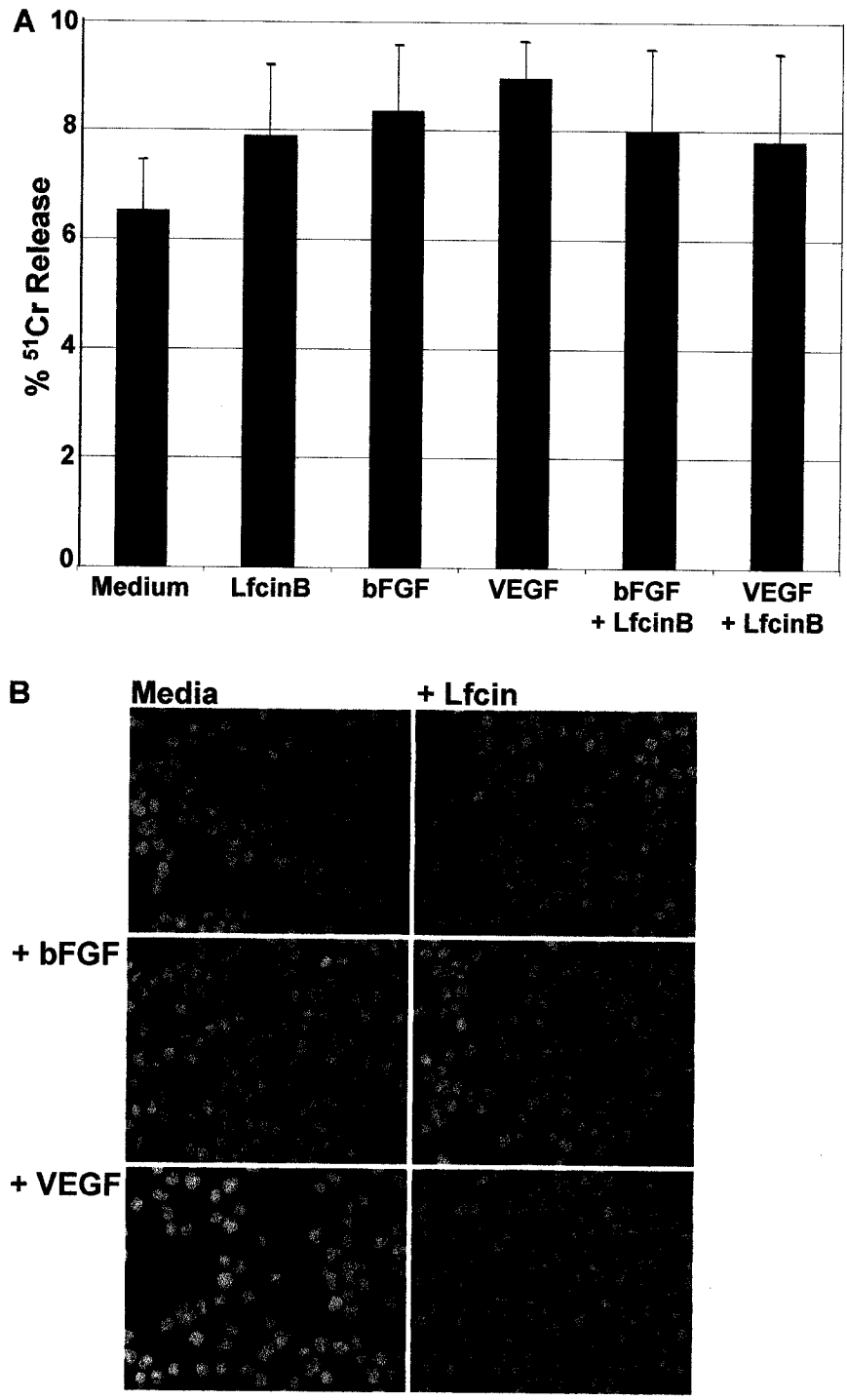

A
10
8
6
4
2
0
% 51Cr Release
Medium
LfcinB
bFGF
VEGF
bFGF + LfcinB
VEGF + LfcinB
B
Media
+ Lfcin
+ bFGF
+ VEGF

Figure 5.4

supernatant. Moreover, ^{51}Cr release was not increased in the presence of combined LfcinB and bFGF or VEGF. Figure 5.4B shows that HUVECs that were cultured for 24 h in the presence of LfcinB without or with bFGF or $VEGF_{165}$ did not exhibit chromatin condensation or nuclear fragmentation by Hoechst staining. I concluded that LfcinB was not inhibiting angiogenesis via a cytotoxic effect on resting or activated endothelial cells.

LfcinB Binding to Immobilized Heparin was Inhibited by bFGF and $VEGF_{165}$.

Both bovine lactoferrin and LfcinB possess heparin-binding activity (Wu *et al.*, 2003; Shimazaki *et al.*, 1998). Figure 5.5A shows that LfcinB bound plastic-immobilized heparin, which confirmed that the heparin-binding activity of bovine lactoferrin is localized to the LfcinB fragment. Moreover, both bFGF and $VEGF_{165}$ inhibited LfcinB binding to immobilized heparin in a dose-dependent fashion. In contrast, there was no inhibitory effect by non-heparin-binding EGF on LfcinB binding to heparin. LfcinB failed to bind immobilized bFGF or $VEGF_{165}$ (Figure 5.5B), ruling out any direct interaction between LfcinB and these growth factors. Since both bFGF and $VEGF_{165}$ must interact with heparin-like heparan sulfate proteoglycans for binding and signaling through their respective receptors (Rapraeger *et al.*, 1991; Gitay-Goren *et al.*, 1992), these data suggest that LfcinB may interfere with endothelial cell responses to bFGF and $VEGF_{165}$ by competing with these growth factors for the same binding sites on cell-surface heparan sulfate proteoglycans.

LfcinB Binding to HUVECs was Heparin-Dependent and Inhibited by bFGF and $VEGF_{165}$.

I next determined whether LfcinB was able to bind to heparin-like structures on HUVEC monolayers. Figure 5.6A shows that biotin-LfcinB bound strongly to HUVECs,

Figure 5.5 *LfcinB binding to immobilized heparin was inhibited by bFGF and $VEGF_{165}$.* **A.** Heparin (10 μg/mL) was immobilized on a flat-bottomed 96-well plates (o/n, 4°C). Plates were blocked with 1 % BSA in PBS, and exposed to biotin-LfcinB (50 μg/mL, 4°C) for 2 h, alone or in combination with bFGF, $VEGF_{165}$, or non-heparin-binding EGF. Plates were then washed and exposed to Streptavidin-HRP. After 2 hours, plates were washed and 1, 2-phenylenediamine substrate was added. Absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.001. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to LfcinB treatment alone). **B.** bFGF (100 ng/mL) or VEGF (100 ng/mL) were immobilized on a flat-bottomed 96-well plates (o/n, 4°C). Plates were blocked with 1 % BSA in PBS, and exposed to biotin-LfcinB (200 μg/mL, 4°C) for 2 h. Plates were then exposed to Streptavidin-HRP (1:1000, 2h, 4°C). Colorimetric substrate was added and analyzed by ELISA plate reader (492 nm). Each value represents mean ± SD. A control assay was performed using the Bradford protein assay, which determined that the growth factors bFGF and $VEGF_{165}$ did bind to 96-well plates.

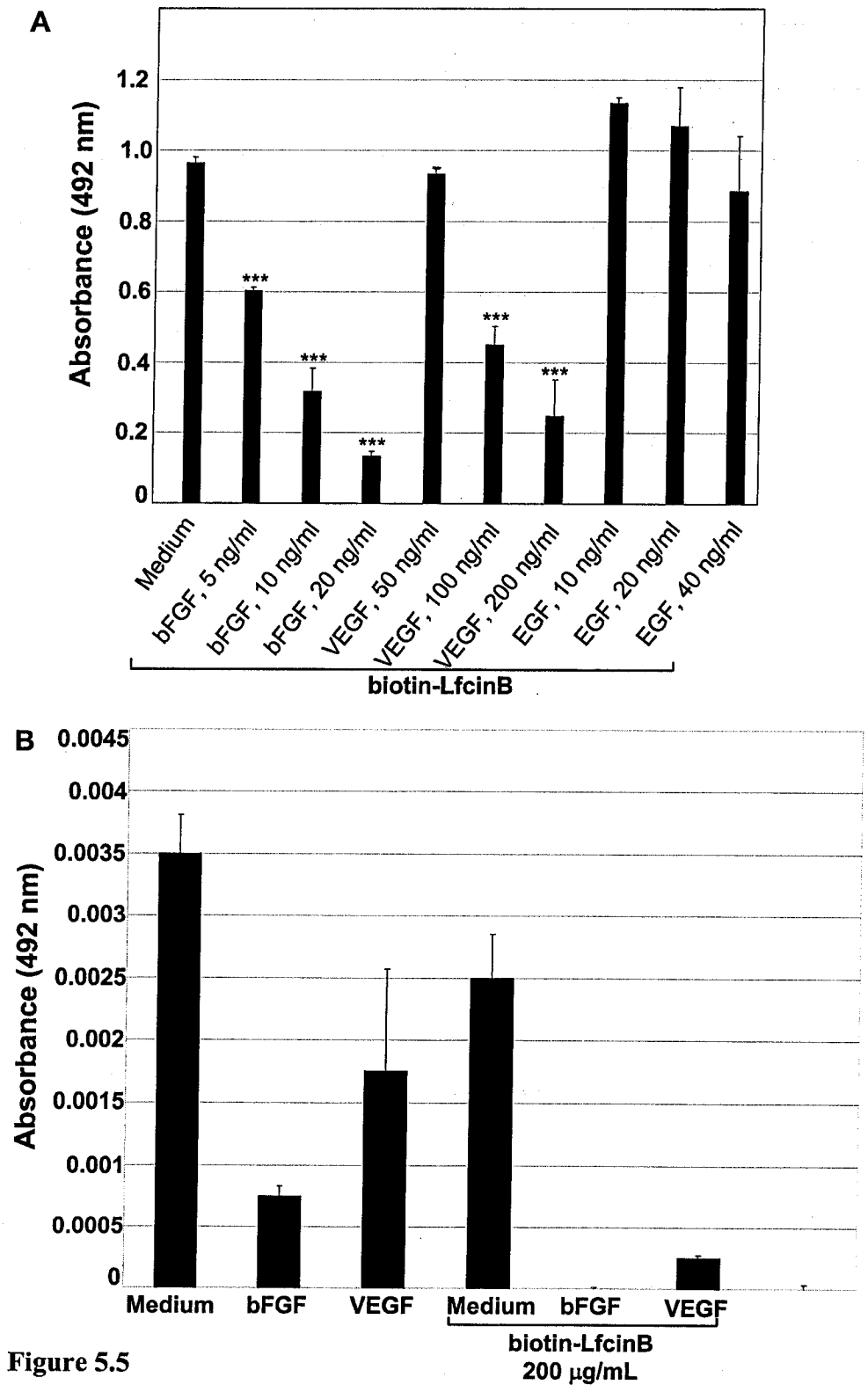
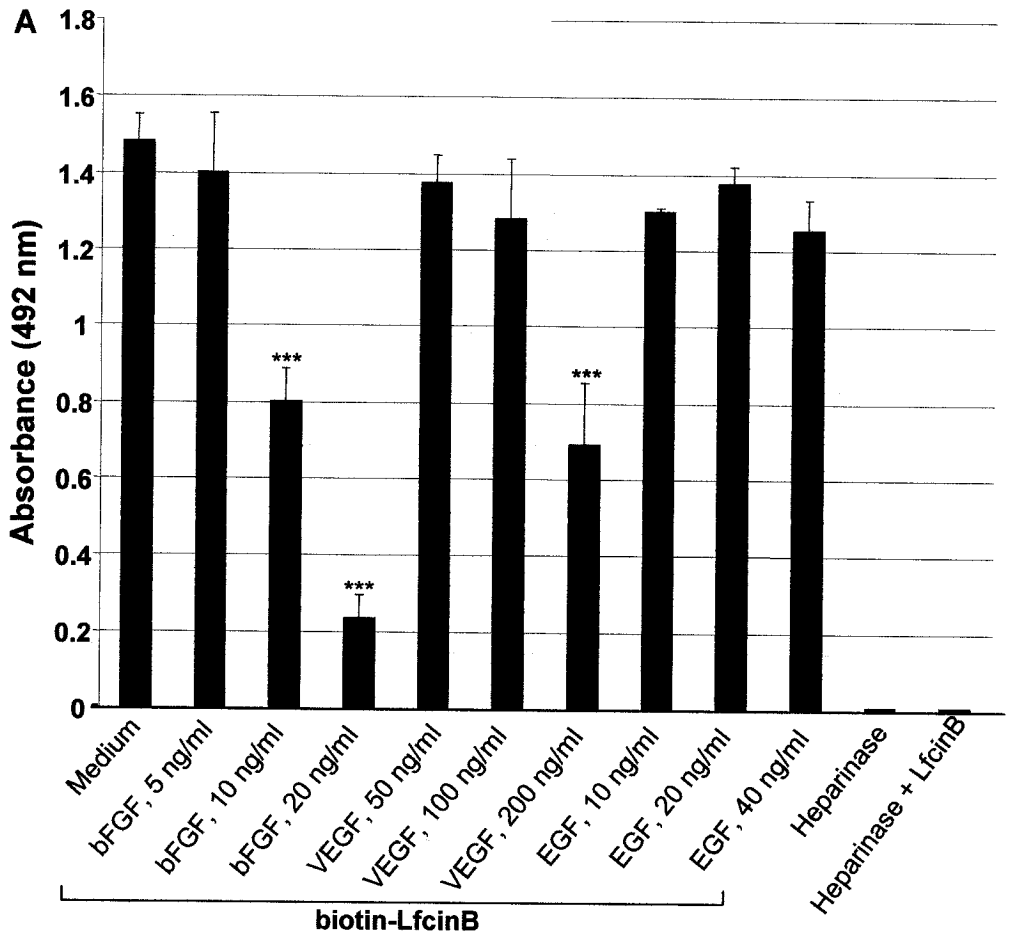

A
Absorbance (492 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0
***
Medium
bFGF, 5 ng/ml
bFGF, 10 ng/ml
bFGF, 20 ng/ml
VEGF, 50 ng/ml
VEGF, 100 ng/ml
VEGF, 200 ng/ml
EGF, 10 ng/ml
EGF, 20 ng/ml
EGF, 40 ng/ml
biotin-LfcinB
B
Absorbance (492 nm)
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Medium
bFGF
VEGF
Medium
bFGF
VEGF
biotin-LfcinB
200 μg/mL

Figure 5.5

Figure 5.6 *LfcinB binding to HUVECs was heparin-dependent and inhibited by bFGF and $VEGF_{165}$.* **A.** Colorimetric analysis of the effect of growth factors on HUVEC-LfcinB interactions. HUVEC monolayers were exposed to biotinylated LfcinB (50 μg/mL) in the absence or presence of the indicated concentrations of bFGF, $VEGF_{165}$, or non-heparin-binding EGF for 2 h. Some cultures were treated with heparinase prior to exposure to biotinylated LfcinB. HUVEC monolayers were then washed and incubated for 2 h with streptavidin-HRP. Following additional washes, 1, 2-phenylenediamine substrate was added and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.001. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to LfcinB treatment alone). **B.** Determination by fluorescent microscopy of bFGF or $VEGF_{165}$ effect on HUVEC-LfcinB interactions. HUVEC monolayers were exposed to medium alone or to biotinylated LfcinB (50 μg/mL) in the absence or presence of bFGF (20 ng/mL) or $VEGF_{165}$ (200 ng/mL) for 2 h. HUVEC monolayers were then washed and incubated with streptavidin-TexasRed. After additional washes, LfcinB binding to HUVECs was visualized by fluorescent microscopy (200× magnification). **C.** Effect of heparinase pretreatment on HUVEC-LfcinB interactions. HUVEC monolayers were pretreated with medium or heparinase, washed, and then incubated with medium or biotinylated LfcinB (50 μg/mL) for 2 h. Following additional washes, HUVEC monolayers were incubated with streptavidin-TexasRed. HUVEC monolayers were then washed LfcinB binding to HUVECs was visualized by fluorescent microscopy (200× magnification).


A
Absorbance (492 nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
Medium
bFGF, 5 ng/ml
bFGF, 10 ng/ml
bFGF, 20 ng/ml
VEGF, 50 ng/ml
VEGF, 100 ng/ml
VEGF, 200 ng/ml
EGF, 10 ng/ml
EGF, 20 ng/ml
EGF, 40 ng/ml
Heparinase
Heparinase + LfcinB
biotin-LfcinB

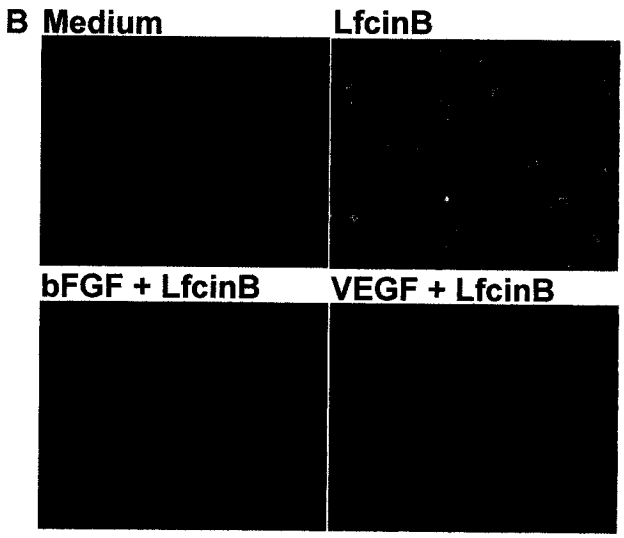

B Medium
LfcinB
bFGF + LfcinB
VEGF + LfcinB

Figure 5.6

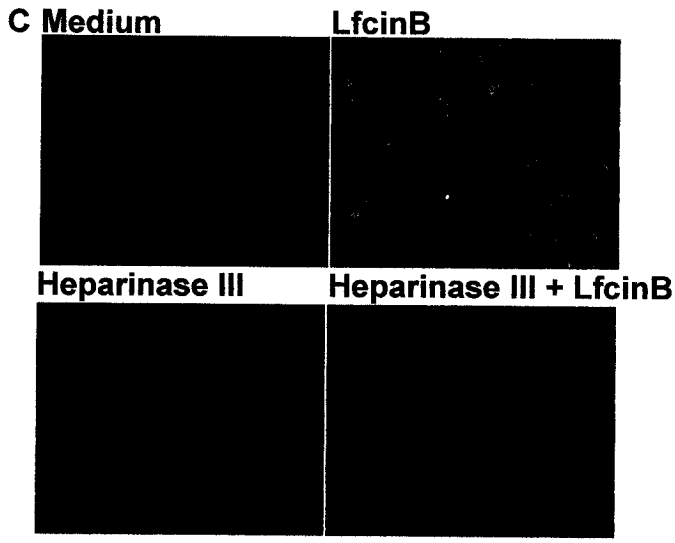


Figure 5.6

and that this interaction was ablated when HUVEC monolayers were pre-treated with heparinase to remove heparan sulfate proteoglycans from the cell surface. Moreover, the heparin-binding growth factors bFGF and $VEGF_{165}$ had a dose-dependent inhibitory effect on LfcinB binding to HUVECs. Non-heparin-binding EGF failed to inhibit LfcinB binding to HUVEC monolayers, which was consistent with heparin-binding activity by LfcinB. Similar results were obtained when fluorescent microscopy was used to determine the effect of bFGF or $VEGF_{165}$, as well as heparinase pre-treatment, on the interaction of LfcinB with HUVECs (Figures 5.6B and 5.6C). Figure 5.7 shows that LfcinB inhibited, in a dose-dependent fashion, bFGF (Figure A) and $VEGF_{165}$ (Figure B) binding to HUVEC monolayers. Collectively, these data suggest that LfcinB interacted with HUVECs via heparin-like heparan sulfate proteoglycans, and that competition for heparin-like binding sites that are associated with bFGF and $VEGF_{165}$ receptors interfered with bFGF and VEGF binding to their respective cell-surface receptors.

Figure 5.7 *bFGF and $VEGF_{165}$ binding to HUVECs was inhibited by LfcinB.* **A.** Colorimetric analysis of the effect of LfcinB on bFGF binding to HUVECs. HUVEC monolayers were exposed to biotinylated bFGF (10 ng/mL) in the absence or presence of the indicated concentrations of LfcinB for 2 h. HUVEC monolayers were then washed and incubated for 2 h with streptavidin-HRP. Following additional washes, 1, 2-phenylenediamine substrate was added and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.002. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to bFGF treatment alone). **B.** Colorimetric analysis of the effect of LfcinB on $VEGF_{165}$ binding to HUVECs. HUVEC monolayers were exposed to biotinylated $VEGF_{165}$ (100 ng/mL) in the absence or presence of the indicated concentrations of LfcinB for 2 h. HUVEC monolayers were then washed and incubated for 2 h with streptavidin-HRP. Following additional washes, 1, 2-phenylenediamine substrate was added and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Statistical significance was determined by Tukey-Kramer multiple comparisons test (*** denotes $p<0.001$ when compared to VEGF treatment alone).

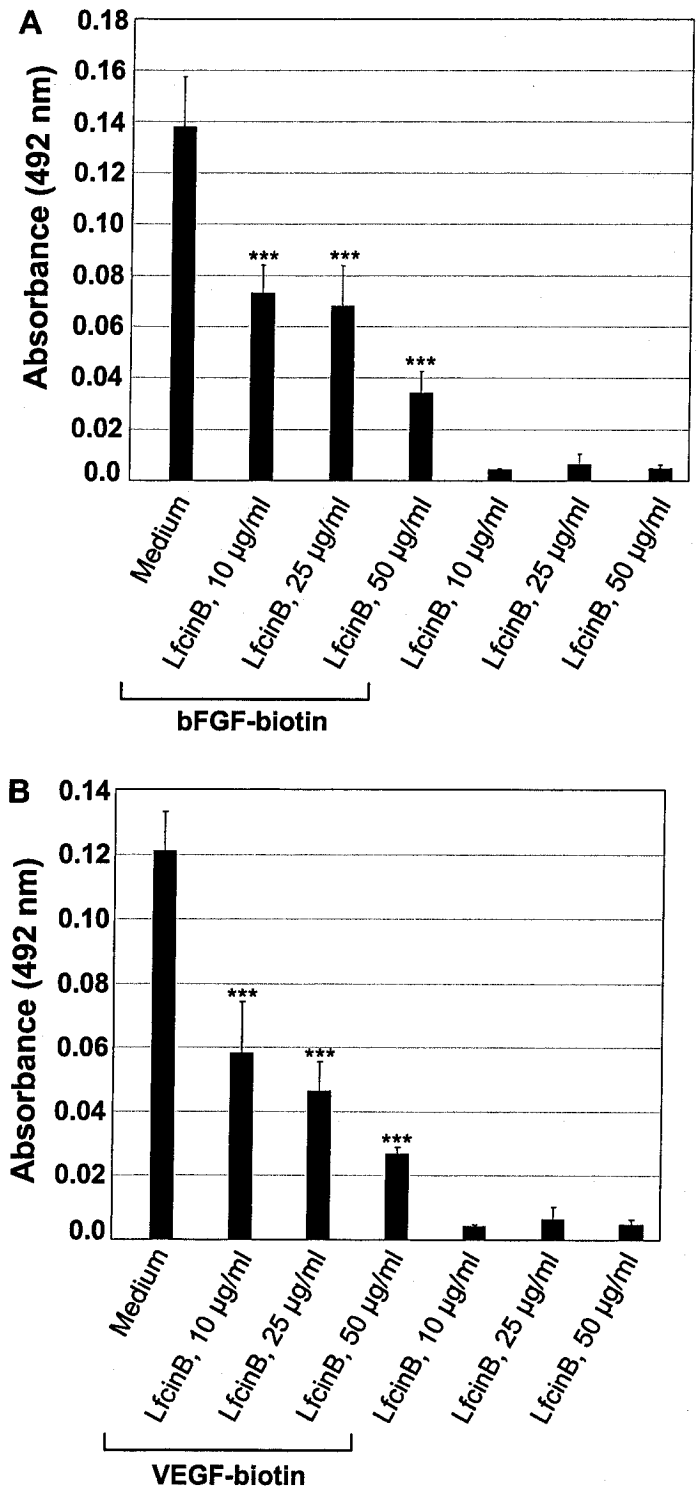

A
Absorbance (492 nm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
***
***
***
Medium
LfcinB, 10 µg/ml
LfcinB, 25 µg/ml
LfcinB, 50 µg/ml
LfcinB, 10 µg/ml
LfcinB, 25 µg/ml
LfcinB, 50 µg/ml
bFGF-biotin
B
Absorbance (492 nm)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
***
***
***
Medium
LfcinB, 10 µg/ml
LfcinB, 25 µg/ml
LfcinB, 50 µg/ml
LfcinB, 10 µg/ml
LfcinB, 25 µg/ml
LfcinB, 50 µg/ml
VEGF-biotin

Figure 5.7

CHAPTER 6

Discussion

Conventional cancer treatment commonly involves chemotherapeutic drug administration, which presents many serious drawbacks including non-specific toxicity associated with the inability of conventional chemotherapeutic agents to discriminate between rapidly dividing healthy cells, such as gut epithelial cells and hematopoietic progenitor cells, and neoplastic cells (Savarese *et al.*, 2003). Additionally, MDR tumour variants often arise through mutations in individual cancer cells, resulting in tumours that are resistant to chemotherapy (Krishna and Mayer, 2000). These restrictions that are associated with existing chemotherapeutic drugs have inspired investigators to seek out new anticancer agents.

Anticancer Activity of LfcinB

Selective Cytotoxicity via Apoptosis

I have determined that LfcinB was cytotoxic for a variety of human leukemia and carcinoma cell lines, including Jurkat T leukemia and MDA-MB-435 breast carcinoma cells. Importantly, LfcinB did not have any adverse effect on the viability of untransformed human primary cells, including lymphocytes, epithelial cells, fibroblasts, and endothelial cells. Even human T cells that were rapidly proliferating in response to mitogenic stimulation were not adversely affected by a concentration of LfcinB that triggered apoptosis in cultures of Jurkat T leukemia cells. The basis for this selectivity and the mechanism of interaction between LfcinB and the tumour cell membrane will be addressed shortly. I first wanted to determine whether LfcinB was inducing apoptosis in human cancer cell cultures. *In vitro* treatment with LfcinB induced DNA fragmentation in breast, colon, and ovarian carcinoma lines, as well as in leukemia and lymphoma cell

lines. Moreover, DNA isolated from Jurkat T leukemia cells exposed to LfcinB yielded a ladder-like pattern on agarose gel electrophoresis, and PS headgroups were externalized from the inner to the outer leaflet of the plasma membrane detected by Annexin V staining, both of which are characteristic of apoptosis (Kerr *et al.*, 1972). Changes in Jurkat T leukemia cell morphology, including membrane blebbing and cell shrinkage, were also observed following LfcinB treatment. Together, these finding indicated that LfcinB was inducing apoptotic cell death (Fleisher, 1997), which agreed with an earlier report that LfcinB triggers apoptosis in cultures of THP-1 monocytic leukemia cells (Yoo *et al.*, 1997b). Of importance, I am the first to show that LfcinB was able to induce apoptosis in carcinoma cell lines derived from malignancies of the breast, colon, and ovary.

Caspase Activation and Death Receptor Expression Following LfcinB Exposure

Inhibition of caspases-9, -2, and -3 activity significantly inhibited LfcinB-induced apoptosis in Jurkat T leukemia and MDA-MB435 breast carcinoma lines, while inhibition of caspases-8 activity did not have any substantial effect on apoptosis triggered by LfcinB. Additionally, immunoblot analysis using antibodies that were specific for activated caspases confirmed the presence of activated caspase-9, -2, and -3 following 2 hour LfcinB exposure. In contrast, there was little or no active caspase-8 was observed at this time point, which argued against an important role for death-receptors in LfcinB-induced apoptosis (Juo *et al.*, 1998). This data suggested that LfcinB-induced apoptosis does not require cell surface death receptor signaling. Flow cytometric analysis did not show any changes in Fas, FasL, DR4, or DR5 expression by Jurkat T leukemia cells

following LfcinB exposure, while TRAIL expression was slightly decreased in cells treated with LfcinB. Moreover, the additional of neutralizing mAb to FasL or TRAIL did not prevent LfcinB-induced apoptosis of Jurkat T leukemia cells. This data is consistent with a report that the Fas-resistant cell line, THP-1, is readily killed by LfcinB (Yoo *et al.*, 1997b). In conclusion, LfcinB exposure did not trigger the death receptor pathway of apoptosis in human cancer cells, which is in contrast to the contribution of death receptor signaling previously reported in cells exposed to various chemotherapeutic drugs (Friesen *et al.*, 1996; Williams *et al.*, 1997).

LfcinB Triggers the Mitochondria-Dependent Pathway of Apoptosis

Caspase-9 and caspase-2 function as initiator caspases in the mitochondria-dependent pathway of apoptosis (Read *et al.*, 2002; Slee *et al.*, 1999). The involvement of caspase-9 and caspase-2 in LfcinB-induced apoptosis, as well as the lack of death receptor involvement, implied that the mitochondria-associated pathway of apoptosis may play an important role in LfcinB-induced destruction of human cancer cells. Dissipation of $\Delta\Psi_m$ is commonly associated with mitochondrial membrane permeability, which results in the release of proapoptotic proteins, including cytochrome c, from the intermembrane space of the mitochondria into the cytoplasmic compartment (Green and Reed, 1998). In fact, cell survival depends on the maintenance of mitochondrial transmembrane potential, which is involved in ATP synthesis and supports oxidative phosphorylation (Mingatto *et al.*, 2002). LfcinB treatment resulted in the dissipation of $\Delta\Psi_m$ in Jurkat T leukemia cells, indicating that LfcinB disrupted the integrity of mitochondrial membranes.

The Mitochondria-Dependent Pathway Induced by LfcinB is Inhibited by Bcl-2 Overexpression

Antiapoptotic Bcl-2 family members, including Bcl-2 and Bcl-x_L, are known to inhibit apoptosis by interacting with the outer mitochondrial membrane and inhibiting the release of proapoptotic proteins through a number of different mechanisms, including the inhibition of proapoptotic Bax translocation to the mitochondria from the cytoplasm, and by trapping activated proapoptotic Bid (Cheng *et al.*, 2001). Proapoptotic Bcl-2 family members, such as Bax and Bid, induce mitochondrial membrane permeability by interacting with membrane permeability transition pore components, leading to pore opening that allows for the transfer of large mitochondrial proteins like cytochrome c into the cytoplasmic compartment (Crompton, 1999). I found that Jurkat T leukemia cells that were engineered to overexpress Bcl-2 were less susceptible to LfcinB-induced apoptosis. However, determining the role of Bcl-2 in LfcinB-induced apoptosis by overexpression may be difficult to interpret. Other studies have shown that overexpression of wild-type Bcl-2 can be toxic to many cells due to a yet undetermined proapoptotic activity of Bcl-2 that manifests during high levels of Bcl-2 protein expression (Pietenpol *et al.*, 1992; Uhlmann *et al.*, 1998; Wang *et al.*, 2001). Therefore, Bcl-2 overexpressing cells that survive may have altered expression of one or more additional proteins, giving them a survival advantage in addition to Bcl-2 (Uhlmann *et al.*, 1998; Wang *et al.*, 2001). Therefore, future studies using immunoblot analysis are needed to determine the possible involvement of other Bcl-2 family members in LfcinB-induced apoptosis.

Kinetic Analysis of Apoptosis Following LfcinB Treatment

Inactive forms of caspase-9 and caspase-2 are present in mitochondria and the cytosol of cells, while inactive caspase-2 also resides in the nucleus (Susin *et al.*, 1999; Zhivotovsky *et al.*, 1999). Kinetic analysis of caspase activity that was performed on isolated cytosolic cell fractions suggested that caspases-2 becomes activated prior to caspases-9 in LfcinB-treated Jurkat T leukemia cells. Interestingly, caspase-2 activity was required for $\Delta\Psi_m$ dissipation following LfcinB treatment in these cells, which is consistent with the recent finding that pro-caspase-2 is cleaved to its active form in advance of cytochrome c release from mitochondria (Robertson *et al.*, 2002). Caspase-2 triggers rapid, concentration-dependent cytochrome c release from isolated mitochondria, liposomes, and permeabilized Jurkat T leukemia cells by directly permeabilizing the outer mitochondrial membrane (Enoksson *et al.*, 2005; Robertson *et al.*, 2004). In this regard, caspase-2 can control the activation of pro-caspase-9, by initiating mitochondrial membrane permeability and the release of cytochrome c involved in the apoptosome complex (Lassus *et al.*, 2002). The low level of caspases-8 activation that was detected following LfcinB treatment in Jurkat T leukemia cells may have been due to a feedback amplification loop initiated by caspase-3 (Souza-Fagundes *et al.*, 2003).

Caspase-3, which is an important executioner caspase (Slee *et al.*, 2001), was present at high levels following LfcinB treatment in Jurkat T leukemia cells. However, inhibition of caspase-3 did not completely prevent LfcinB-induced apoptosis in these cells. This may be due to the involvement of other executioner caspases, such as caspase-6 or caspase-7 in LfcinB-induced apoptosis. Alternatively, LfcinB may induce the parallel involvement of proapoptotic AIF, which is released from the mitochondria

following mitochondrial membrane permeability and mediates apoptosis by localizing to the nucleus and inducing DNA fragmentation and chromatin condensation in a caspase-independent manner (Susin *et al.*, 1999; Cande *et al.*, 2002). Both scenarios are consistent with the finding that LfcinB can induce apoptosis in MCF-7 breast carcinoma cells, which lack pro-caspase-3, and also undergo apoptosis independent of cytochrome c (Blanc *et al.*, 2000; Li *et al.*, 1997).

ROS Activation in LfcinB-Treated Cancer Cells

ROS, including the superoxide anion, hydroxyl radicals, and hydrogen peroxide, are normal byproducts of oxidative respiration (England and Cotter, 2005). Cells are therefore equipped with antioxidants, such as GSH, that intercept ROS before they can cause harm to the cell (McEligot *et al.*, 2005). Apoptosis is often associated with oxidative stress due to excessive depletion of GSH and unchecked production of ROS by the mitochondrial electron transport chain (Tan *et al.*, 1998). ROS production occurs at an early stage in LfcinB-induced apoptosis of Jurkat T leukemia cells. Jurkat T leukemia cells exposed to LfcinB displayed increased production of ROS as early as 1 h following LfcinB treatment. In addition, pre-treating Jurkat cells with the exogenous antioxidants NAC or GSH interfered with LfcinB-induced apoptosis. However, the broad spectrum caspase inhibitor, Z-VAD-FMK had no effect on ROS generation in response to LfcinB, indicating that ROS production preceded caspase activation. These results are consistent with previous findings that implicate ROS in the induction of mitochondria-dependent apoptosis (Susuki *et al.*, 1998). My findings are also in agreement with an earlier report that the addition of antioxidants prior to LfcinB treatment inhibited LfcinB-induced apoptosis of THP-1 human monocytic leukemia cells (Yoo *et al.*, 1997b). However, the

downstream effect of ROS on the process of apoptosis was not characterized by Yoo *et al.* (1997b). In this regard, my studies showed that the dissipation of $\Delta\Psi_m$ in LfcinB-treated Jurkat T leukemia cells was at least in part a result of oxidative stress caused by LfcinB exposure. I speculate that early ROS generation was caused by mitochondrial membrane permeabilization that resulted from LfcinB entering Jurkat T leukemia cells and permeabilizing the negatively charged mitochondrial membrane (Eliassen *et al.*, 2002). This in turn led to ROS-induced cleavage of pro-caspase-2 (Takahashi *et al.*, 2004), followed by further destabilization of the outer mitochondrial membrane, which amplified ROS production and the caspase-2-regulated release of cytochrome c that is required for the activation of pro-caspase-9 and downstream executioner caspases (Robertson *et al.*, 2002). This series of events is consistent with the finding that NAC almost completely ablated ROS and LfcinB-induced apoptosis in Jurkat T leukemia cells, while only partially inhibiting dissipation of $\Delta\Psi_m$. The series of apoptotic events following LfcinB treatment in Jurkat T leukemia cells is schematically represented in Figure 6.1.

The Anticancer Effect of Linear LfcinB and LfcinB Derivatives

Pepsin-generated LfcinB has a cyclic structure formed by an internal disulfide bond between cysteine residues found at positions 3 and 20 of the peptide (Bellamy *et al.*, 1992). Cyclic and linear forms of LfcinB are equally effective in killing various microbes (Hoek *et al.*, 1997). Moreover, the cyclic structure was not required for LfcinB to induce apoptosis in human cancer cell lines since pepsin-generated LfcinB and synthetic LfcinB that lacked the disulfide bond displayed equivalent anticancer activity against Jurkat T leukemia and MCF-7 breast carcinoma cells. Additionally, pre-treatment with 2-ME,

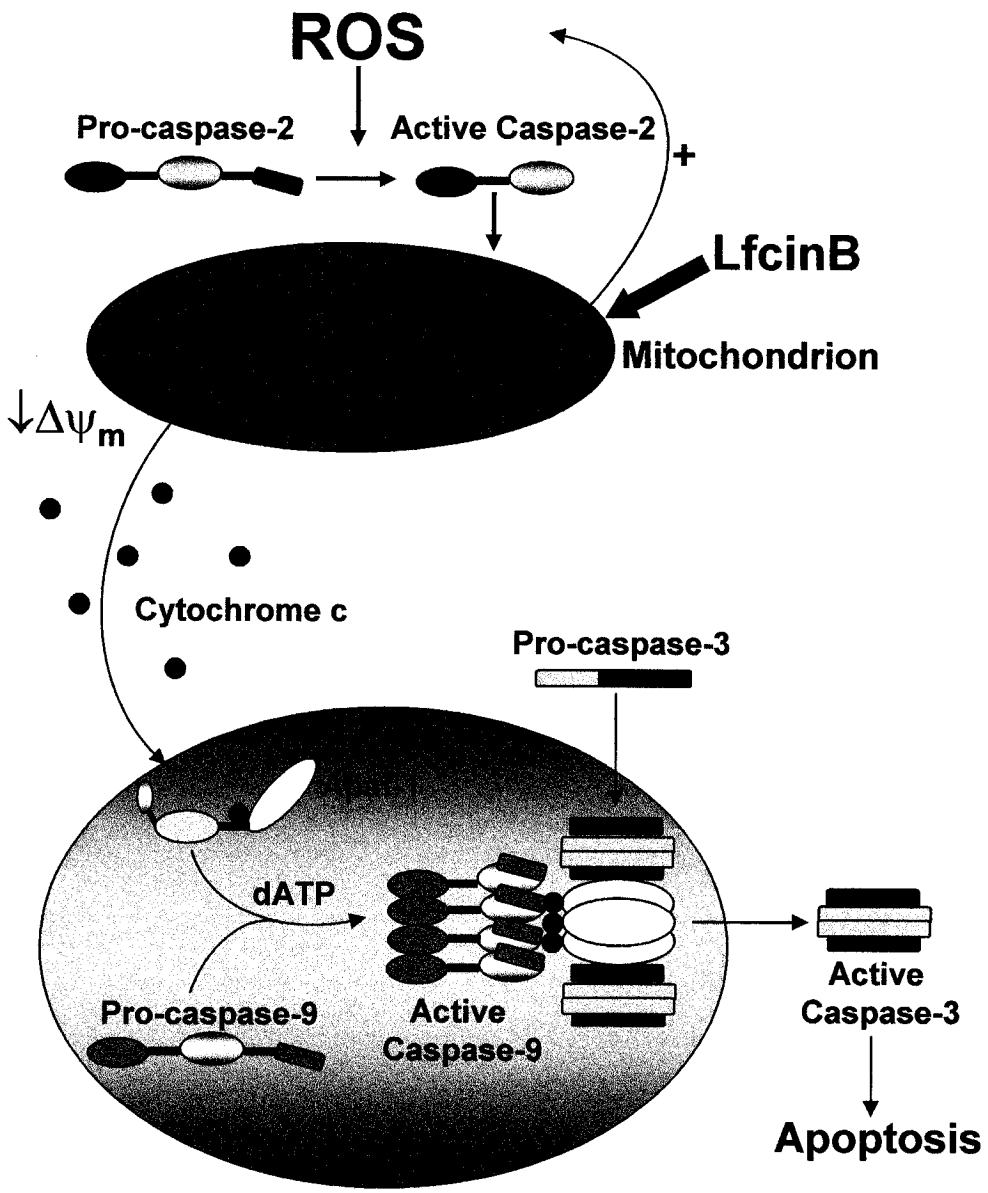


Figure 6.1 *Model of LfcinB-induced apoptosis in Jurkat T leukemia cells.* LfcinB caused an increase in ROS production, which activates caspase-2. Activated caspase-2 and ROS then lead to mitochondrial membrane potential dissipation. Cytochrome c is released from the mitochondria and forms the apoptosome complex with Apaf-1 and pro-caspase-9 to activate the executioner caspase-3.

which cleaves disulfide bonds that may have spontaneously formed in linear LfcinB, had no effect on LfcinB-induced DNA fragmentation in Jurkat T leukemia cells. However, 2-ME is a volatile chemical that may breakdown over the course of the assay. Future experiments will be necessary to examine the requirement for disulfide bond formation in LfcinB-induced cytotoxicity by substituting the two cysteine residues in the LfcinB structure with serine residues (Regeimbal and Bardwell, 2002), and/or by blocking disulfide bond formation with iodoacetamide, which alkylates free sulfhydryl groups (Whitley and van Heijne, 1993). In contrast to my findings, one report suggested that linear LfcinB was not effective in killing Meth A fibrosarcoma or B16F10 murine melanoma cell lines (Eliassen *et al.*, 2002). This discrepancy may be explained by genetic and/or membrane differences between murine and human tumour cell lines (Jaworski *et al.*, 2005), which would result in differential susceptibility to linear LfcinB-induced apoptosis.

The anticancer and antimicrobial activities of LfcinB are not associated with precisely the same amino acid sequence because a 10-amino acid derivative (FKCRRWQWRM) and a 6-amino acid derivative (RRWQWR) of LfcinB that form the antimicrobial centre of the peptide (Schibli *et al.*, 1999; Hoek *et al.*, 1997) exhibited significantly less and no cytotoxic activity, respectively, against Jurkat T leukemia cells. The 6-mer peptide is also significantly less lytic in model membrane studies in comparison to the intact 25-mer, most likely due to an inability of the 6-mer to partition deep in the membrane bilayer as a result of its small size and lack of hydrophobic residues other than Trp (Schibli *et al.*, 2002). Interestingly, when the 6-mer or 10-mer derivatives of LfcinB were delivered to the cytoplasmic compartment of Jurkat T

leukemia cells via fusogenic liposomes I found that both the 10-mer and the 6-mer were cytotoxic to Jurkat T leukemia cells (see Appendix A, Figure 1B). The ability of 10-mer and 6-mer derivatives of LfcinB to kill Jurkat T leukemia cells once inside the cytosol suggested that the resistance to free 6-mer and 10-mer was governed at the level of the cell membrane, possibly due to the inability of these small peptides to span or destabilize the tumour cell membrane. It is also possible that, when LfcinB or its derivatives were delivered directly into the cytosol of cancer cells, the ratio of peptide molecules to the intracellular target was much greater than that of ratio between the peptide molecules and the plasma membrane in the extracellular environment. Additionally, once internalized into cells, the 6-mer and 10-mer peptides may target the negatively charged mitochondrial membrane which lacks cholesterol, and may therefore be easier to destabilize (Ellerby *et al.*, 1999). A recent report determined that peptides shorter than 23 amino acids are unable to span cell membranes by the barrel-stave destabilization model (Leuschner and Hansel, 2004). In light of this finding, my observation that non-encapsulated 10-mer was able to kill Jurkat T leukemia cells (30% ± 5 at 63 μM) suggested that the interaction between the 10-mer peptide and the tumour cell membrane may be via the carpet model. Further study is necessary to determine whether this is the case, and to understand whether the internalized 10-mer and 6-mer peptides target organelles within cancer cells.

In Vivo Anticancer Effect of LfcinB

In order to develop new anticancer drugs it is necessary to test their efficacy *in vivo*. I therefore analyzed the ability of LfcinB to increase survival time in

immunodeficient SCID/beige mice bearing disseminated Ramos B lymphoma cells. Mice that were treated with LfcinB on day 1 after lymphoma cell injection showed a 1.4-fold increase in median survival compared to controls, whereas mice that were given LfcinB on days 1 and 3 after lymphoma cell injection showed a 1.9-fold increase in median survival, as well as an increased proportion (60%) of long-term survivors. Additionally, mice treated with LfcinB showed steady weight gain over the course of the experiment, while control mice experienced dramatic weight loss. Of interest, LfcinB-induced cytotoxic activity against Ramos and Raji B lymphoma cells was impaired at higher serum concentrations *in vitro*. This may have been the result of cationic LfcinB binding to negatively charged serum proteins. Alternatively, this inhibitory effect may have been due to the ability of serum albumin to block $\Delta\Psi_m$, thereby inhibiting apoptosis (Gallego-Sandin *et al.*, 2005). Regardless of the mechanism, the *in vivo* data suggests that negatively charged serum components do not have the ability to block the anticancer effects of LfcinB as long as a sufficient quantity is administered *in vivo*. Therefore, LfcinB may be an effective treatment for B-cell lymphoma.

Structure-Function Relationship in the Anticancer Activity of LfcinB

In addition to considerable antimicrobial activity, LfcinB has cytotoxic activity against many different human cancer cells both *in vitro* and *in vivo* (Yoo *et al.*, 1997a and b; Roy *et al.*, 2002; Mader *et al.*, 2005), while having no cytotoxic effect against normal human cells (Mader *et al.*, 2005). Interestingly, LfcinB displays a distinct structure composed of many asymmetrically clustered basic amino acid residues, giving the peptide a distinct net positive charge of 7.85 at pH 7.0 (Eliassen *et al.*, 2002). Although

the molecular basis of the selectivity of LfcinB for transformed cells is not yet clear, one possibility is that the cationic, amphipathic structure of LfcinB allows the peptide to interact with the negatively charged outer membrane of the cancer cell, resulting in plasma membrane destabilization, and cell death (Ellerby *et al.*, 1999; Chan *et al.*, 1998; Conner *et al.*, 1989). Model membrane analysis has shown that LfcinB preferentially binds to cancer cell membranes with a net negative charge but not to cell membranes of untransformed eukaryotic cells that have a net neutral charge (Vogel *et al.*, 2002). This is consistent with antimicrobial studies where LfcinB was shown to interact more strongly with phosphatidylglycerol (PG) headgroups of multilamellar vesicles than with phosphatidylethanolamine (PE) and phosphatidylcholine (PC) headgroups (Vogel *et al.*, 2002), which may explain why LfcinB prefers to bind to the membrane of bacterial cells but not to that of healthy eukaryotic cells. Bacterial cell membranes have an abundance of PG and PE, while eukaryotic cells display PC on their membranes. Both PE and PC are zwiterrionic and consequently carry no net charge, while PG has an overall net negative charge. The negative charge found on the surface of cancer cells can be attributed to membrane phospholipids like PS, and negatively charged glycoproteins, such as O-glycosylated mucins, that are overexpressed in many cancer cells, including breast and prostate carcinomas (Rakha *et al.*, 2005; Moniaux *et al.*, 2004). In addition to a higher content of anionic phospholipids, the protein content and fluidity of many tumour cell membranes are different from that of normal human cells (Matsuzaki, 1997). Furthermore, cancer cell membranes tend to express abundant microvilli, which can increase cell surface area, and thus the amount of membrane-bound peptide (Ren, 1991). Together, the differences between normal and tumour cell membranes may explain why

the cytotoxic activity of LfcinB is selective for cancer cells. In this regard, fluorescent microscopy analysis showed that LfcinB avidly bound to the surface of Jurkat T leukemia cells as early as 5 min post exposure to the peptide, while LfcinB did not bind to the surface of normal human T cells. This is consistent with the finding that LfcinB was not cytotoxic to normal human T cells. In addition, the mouse homologue of Lfcin, LfcinM, which has a net charge of 3.85 at pH 7, has no cytotoxic effect against Meth A fibrosarcoma cells *in vitro*, which further supports the hypothesis that electrostatic interactions between the highly cationic LfcinB peptide and negatively-charged cancer cells are important for its anticancer activity (Eliassen *et al.*, 2002).

Interestingly, I have found that LfcinB was unable to kill certain human cancer cell types, including the PC3 prostate carcinoma cell line, even though LfcinB maintained the ability to bind to PC3 cell membranes (see Appendix A, Figure 2). One possible explanation for this surprising finding is that a variation exists at the level of the cell membrane that determines whether a cancer cell is sensitive to LfcinB toxicity. Alternatively, more resistant tumour cell lines may overexpress antiapoptotic proteins, such as Bcl-x_L which has already been shown to be overexpressed in Skov3 ovarian carcinoma cells (Liu *et al.*, 1998). Future studies are needed to examine additional LfcinB-resistant tumour cell lines for phenotypic and/or genotypic differences that may explain the decreased sensitivity, and to establish whether LfcinB is equally effective against cancer cells that express multidrug resistance proteins.

Receptor Analysis of the Interaction between LfcinB and Cancer Cells

I wanted to understand whether the anticancer activity of LfcinB was initiated through receptor binding and subsequent endocytosis, since eukaryotic cells require vesicular-mediated endocytosis for the uptake of many extracellular components (Strømhaug *et al.*, 1997). I found that inhibitors of receptor-mediated endocytosis, dansylcadaverine and cytochalasin D, failed to prevent LfcinB cytotoxicity in T leukemia cell cultures. This was consistent with previous results indicating that LfcinB entry into Vero cells is independent of receptor-mediated endocytosis (Andersen *et al.*, 2004). In light of these findings, and the knowledge that LfcinB binds to negatively charged membrane components, I believe that LfcinB does not require receptor binding to induce apoptosis in Jurkat T leukemia cells. Although LfcinB receptors have not been identified, lactoferrin is known to interact with a functional lactoferrin receptor (Legrand *et al.*, 1992), the low density lipoprotein receptor-related protein (Ziere *et al.*, 1993), cell surface nucleolin protein (Legrand *et al.*, 2004), and cell membrane proteoglycans (Wu *et al.*, 1995). However, the cationic nature of lactoferrin results in a tendency to bind different anionic molecules and makes identification of specific cell surface receptors difficult. Yazidi-Belkoura *et al.* (2001) determined that lactoferrin predominantly binds to membrane-associated proteoglycans, which questions the presence of a functional lactoferrin receptor. The interaction between lactoferrin and cell-surface proteoglycans on human HT-29 colorectal carcinoma cells is mediated through the N-terminal portion of the peptide, which contains the amino acids that make up LfcinB (Yazidi-Belkoura *et al.*, 2001).

LfcinB Conforms to the Barrel-Stave Model of Membrane Destabilization

Amphipathic peptides generally employ the barrel-stave model of membrane interaction, while hydrophobic peptides tend to destabilize the cell membrane through the carpet model (Ye *et al.*, 2004). Additionally, peptides shorter than 23 amino acids are believed to be incapable spanning the human cell membrane (Leuschner and Hansel, 2004). It is therefore hypothesized that because LfcinB is an amphipathic peptide comprised of 25 amino acid residues, LfcinB employs the barrel-stave model of membrane destabilization in killing human tumour cells (Strøm *et al.*, 2002). I have determined that intracellular ^{51}Cr was released from Jurkat T leukemia cells as early as 5 min following LfcinB treatment, and that this event preceded DNA fragmentation. Since DNA fragmentation is a hallmark of early apoptosis (Wyllie, 1980), this data suggested that LfcinB compromised the cell membrane prior to the initiation of apoptosis. LfcinB treatment also caused a flux in intracellular calcium when Jurkat T leukemia cells were suspended in calcium rich buffer; however, this calcium flux was ablated when Jurkat cells were suspended in calcium-free HEPES tyrodes buffer. This observation suggests that calcium was entering Jurkat cells upon exposure to LfcinB, possibly through LfcinB-vented pores. However, had LfcinB exposure resulted in Ca^{2+} entry from the extracellular milieu into the cell cytosol via the formation of membrane pores, I would expect that the flux would continue to increase in a linear manner over time. Because the Ca^{2+} flux ended rapidly, it is possible that LfcinB treatment was causing Jurkat T leukemia cell agglutination, resulting in an increase in fluorescence. In this regard, various cations have been shown to cause cellular agglutination (Utsumi and Oda, 1973) through a mechanism that involves intracellular crossbridge formation through their interaction with membrane carbohydrates (Curtis, 1973; Terry and Culp, 1974). Therefore, the possible formation of

membrane pores in Jurkat T leukemia cell membranes via LfcinB insertion will need to be further examined. This will be accomplished using various techniques, including membrane current measurements utilizing planar bilayers, which can determine the conductance through and size of membrane pores. This technique has been used previously to determine membrane pore formation by other CAPs, such as the cecropins (Christensen et al., 1988). Differential scanning calorimetry, which measures membrane phase transitions indicative of pore formation, will also be employed to analyze the potential pore forming activity of LfcinB. This method has been used by several researchers to analyze magainin-2 pore formation (Matsuzaki et al., 1995; Ludtke et al., 1996). Further studies that I conducted determined that LfcinB entered Jurkat T leukemia cells and was present in the cytosol prior to cell death. Moreover, LfcinB entry into the cytosol was independent of receptor-mediated endocytosis. This observation is consistent with a recent report that *in vitro* exposure to LfcinB causes murine Meth A fibrosarcoma cells to lose membrane integrity and eventually lyse (Eliassen *et al.*, 2002). CAPs that utilize the barrel-stave model of membrane destabilization are known to enter the cytoplasmic compartment of a cell, whereas CAPs that employ other methods of membrane destabilization do not enter the cell. My data therefore support the hypothesis that LfcinB forms pores in the tumour cell membrane, allowing the peptide to gain access to the intracellular environment.

LfcinB Interacts with Mitochondria

Once inside the cell, LfcinB may interact with cellular components, such as the mitochondrial membrane and DNA (Britigan *et al.*, 2001). Previous evidence suggests

that lactoferrin can enter microbial cells and tumour cells, and may have cytotoxic activity linked to its interaction with intracellular components (He and Furmanski, 1995; Haukland *et al.*, 2001). In this regard, lactoferrin enters K562 human myelogenous leukemia cells and localizes to the nucleus, where it affects gene transcription by binding to nuclear DNA (He and Furmanski, 1995). The DNA-binding properties of lactoferrin reside in the N-terminal region, specifically within the amino acid sequence that contains LfcinB (Kanyshkova *et al.*, 1999). Since I have determined that LfcinB can enter Jurkat T leukemia cells to reside in the cytoplasmic compartment, it is possible that LfcinB targets intracellular components, including mitochondria and nucleic acids. The common ancestry of prokaryotic cells and the mitochondria of eukaryotic cells, and the knowledge that they both have large transmembrane potentials and are composed of a high content of anionic phospholipids suggests that intracellular LfcinB may interact with mitochondrial membranes (Gray *et al.*, 1999; Risso *et al.*, 2002; Dekroon *et al.*, 1997; Matsuzaki, 1995). Preliminary transmission electron microscopy experiments determined that LfcinB avidly bound to mitochondria isolated from Jurkat T leukemia cells. Further experiments are needed to determine whether this interaction occurs when LfcinB enters intact cells and whether the binding of LfcinB to the mitochondrial membrane causes membrane destabilization, allowing for ROS release prior to $\Delta\Psi_m$. In addition, it is possible that LfcinB may interact with other organelles once inside the cell. Interactions with endoplasmic reticulum and lysosomes may result in the release of Ca^{2+} and cathepsin B, respectively, into the cytosol, each of which have been shown to have proapoptotic activity (Ferri and Kroemer, 2001; Savino *et al.*, 2006). Additionally, recent evidence suggests that endoplasmic reticular stress can lead to the activation of caspase-2

(Dahmer, 2005). I have shown that caspase-2 is required for the $\Delta\Psi_m$ observed during early LfcinB-induced apoptosis in Jurkat T leukemia cells. Although caspase-2 activation was shown to be induced in part by ROS, it is possible that endoplasmic reticular stress may have also contributed to caspase-2 activation.

Administration of LfcinB may be Achieved by Encapsulating the Peptide in a Liposomal Vehicle

Delivery of LfcinB to the tumour site may be difficult due to the likelihood of enzymatic degradation and inactivation of the peptide by serum components (Papo and Shai, 2003). One method to avoid this problem is through the use of targeted liposomal vehicles, which would allow for the encapsulation and delivery of LfcinB via the intravenous route, preventing peptide degradation and inactivation, and enhancing drug activity, while decreasing any toxic side effects (Yuan *et al.*, 1994; Yamada *et al.*, 2005; Swenson *et al.*, 2004). Furthermore, tumour-associated blood vessels have increased vascular permeability in comparison to their normal counterparts (Jain, 2002), which would allow for the delivery of liposome-encapsulated LfcinB directly to the tumour site via leakage from tumour-associated vasculature. In this study, I have shown that LfcinB delivered via fusogenic liposomal vehicles is able to kill Jurkat T leukemia cells *in vitro*, and that this effect was not due to leakage of LfcinB from extracellular liposomes (see Appendix A, Figure 1A). Interestingly, LfcinB delivered directly to the cytoplasmic compartment of LfcinB-resistant HUVECs and human primary fibroblasts via fusogenic liposomes resulted in significant cell death, which suggests that the selective anticancer activity displayed by LfcinB is determined at the level of the cell membrane and not by intracellular biochemical processes. Furthermore, liposome-mediated delivery of the 6-

mer derivative of LfcinB was cytotoxic to both HUVEC and Jurkat T leukemia cells, both of which are both resistant to free extracellular 6-mer.

As mentioned above, one potential difficulty in administering LfcinB is its possible inactivation by negatively charged serum components. I have determined that increasing concentrations of BSA, a serum component displaying a high net negative charge, was able to inhibit LfcinB-induced cell death in Jurkat T leukemia cells. Initially, I believed that this effect was due to an electrostatic interaction between BSA and LfcinB, which in turn reduced the amount of LfcinB that was available to bind to human tumour cells. However, fluorescent microscopy analysis revealed substantial LfcinB binding to Jurkat T leukemia cells in the presence of high concentrations of BSA. Recent evidence suggests that BSA may prevent mitochondrial membrane potential destabilization (Gallego-Sandin *et al.*, 2005), which I know to be a necessary step in LfcinB-induced cell death (Mader *et al.*, 2005). Other reports suggest that albumin may also have cytoprotective effects through an antioxidant function that prevents free radical accumulation within the cell (Gum *et al.*, 2004; Moran *et al.*, 2002). Therefore, the inhibitory effect of BSA on LfcinB-induced tumour cell death may have been due to its ability to block $\Delta\Psi_m$ and/or ROS accumulation, rather than by interfering with LfcinB-cell membrane interactions. Future analysis via flow cytometry will quantitate the binding of LfcinB to human cancer cells under low and high serum conditions.

Use of Inhibitors to Block Tumour-Associated Angiogenesis

Novel treatment strategies based on the inhibition of tumour-associated blood vessel development have gained considerable attention in recent years due to the intrinsic

advantages that antiangiogenic therapy has over conventional chemotherapy. Unlike most tumour cells, ECs are readily accessible from the vascular circulation, and as genetically stable, homogenous cells they are not likely to develop resistance to antiangiogenic agents (Kerbel and Folkman, 1997). Also, antiangiogenic drugs selectively target tumour-associated vasculature, which results in comparatively few adverse effects, while endothelial cells that form the tumour vasculature are easily accessible to antiangiogenic agents delivered via the blood (Fayette *et al.*, 2005). In healthy adults, endothelial cells are normally very stable with a turnover rate measured in months or years (Han and Liu, 1999), and angiogenesis is only observed in the physiological processes of wound repair (Hunt *et al.*, 1984), and during the female reproductive cycle (Reynolds *et al.*, 1992). However, angiogenesis takes place in certain pathological conditions, such as rheumatoid arthritis (Walsh, 1999), and solid tumour growth (Folkman, 1971).

Role of LfcinB as an Antiangiogenic Agent

Various angiogenesis inhibitors are presently undergoing clinical trials or are being used in clinical practice (Kerbel and Folkman, 2002; Fayette *et al.*, 2005). At the same time, the search continues for novel antiangiogenic agents that might be valuable in the treatment of different cancers. A potential antiangiogenic role for LfcinB was suggested by the finding that systemic administration of LfcinB to tumour-bearing mice caused a reduction in the number of tumour-associated blood vessels (Yoo *et al.*, 1997a). Treatment of tumour-bearing mice with bovine lactoferrin led to similar results. In addition, recent evidence supports an antiangiogenic role for lactoferrin since oral administration of lactoferrin inhibited VEGF-induced angiogenesis in a mesenteric-

window assay performed in rats (Norrby *et al.*, 2001), and Lewis lung carcinoma-induced angiogenesis in a dorsal air sack assay in mice (Shimamura *et al.*, 2004). My observation that LfcinB inhibited bFGF- and $VEGF_{165}$-induced angiogenesis *in vivo*, as well as bFGF- and $VEGF_{165}$-induced the proliferation and migration of HUVECs *in vitro*, provides convincing evidence that the antiangiogenic activity of bovine lactoferrin resides within the LfcinB sequence located proximal to the N-terminus of the parent protein. Of interest, LfcinB is produced in significant quantities in the stomach of rats and humans following bovine lactoferrin ingestion (Kuwata *et al.*, 1998; Kuwata *et al.*, 2001). A recent study failed to demonstrate the presence of dietary bovine lactoferrin or its functional fragments in the portal blood of rats (Wakabayashi *et al.*, 2004). However, the systemic antiangiogenic activity of ingested bovine lactoferrin in both rats and mice (Norrby *et al.*, 2001; Shimamura *et al.*, 2004) supports the belief that LfcinB is transferred into circulating blood following ingestion.

The proliferation and migration of endothelial cells are important components of the angiogenic process, which is strictly regulated under normal conditions by a balance of proangiogenic and antiangiogenic factors. However, under pathological conditions such as tumour growth, angiogenesis can become dysregulated (Carmeliet and Jain, 2000). Growth factors that promote angiogenesis include bFGF and $VEGF_{165}$ (Javerzat *et al.*, 2002; Ribatti *et al.*, 2005), both of which are produced by neoplastic cells (Yoshiji *et al.*, 1996; Cronauer *et al.*, 1997; Birck *et al.*, 1999). While LfcinB significantly inhibited bFGF- and $VEGF_{165}$-induced HUVEC proliferation, LfcinB did not affect the ability of non-heparin-binding EGF to stimulate HUVEC proliferation. Of interest, when LfcinB was administered in combination with growth factors bFGF or $VEGF_{165}$ there was a

significant reduction in HUVEC proliferation compared to LfcinB treatment alone for reasons that are not clear at this time. In addition, bFGF- and $VEGF_{165}$-, but not non-heparin-binding EGF-, induced angiogenesis in the Matrigel plug assay was inhibited by LfcinB. Importantly, LfcinB was not cytotoxic against HUVECs, eliminating the possibility that HUVECs are undergoing apoptosis when exposed to LfcinB, as occurs when various cancer cell lines are treated with LfcinB (Yoo *et al.*, 1997b; Mader *et al.*, 2005). Together with the knowledge that both bovine lactoferrin and LfcinB are known to bind heparin (Wu *et al.*, 2003; Shimazaki *et al.*, 1998), these findings led me to hypothesize that LfcinB competed with bFGF and $VEGF_{165}$ for heparin-like binding sites on heparan sulfate proteoglycans on the surface of HUVECs.

Heparan sulfate proteoglycans are necessary for bFGF and $VEGF_{165}$ binding and subsequent signaling through their respective cell-surface receptors (Rapraeger *et al.*, 1991; Gitay-Goren *et al.*, 1992). Consistent with earlier reports (Shimazaki *et al.*, 1998), I found that LfcinB bound to immobilized heparin. Moreover, LfcinB-heparin binding was reduced in the presence of bFGF or $VEGF_{165}$, suggesting that LfcinB, bFGF, and $VEGF_{165}$ may interact with and compete for the same heparin-like binding sites on cell-surface heparan sulfate proteoglycans. This data is further supported by the finding that HUVECs that were pre-treated with heparinase III to remove cell-surface heparan sulfate proteoglycans, failed to bind LfcinB. Furthermore, LfcinB and bFGF or $VEGF_{165}$, but not non-heparin-binding EGF, showed competitive binding to the surface of HUVECs. Collectively, these data support my belief that LfcinB exerts its antiangiogenic activity by interfering with heparan sulfate proteoglycan-dependent bFGF and $VEGF_{165}$ binding and signaling through their respective cell-surface receptors.

Cell-surface heparan sulfate proteoglyans have many important functions including cell attachment, migration, and proliferation by serving as co-receptors for many soluble ligands including growth factors (bFGF and $VEGF_{165}$), and insoluble ligands such as extracellular matrix molecules (Bernfield *et al.*, 1999). Although the mechanism of interaction between LfcinB and heparin-like molecules has not yet been elucidated, it is known that the net charge of LfcinB is nearly 8 (Jenssen *et al.*, 2004), while both heparin and heparan sulfate proteoglycans are negatively charged molecules (Stuckey *et al.*, 1992; Sunnergren *et al.*, 1987). This suggests that the interaction between LfcinB and heparin-like molecules may involve electrostatic interactions, which is consistent with recent evidence that $VEGF_{165}$ binds to a long stretch of anionic residues in heparan sulfate molecules (Freeman *et al.*, 2005). Interestingly, recent studies indicate that LfcinB inhibits herpes simplex virus and adenovirus infection in target cells by competing for heparan sulfate proteoglycans that function as viral attachment sites (Lindahl *et al.*, 1994; Andersen *et al.*, 2004; Di Biase *et al.*, 2003). It was determined that hydrophobicity, and spatial distribution of charged and lipophilic amino acids are important in the binding of LfcinB analogues to heparin-like proteoglycans, since peptides of similar net charge do not bind HS to the same extent as LfcinB (Jenssen *et al.*, 2004). This suggests that LfcinB is not binding non-specifically to anionic membrane molecules through electrostatic interactions.

My findings support the potential therapeutic application of LfcinB as an antiangiogenic agent in the treatment of human cancers. Further investigation is necessary to understand why LfcinB did not kill HUVECs, as it does neoplastic cells, when bound to the cell surface (Eliassen *et al.*, 2002; Yoo *et al.*, 1997b; Mader *et al.*,

2005). Perhaps LfcinB that has bound cell-surface heparan sulfate proteoglycans is not in close enough proximity to the endothelial cell surface to disrupt membrane integrity and trigger apoptosis. Interestingly, in contrast to normal vascular endothelium, anionic phospholipids such as PS are exposed on the surface of tumour endothelium (Augustin *et al.*, 1995; Ran *et al.*, 2002; Ran and Thorpe, 2002). PS is a phospholipid that normally resides almost exclusively in the inner leaflet of the plasma membrane (Williamson and Schlegel, 1994). Loss of PS asymmetry is observed in different physiological and pathological settings, including apoptosis (Kerr *et al.*, 1972), cell aging (Herrmann and Devaux, 1990), cell migration (Vogt *et al.*, 1996), and viral infection (van Geelen *et al.*, 1995). Spontaneous PS translocation to the outer leaflet of the plasma membrane has also been observed in malignant cells in the absence of exogenous cell injury or activators (Utsugi *et al.*, 1991). This observation suggests the exciting possibility that, in addition to blocking angiogenesis induced by heparin-binding growth factors, positively charged LfcinB may be able to bind anionic phospholipids on the plasma membrane of tumour-associated vascular endothelial cells in close enough proximity and sufficient quantity to cause membrane destabilization and apoptosis, as occurs when neoplastic cells are exposed to LfcinB (Eliassen *et al.*, 2002; Yoo *et al.*, 1997b; Mader *et al.*, 2005). Future studies will determine whether this is in fact the case.

Overall Summary

LfcinB selectively induces apoptosis in a range of human leukemia and carcinoma cell lines via a reactive oxygen species-, caspase-2-dependent loss of mitochondrial transmembrane potential and the sequential activation of caspase-9, and caspase-3.

LfcinB interacts with the outer membrane leaflet of Jurkat T leukemia cells in a receptor-independent manner and may enter these cells through the formation of membrane pores. Once inside the tumour cell, LfcinB may interact with the negatively charged mitochondrial membranes since I have determined that LfcinB interacts with, and enters isolated mitochondria. The selective cytotoxic activity of LfcinB for cancer cells is likely associated with differences in membrane composition between neoplastic and normal human cells, which suggests that LfcinB may have utility in the treatment of certain human cancers without the nonspecific toxicity associated with conventional chemotherapeutic agents. Additionally, the ability of LfcinB to inhibit heparin-binding growth factor-induced angiogenesis by blocking growth factor interaction with heparin-like molecules supports a dual anticancer role for this peptide. The ability of LfcinB to block tumour growth directly by killing tumour cells, and indirectly by blocking tumour-associated angiogenesis, would make LfcinB a very strong candidate peptide for cancer therapy.

References

Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE. (2002) Endostatin's antiangiogenic signaling network. Mol. Cell 13: 649-663.

Aguilera O, Ostolaza H, Quiros LM, Fierro JF. (1999) Permeabilizing action of an antimicrobial lactoferricin-derived peptide on bacterial and artificial membranes. FEBS Lett., 462: 273-277.

Ahne W, Thomsen I, Winton J. (1987) Isolation of a reovirus from the snake, Python regius. Arch. Virol., 94: 135–139.

Akhtar N, Dickerson EB, Auerbach R. (2002) The sponge/Matrigel angiogenesis assay. Angiogenesis 5: 75-80.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. (1996) Human ICE/CED-3 protease nomenclature. Cell 87: 171.

Andersen, J.H., Osbakk, S.A., Vorland, L.H., Traavik, T., Gutteberg, T.J. (2001) Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. Antiviral Res., 51: 141-149.

Andersen JH, Jenssen H, Sandvik K, Gutteberg TJ. (2004) Anti-HSV activity of lactoferrin and lactoferricin is dependent on the presence of heparan sulphate at the cell surface. J. Med. Virol., 74: 262-271.

Anderson BF, Baker HM, Norris GE, Rice DW, Baker EN. (1989) Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 A resolution. J. Mol. Biol., 209: 711-734.

Antonsson B, Motessuit S, Lauper S, Eskes R, Martinou JC. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem. J., 345: 271-278.

Arnold RR, Cole MF, Mcghee JR. (1997) A bactericidal effect for human lactoferrin. Science 197: 263-265.

Ashikari-Hada S, Habuchi H, Kariya Y, Kimata K. (2005) Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. J. Biol. Chem., 280: 31508-31515.

Ashkenazi A. (2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat. Rev. Cancer 2: 420-430.

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W. (1995) Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am. J. Pathol., 147: 339-351.

Baker EN, Anderson BF, Baker HM, Day CL, Haridas M, Norris GE, Rumball SV, Smith CA, Thomas DH. (1994) Three-dimensional structure of lactoferrin in various functional states. Adv. Exp. Med. Biol., 357: 1-12.

Baker EN, Baker HM, Kidd RD. (2002) Lactoferrin and transferrin: functional variations on a common structural framework. Biochem. Cell Biol., 80: 27-34.

Bar-Eli M. (1999) Role of interleukin-8 in tumor growth and metastasis of human melanoma. Pathobiol., 67: 12-18.

Barry MA, Reynolds JE, Eastman A. (1993) Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. Cancer Res., 53: 2349-2357.

Bellamy W, Takase M, Wakabayashi H, Kawase K, Tomita M. (1992) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J. Appl. Bacteriol., 73: 472-479.

Bennelli R, Morini M, Carrozzino F, Ferrari N, Minghelli S, Santi L, Cassatella M, Noonan DM, Albini A. (2002) Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J., 16: 267-269.

Bernfield M, Sanderson RD. (1990) Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. Philos. Trans. R. Soc. Lond. B. Biol. Sci., 327: 171-186.

Bezault J, Bhimani R, WiprovnickJ, Furmanski P. (1994) Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res., 54: 2310-2312.

Birck A, Kirkin AF, Zeuthen J, Hou-Jensen K. (1999) Expression of basic fibroblast growth factor and vascular endothelial growth factor in primary and metastatic melanoma from the same patients. Melanoma Res., 9: 375-381.

Blanc C, Deveraux QL, Krajewski S, Janicke RU, Porter AG, Reed JC, Jaggi R, Marti A. (2000) Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res., 60: 4386-4390.

Blanchard H, Kodandapani L, Mittl PR, Di Marco S, Krebs JF, Wu JC, Tomaselli KJ, Grutter M. (1999) The three-dimensional structure of caspase-8: an initiator enzyme in apoptosis. Structure 7: 1125-1133.

Bleicher RJ, Cabot MC. (2002) Glucosylceramide synthase and apoptosis. Biochim. Biophys. Acta., 1585: 172-178.

Blok J, Scheven BA, Mulder-Stxpel AA, Ginsel LA, Daems WT. (1982) Endocytosis in absorptive cells of cultured human small-intestinal tissue: effect of cytochalasin B and D. Cell Tissue Res., 222: 113-126.

Bos R, van Diest PJ, de Jong JS, van der Groep P, van der Valk P, van der Wall E. (2005) Hypoxia-inducible factor-1alpha is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. Histopathology 46: 31-36.

Bratton SB, Lewis J, Butterworth M, Duckett CS, Cohen GM. (2002) XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis. Cell Death Differ., 9: 881-92.

Breier G., Risau W. (1996) The role of vascular endothelial growth factor in blood vessel formation. Trends Cell. Biol., 6: 454-456.

Brenner C, Cadiou H, Viera HL, Zamzami N, Marzo I, Xie Z, Leber B, Andrews D, Duclohier H, Reed JC, Kroemer G. (2000) Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene 19: 329-336.

Britigan BE, Lewis TS, Waldschmidt M, McCormick ML, Krieg AM. (2001) Lactoferrin binds CpG-containing oligonucleotides and inhibits their immunostimulatory effects on human B cells. J. Immunol., 167: 2921-2928.

Brock J. (1995) Lactoferrin: a multifunctional immunoregulatory protein? Immunol. Today 16: 417-419.

Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B. (1999) Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J. Clin. Invest., 104: 263-269.

Buschmann T, Potapova O, Bar-Shira A, Ivanov VN, Fuchs SY, Henderson S, Fried VA, Minamoto T, Alarcon-Vargas D, Pincus MR, Gaarde WA, Holbrook NJ, Shiloh Y, Ronai Z. (2001) Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol. Cell Biol., 8: 2743-2754.

Cain K, Bratton SB, Langlais C, Walker G, Brown DG, Sun XM, Cohen GM. (2000) Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. J. Biol. Chem., 275: 6067-6070.

Cande C, Cecconi F, Dessen P, Kroemer G. (2002) Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J. Cell Sci., 115: 4727-4734.

Carmeliet P. (1999) Controlling the cellular brakes. Nature 401: 657-658.

Carmeliet, P., Jain R.K. (2000) Angiogenesis in cancer and other diseases. Nature 407: 249-257.

Chan SC, Yau WL, Wang W, Smith DK, Sheu FS, Chen HM. (1998) Microscopic observations of the different morphological changes caused by anti-bacterial peptides on Klebsiella pneumoniae and HL-60 leukemia cells. J. Pep. Sci., 4: 413-425.

Chapple DS, Hussain R, Joannou CL, Hancock REW, Odell E, Evans RW, Siligardi G. (2004) Structure and association of human lactoferrin peptides with Escherichia coli lipopolysaccharide. Antimicrob. Agents Chemother., 48: 2190-2198.

Chavakis T, Cines DB, Rhee JS, Liang OD, Schubert U, Hammes HP, Higazi AA, Nawroth PP, Preissner KT, Bdeir K. (2004) Regulation of neovascularization by human neutrophil peptides (alpha-defensins): a link between inflammation and angiogenesis. FASEB J., 18: 1306-1308.

Chen HM, Wang W, Smith D, Chan SC. (1997) Effects of the anti-bacterial peptide cecropin B and its analogs, cecropins B-1 and B-2, on liposomes, bacteria, and cancer cells. Biochem. Biophys. Acta., 1336: 171-179.

Chen YH, Wu HL, Chen CK, Huang YH, Yang BC, Wu LW. (2003) Angiostatin antagonizes the action of VEGF-A in human endothelial cells via two distinct pathways. Biochem. Biophys. Res. Commun., 310: 804-10.

Chen J, Xu X-M, Underhill CB, Yang S, Wang L, Chen Y, Hong S, Creswell K, Zhang L. (2005) Tachyplesin activates the classic complement pathway to kill tumor cells. Cancer Res., 65: 4614-4622.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell., 8: 705-11.

Cho E, Smith-Warner SA, Spiegelman D, Beeson WL, van den Brandt PA, Colditz GA, Folsom AR, Fraser GE, Freudenheim JL, Giovannucci E, Goldbohm RA, et al. (2004) Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. J. Natl. Cancer Inst., 96: 1015-1022.

Christensen B, Juergen F, Merrifield RB, Mauzerall D. (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc. Natl. Acad. Sci., 85: 5072-5076.

Clapham DE. (1995) Calcium signaling. Cell 80: 259-268.

Colville-Nash PR, Scott DL. (1992) Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. Ann. Rheum. Dis., 51: 919-925.

Conner J, Bucana C, Fidler IJ, Schroit AJ. (1989) Differentiation dependent expression of phosphatidyl serine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombin complex formation. Proc. Natl. Acad. Sci. USA 86: 3184-3188.

Corcoran JA, Duncan R. (2004) Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion. J. Virol., 78: 4342–4351.

Costantini P, Belzacq AS, Vieira HL, Larochette N, de Pablo MA, Zamzami N, Susin SA, Brenner C, Kroemer G. (2000) Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene 19: 307-314.

Cox TM, Mazurier J, Spik G, Montreuil J, Peters TJ. (1979) Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine. Biochem. Biophys. Acta., 588: 120-128.

Crompton M. (1999) The mitochondrial permeability transition pore and its role in cell death. Biochem. J., 341: 233-249.

Cronauer MV, Hittmair A, Eder IE, Hobisch A, Culig Z, Ramoner R, Zhang J, Bartsch G, Reissigl A, Radmayr C, Thurnher M, Klocker H. (1997) Basic fibroblast growth factor levels in cancer cells and in sera of patients suffering from proliferative disorders of the prostate. Prostate 31: 223-233.

Curtis ASG. (1973) Cell adhesion. Prog. Biophys. Mol. Biol., 27: 315-386.

Dahmer MK. (2005) Caspases-2, -3, and -7 are involved in thapsigargin-induced apoptosis of SH-SY5Y neuroblastoma cells. J. Neurosci. Res., 80: 576 – 583.

Daugas E, Susin SA, Zamzami N, Ferri K, Irinopoulos T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G. (2000) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J., 14: 729-739.

Dawe S, Boutilier J, Duncan R. (2002) Identification and characterization of a baboon reovirus-specific nonstructural protein encoded by the bicistronic s4 genome segment. Virology 304: 44-52.

Dechecchi MC, Melotti P, Bonizzato A, Santacatterina M, Chilosi M, Cabrini G. (2001) Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenoviraus type 2 and 5. J. Virol., 75: 8772-8780.

de Figueiredo RC, Soares MJ. (2000) Low temperature blocks fluid-phase pinocytosis and receptor-mediated endocytosis in Trypanosoma cruziepimastigotes Parasitol. Res., 86: 413-418.

Dekroon A, Dolis D, Mayer A, Lill R, De Kruijff B. (1997) Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and neurospora crassa. Is cardiolipin present in the mitochondrial outer membrane? Biochem. Biophys. Acta., 1325: 108-116.

De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R. (1997) Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science 277: 1652-1655.

Deng Y, Ren X, Yang L, Lin Y, Wu X. (2003) A JNK-dependent pathway is required for TNFα-induced apoptosis. Cell 115: 61-70.

Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol., 144: 891-901.

Deshmukh M, Kuida K, Johnson EM Jr. (2000) Caspase inhibition extends the commitment to neuronal death beyond cytochrome c release to the point of mitochondrial depolarization. J. Cell Biol., 150: 131-143.

Di Biase AMD, Pietrantoni A, Tinari A, Siciliano R, Valenti P, Antonini G, Seganti L, Superti F. (2003) Heparin-interacting sites of bovine lactoferrin are involved in anti-adenovirus activity. J. Med. Virol., 69: 495-502.

Dong C, Davis RJ, Flavell RA. (2002) MAP kinases in the immune response. Annu. Rev. Immunol., 20: 55-72.

Dudkina NV, Eubel H, Keegstra W, Boekema EJ, Braun HP. (2005) Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. Proc. Natl. Acad. Sci. USA 102: 3225-3229.

Earnshaw WC, Martins LM, Kaufmann SH. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem., 68: 383-424.

Ehrenstein G, Lecar H. (1977) Electrically gated ionic channels in lipid bilayers. Q. Rev. Biophys., 10: 1-34.

Elass-Rochard E, Roseanu A, Legrand D, Trif M, Salmon V, Motas C, Montreuil J, Spik G. (1995) Lactoferrin-lipopolysacchasride interaction: Involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. Biochem. J., 312: 839-845.

Eliassen LT, Berge G, Sveinbjørnsson B, Svendsen JS, Vorland LH, Rekdal Ø. (2002) Evidence for a direct antitumor mechanism of action of bovine lactoferricin. Anticancer Res., 22: 2703-2710.

Eliceiri BP, and Cheresh DA. (1999) The role of α_v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J. Clin. Invest., 103: 1227-1230.

Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Rio GD, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R. (1999) Anti-cancer activity of targeted pro-apoptotic peptides. Nat. Med., 5: 1032-1038.

England K, Cotter TG. (2005) Direct oxidative modifications of signalling proteins in mammalian cells and their effects on apoptosis. Redox Rep., 10: 237-45.

Enoksson M, Robertson JD, Gogvadze V, Bu P, Kropotov A, Zhivotovsky B, Orrenius S. (2005) Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. J. Biol. Chem., 279: 49575-49578.

Farkas-Himsley H, Hill R, Rosen B, Arab S, Lingwood CA. (1995) The bacterial colicin active against tumor cells in vitro and in vivo is verotoxin 1. Proc. Natl. Acad. Sci. USA 92: 6996-7000.

Farnaud S, Spiller C, Moriarty LC, Patel A, Gant V, Odell EW, Evans RW. (2004) Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. FEMS Microbiol. Lett., 233: 193-199.

Faulkner G, Garduno RA. (2002) Ultrastructural analysis of differentiation in Legionella pneumophila. J. Bacteriol., 184: 7025-7041.

Fesik SW. (2000) Insights into programmed cell death through structural biology. Cell 103: 273-282.

Fayette J, Soria JC, Armand JP. (2005) Use of angiogenesis inhibitors in tumour treatment. Eur. J. Cancer 41: 1109-1116.

Ferrara N. (1993) Vascular endothelial cell growth factor. Trends Cardiovasc. Med., 3: 244-250.

Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat. Med., 4: 336-340.

Ferri KF, Kroemer G. (2001) Organelle-specific initiation of cell death pathways. Nature Cell Biology 3: E255 - E263.

Fesik SW, Shi Y. (2001) Structural biology. Controlling the caspases. Science 294: 1477-1478.

Fleisher TA. (1997) Apoptosis. Ann. Allergy Asthma Immunol., 78: 245-249.

Folkman J. (1971) Tumour angiogenesis: therapeutic implications. New Engl. J. Med., 285: 1182-1186.

Folkman J, Klagsbrun M, Sasse J, Wadzinski M, IngberD, Vlodavsky I. (1988) A heparin-binding angiogenic protein–basic fibroblast groth factor–is stored within basement membrane. Am. J. Pathol., 130: 393-400.

Folkman, J. (1992) The role of angiogenesis in tumor growth. Semin. Cancer Biol., 3: 65-71.

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid, and other disease. Nat. Med., 1: 27-31.

Folkman J. (2006) Angiogenesis. Annu. Rev. Med., 57: 1-18.

Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. (2000) Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology 141: 995-1000.

Freeman C, Liu L, Banwell MG, Brown KJ, Bezos A, Ferro V, Parish CR. (2005) Use of sulfated linked cyclitols as heparan sulfate mimetics to probe the heparin/heparan sulfate binding specificity of proteins. J. Biol. Chem., 280: 8842-8849.

Friesen C, Herr I, Krammer PH, Debatin KM. (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med., 2: 574-577.

Furlong SJ, Mader JS, and Hoskin DW. (2006) Lactoferricin-induced apoptosis in estrogen-nonresponsive MDA-MB-435 breast cancer cells is enhanced by C_6 ceramide or tamoxifen. Oncol. Rep., in press.

Gabriel B, Sureau F, Casselyn M, Teissie J, Petit PX. (2003) Retroactive pathway involving mitochondria in electroloaded cytochrome c-induced apoptosis. Protective properties of Bcl-2 and Bcl-XL. Exp. Cell Res., 28: 195-210.

Gahr M, Speer CP, Damerau B, Sawatzki G. (1991) Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes. J. Leukoc. Biol., 49: 427-433.

Gallego-Sandin S, Novalbos J, Rosado A, Cano-Abad MF, Arias E, Abad-Santos F, Garcia AG. (2005) Albumin prevents mitochondrial depolarization and apoptosis elicited by endoplasmic reticulum calcium depletion of neuroblastoma cells. Eur. J. Pharm., 520: 1-11.

Garcia-Ruiz C, Colell A, Paris R, Fernandez-Checa JC. (2000) Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. FASEB J., 14: 847-858.

Garre C, Bianchi-Scarra G, Sirito M, Musso M, Ravazzolo R. (1992) Lactoferrin binding sites and nuclear localization in K562(S) cells. J. Cell Physiol., 153: 477-82.

Gazit E, Lee WJ, Brey PT, Shai Y. (1994) Mode of action of the antibacterial cecropin B2: a spectrofluorometric study. Biochemistry 33: 10681-10692.

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, and Ferrara N. (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med., 5: 623-628.

Gershenwald JE, Fidler IJ. (2002) Cancer. Targeting lymphatic metastasis. Science 296: 1811-1812.

Gitay-Goran H, Soker S, Vlodavsky I, Neufeld G. (1992) The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J. Biol. Chem., 267: 6093-6098.

Gohji K, Fidler IJ, Tsan R, Radinsky R, von Eschenbach AC, Tsuruo T, Nakajima M. (1994) Human recombinant interferons-beta and –gamma decrease gelatinase production and invasion by human KG-2 renal-carcinoma cells. Int. J. Cancer 58: 380-384.

Gospodarowicz D, Abraham JA, Schilling J. (1989) Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc. Natl. Acad. Sci. USA 86: 7311-7315.

Gottesman MM, Fojo T, Bates SE. (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2: 48-58.

Gottlieb E, Armour SM, Harris MH, Thompson CB. (2003) Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. Cell Death Differ., 10: 709-717.

Gray MW, Burger G, Lang BF. (1999) Mitochondrial evolution. Science 283: 1476-1481.

Green DR, Reed JC. (1998) Mitochondria and apoptosis. Science 281: 1309-1312.

Griffiths CE, Cumberbatch M, Tucker SC, Dearman RJ, Andrew S, Headon DR, Kimber I. (2001) Exogenous topical lactoferrin inhibits allergen-induced Langerhans cell migration and cutaneous inflammation in humans. Br. J. Dermatol., 144: 715-25.

Griffiths GL, Mattes MJ, Stein R, Govindan SV, Horak ID, Hansen HJ, Goldenberg DM. (2003) Cure of SCID mice bearing human B-lymphoma xenografts by an anti-CD74 antibody-anthracycline drug conjugate. Clin. Cancer Res., 9: 6567-6571.

Griscelli F, Li H, Bennaceur, Sorio J, Opolon P, Soria C, Perricaudet M, Yeh P, Lu H. (1998) Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. Proc. Natl. Acad. Sci. USA, 95: 6367-6372.

Grooten J, Gossens V, Vanhaesebroeck B, Fiers W. (1993) Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. Cytokine 5: 546-555.

Gross A, Jockel J, Wei MC, Korsmeyer SJ. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J., 17: 3878-3885.

Gross A, McDonnell JM, Korsmeyer SJ. (1999) Bcl-2 family members and the mitochondria in apoptosis. Genes Dev., 13: 1899-1911.

Gum ET, Swanson RA, Alano C, Liu J, Hong S, Weinstein PR, Panter SS. (2004) Human serum albumin and its N-terminal tetrapeptide (DAHK) block oxidant-induced neuronal death. Stroke 35: 590-595.

Gutterman JU. (1994) Cytokine therapeutics: lessons from interferon alpha. Proc. Natl. Acad. Sci. USA, 91: 1198-1205.

Haigler HT, Maxfield FR, Willingham MC, Pastan I. (1980) Dansylcadaverine inhibits internalization of 125I-epidermal growth factor in BALB 3T3 cells. J. Biol. Chem., 255: 1239-1241.

Hainfeld JF, Powell RD. (2000) New frontiers in gold labeling. J. Histochem. Cytochem., 48: 471-480.

Hajnoczky G, Davies E, Madesh M. (2003) Calcium signaling and apoptosis. Biochem. Biophys. Res. Commun., 304: 445-454.

Han ZC, Liu Y. (1999) Angiogenesis: state of the art. Int. J. Haematol., 70: 68-82.

Hancock RE, Diamond G. (2000) The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol., 8: 402-410.

Haug BE, Skar ML, Svendsen JS. (2001) Bulky aromatic amino acids increase the antibacterial activity of 15-residue bovine lactoferricin derivatives. J. Pept. Sci., 7: 425-432.

Haukland HH, Ulvatne H, Sandvik K, Vorland LH. (2001) The antimicrobial peptides lactoferricin B and magainin 2 cross over the bacterial cytoplasmic membrane and reside in the cytoplasm. FEBS Lett., 508: 389-393.

Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. (2002) Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappaB. Cell. Immunol., 220: 83-95.

He J, Furmanski P. (1995) Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature 373: 721-724.

Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES. (2002) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J. Biol. Chem., 277: 432-438.

Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR, Bleackley RC. (2000) Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J. Exp. Med., 192: 1391-1402.

Herrmann A, Devaux PF. (1990) Alteration of the aminophospholipid translocase activity during in vivo and artificial aging of human erythrocytes. Biochem. Biophys. Acta., 1027: 41-46.

Hickman JA, Potten CS, Merritt AJ, Fisher TC. (1994) Apoptosis and cancer chemotherapy. Philos. Trans. R. Soc. Lond. B. Biol. Sci., 345: 319-325.

Hidalgo M, Eckhardt SG. (2001) Development of matrix metalloproteinase inhibitors in cancer therapy. J. Natl. Cancer Inst., 93: 178-193.

Ho G, Bronze GJ Jr, Schwartz AL. (1997) Role of heparan sulfate proteoglycans in the uptake and degradation of tissue factor pathway inhibitor-coagulation factor Xa complexes. J. Biol. Chem., 272: 16838-16844.

Hoek KS, Milne JM, Grieve PA, Dionysius DA, Smith R. (1997) Antibacterial activity of bovine lactoferrin-derived peptides. Antimicrob. Agents Chemother., 41: 54-59.

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. (2002) VEGF-Trap: A VEGF blocker with potent antitumor effects. PNAS 99: 11393-11398.

Hommes DW, Peppelenbosch MP, van Deventer SJ. (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. Gut 52: 144-151.

Horwitz DA, Bakke AC, Abo W, Nishiya K. (1984) Monocyte and NK cell cytotoxic activity in human adherent cell preparations: discriminating effects of interferon and lactoferrin. J. Immunol., 132: 2370-2374.

Huang TT, Carlson EJ, Raineri I, Gillespie AM, Kozy H, Epstein CJ. (1999) The use of transgenic and mutant mice to study oxygen free radical metabolism. Ann. NY Acad. Sci. 893: 95-112.

Huang S, Mills L, Mian B, Tellez C, McCarty M, Yang XD, Gudas JM, and Bar-Eli M. (2002) Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis on human melanoma. Am. J. Pathol., 161: 125-134.

Hunt TK, Knighton DR, Thakral KK, Goodson WH, Andrews WS. (1984) Studies on inflammation and wound healing: angiogenesis and collagen synthesis stimulated in vivo by resident and activated wound macrophages. Surgery 96: 48-54.

Hwang PM, Zhou N, Arrowsmith CH, Vogel HJ. (1998) Three-dimensional solution structure of lactoferricin B, as antimicrobial peptide derived from bovine lactoferrin. Biochemistry 37: 4288-4298.

Iigo M, Kuhara T, Ushida Y, Sekine K, Moore MA, Tsuda H. (1999) Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. Clin. Exp. Metastasis 7: 35-40.

Ip Y, Davis R. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr. Opin. Cell Biol., 2: 205-219.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, BOdmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388: 190-195.

Jacob L, Zasloff M. (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. Ciba. Found. Symp., 186: 197-216.

Jain RK. (2002) Tumor angiogenesis and accessibility: role of vascular endothelial cell growth factor. Semin. Oncol., 29: 3-9.

Javerzat S, Auguste P, Bikfalvi A. (2002) The role of fibroblast growth factors in vascular development. Trends Mol. Med., 8: 483-489.

Jaworski M , Hailfinger S, Buchmann A, Hergenhahn M, Hollstein M, Ittrich C, Schwarz M. (2005) Human p53 knock-in (hupki) mice do not differ in liver tumor response from their counterparts with murine p53. Carcinogenesis 26: 1829-1834.

Jenssen H, Anderson JH, Uhlin-Hansen L, Gutteberg TJ, Rekdal Ø. (2004) Anti-HSV activity of lactoferricin analogues is only partly due to their affinity for heparan sulfate. Antiviral Res., 61: 101-109.

Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nature Med., 6: 41-48.

Johnstone RW, Ruefli AA, Lowe SW. (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108: 153-164.

Juo P, Kua CJ, Yuan J, Blenis J. (1998) Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr. Biol., 8: 1001-8.

Kang JH, Lee MK, Km KL, Hahm K. (1996) Structure-biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin. Int. J. Pept. Res., 48: 357-363.

Kanyshkova TG, Semenov DV, Buneva VN, Nevinsky GA. (1999) Human milk lactoferrin binds two DNA molecules with different affinities. FEBS Lett., 451: 235-237.

Kanyshkova TG, Buneva VN, Nevinsky GA. (2001) Lactoferrin and its biological functions. Biochemistry 66: 1-7.

Karameris A, Panagou P, Tsilalis T, Bouros D. (1997) Association of expression of metalloproteinases and their inhibitors with the metastatic potential of squamous-cell lung carcinomas. A molecular and immunohistochemical study. Am. J. Respir. Crit. Care Med., 156: 1930-1936.

Kaufmann SH, Hengartner MO. (2001) Programmed cell death: alive and well in the new millennium. Trends Cell Biol., 11: 526-534.

Kaufmann M, Bozic D, Briand C, Bodmer JL, Zerbe O, Kohl A, Tschopp J, Grutter MG. (2002) Identification of a basic surface area of the FADD death effector domain critical for apoptotic signaling. FEBS Lett., 527: 250-254.

Kawakami H, Lonnerdal B. (1991) Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. Am. J. Physiol., 261: G841-846.

Kerbel R, Folkman J. (2002) Clinical translation of angiogenesis inhibitors. Nat. Rev. Cancer 2: 727-739.

Kerr JFR, Wyllie AH, Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26: 239-257.

Kharbanda S, Saxena S, Yoshida K, Pandey P, Kaneki M, Wang Q, Cheng K, Chen YN, Campbell A, Sudha T, Yuan ZM, Narula J, Weichselbaum R, Nalin C, Kufe D. (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. J. Biol. Chem., 275: 322-327.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature 362: 841-844.

Kim YM, Hwang S, Kim YM, Pyun BJ, Kim TY, Lee ST, Gho YS, Kwon YG. (2002) Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J. Biol. Chem., 277: 27872-27879.

Kim JS, He L, Lemasters JJ. (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem. Biophys. Res. Commun., 304: 463-470.

Kimber I, Cumberbatch M, Darman RJ, Headon DR, Bhushan M, Griffiths CE. (2002) Lactoferrin: influences on Langerhans cells, epidermal cytokines, and cutaneous inflammation. Biochem. Cell Biol., 80: 103-107.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J., 14: 5579-5588.

Klagsbrun M, D'Amore P. (1991) Regulators of angiogenesis. Annu. Rev. Physiol., 53: 217-239.

Kobayashi M, Wood PA, Hrushesky WJ. (2002) Circadian chemotherapy for gynecological and genitourinary cancers. Chronobiol. Int., 19: 237-251.

Koch AE, Polverini PJ, and Leibovich SJ. (1986) Stimulation of neovascularization by human rheumatoid synovial tissue macrophages. Arth. Rheum., 29: 471-479.

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SJ, and Strieter RM. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258: 1798-1801.

Kodate M, Kasai T, Hashimoto H, Yasumoto K, Iwata Y, Manabe H. (1997) Expression of matrix metalloproteinase (gelatinase) in T1 adenocarcinoma of the lung. Pathol. Int., 47: 461-469.

Kolesnick RN, Kronke M. (1998) Regulation of ceramide production and apoptosis. Annu Rev. Physiol., 60: 643-65.

Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84: 1415-1420.

Krammer PH. (1999) CD95 (APO-1/Fas)-mediated apoptosis: live and let die. Adv. Immunol., 71: 163-210.

Krishna R, Mayer LD. (2000) Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur. J. Pharm. Sci., 11: 265-83.

Kroemer G, Reed JC. (2000) Mitochondrial control of cell death. Nat. Med., 6: 513-515.

Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. (2001) Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem., 276: 20633-20640.

Kubo H, Matsumoto K, Funahashi M, Takagi H, Kitajima Y, Taniguchi S, Saida T. (2000) Sequential chemoimmunotherapy with cisplatin, interferon-beta and interleukin-2 inhibits the growth of B16-F1 melanoma in syngeneic mice. Melanoma Res., 10: 223-229.

Kuwata H, Yip TT, Yamauchi K, Teraguchi S, Hayasawa H, Tomita M, Hutchens TW. (1998) The survival of ingested lactoferrin in the gastrointestinal tract of adult mice. Biochem. J., 334: 321-323.

Kuwata H, Yamauchi K, Teraguchi S, Ushida Y, Shimokawa Y, Toida T, Hayasawa H. (2001) Functional fragments of ingested lactoferrin are resistant to proteolytic degredation in the gastrointestinal tract of adult rats. J. Nutr., 131: 2121-2127.

Lassus P, Opitz-Araya X, Lazebnik Y. (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297: 1352-1354.

Legrand D, Mazurier J, Elass A, Rochard E, Vergoten G, Maes P, Montreuil J, Spik G. (1992) Molecular interactions between human lactotransferrin and the phytohemagglutinin-activated human lymphocyte lactotransferrin receptor lie in two loop-containing regions of the N-terminal domain of human lactotransferrin. Biochemistry 31: 9243-9251.

Legrand D, Vigie′ K, Said EA, Elass E, Masson M, Slomianny M-C, Carpentier M, Briand J-P, Mazurier JI, Hovanessian AG. (2004) Surface nucleolin participates in both the binding and endocytosis of lactoferrin in target cells. Eur. J. Biochem., 271: 303–317.

Letai A, Bassik M, Walensky L, Sorcinelli M, Weiler S, Korsmeyer S. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2: 183-192.

Leuschner C, Hansel W. (2004) Membrane disrupting lytic peptides for cancer treatments. Curr. Pharm. Des., 10: 2299-2310.

Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz LC. (1997) Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. J. Biol. Chem., 272: 30299-305.

Li LY, Luo X, Wang X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412: 95-99.

Lichtenstein A, Ganz T, Selsted ME, Lehrer RI. (1986) In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes. Blood 68: 1407-1410.
Lindahl U, Lidholt K, Spillmann D, Kjellen L. (1994) More to "heparin" than anticoagulation. Thromb. Res., 75: 1-32.

Liu MY, Colombini M. (1992) Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC. Biochem. Biophys. Acta., 1098: 255-260.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86: 147-157.

Liu JR, Fletcher B, Page C, Hu C, Nunez G, Baker V. (1998) Bcl-xL is expressed in ovarian carcinoma and modulates chemotherapy-induced apoptosis. Gynecol. Oncol., 70: 398-403.

Lockshin RA, Zakeri Z. (2002) Caspase-independent cell deaths. Curr. Opin. Cell Biol., 14: 727-733.

Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW. (1996) Membrane Pores Induced by Magainin. Biochemistry 35: 13723-13728.

Mader JS, Salsman J, Conrad DM, Hoskin DW. (2005) Bovine lactoferricin induces apoptosis in human leukemia and carcinoma cell lines. Mol. Cancer Ther., 4: 612-624.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277: 55-60.

Makrigiannis AP, Blay J, Hoskin DW. (1994) Cyclosporin A inhibits 2-chloroadenosine-induced DNA cleavage in mouse thymocytes. Int. J. Immunopharmacol., 16: 995-1001.

Mantel C, Miyazawa K, Broxmeyer HE. (1994) Physical characteristics and polymerization during iron saturation of lactoferrin, a myelopoietic regulatory molecule with suppressor activity. Adv. Exp. Med. Biol., 357: 121-132.

Marchetti M, Pisani S, Antonini G, Valenti P, Seganti L, Orsi N. (1998) Metal complexes of bovine lactoferrin inhibit in vitro replication of herpes simplex virus type 1 and 2. Biometals 11: 89-94.

Marler JJ, Rubin JB, Trede NS, Conners S, Grier H, Upton J, Mulliken JB, Folkman J. (2002) Successful antiangiogenic therapy of giant cell angioblastoma with interferon alfa 2b: report of 2 cases. Pediatrics 109: E37.

Marshall SH, Arenas G. (2003) Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. J. Biotech., 6: 271-282.

Martin E, Gantz T, Lehrer R. (1995) Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukoc. Biol., 58: 128-136.

Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science 281: 2027-2031.

Masson PL, Heremans JF, Schonne E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leukocytes. J. Exp. Med., 130: 643-658.

Mathias S, Pena LA, Kolesnick RN. (1998) Signal transduction of stress via ceramide. Biochem. J., 335: 465-480.

Matsuzaki K, Murase O, Fujii N, Miyajima K. (1995) Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. Biochemistry 34: 6521-6526.

Matsuzaki K. (1997) Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. Biochem. Biophys. Acta., 1327: 119-130.

Matsuzaki K. (1999) Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochem. Biophys. Acta., 1462: 1-10.

Mattsby-Baltzer I, Roseanu A, Motas C, Elverfors J, Engberg I, Hanson LÅ. (1996) Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. Pediatr. Res., 40: 257-262.

Matzinger, P. (1991) The JAM test: A simple assay for DNA fragmentation and cell death. J. Immunol. Met., 145: 185-195.

Matzinger P. (1999) The JAM test. A simple assay for DNA fragmentation and cell death. J. Immunol. Methods 145: 185-192.

Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc. Natl. Acad. Sci. U S A 94: 8104-8109.

McEligot AJ, Yang S, Meyskens FL Jr. (2005) Redox regulation by intrinsic species and extrinsic nutrients in normal and cancer cells. Annu. Rev. Nutr., 25: 261-295.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J., 16: 2794-2804.

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez, R, Moller NP, Risau W, Ullrich A. (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72: 835-846.

Mingatto FE, Rodrigues T, Pigosso AA, Uyemura SA, Curti C, Santos AC. (2002) The critical role of mitochondrial energetic impairment in the toxicity of nimesulide to hepatocytes. J. Pharmacol. Exp. Ther., 303: 601-607.

Miyauchi H, Hashimoto S, Nakajima M, Shinoda I, Fukuwatari Y, Hayasawa H. (1998) Bovine lactoferrin stimulates the phagocytic activity of human neutrophils: identification of its active domain. Cell. Immunol., 187: 34-37.

Miyazawa K, Mantel C, Lu L, Morrison DC, Broxmeyer HE. (1991) Lactoferrin-lipopolysaccharide interactions. Effect on lactoferrin binding to monocyte/macrophage-differentiated HL-60 cells. J. Immunol., 146: 723-729.

Monaco V, Formaggio F, Crisma M, Toniolo C, Hanson P, Millhauser GL. (1999) Orientation and immersion depth of a helical lipopeptaibol in membranes using TOAC as an ESR probe. Biopolymers 50: 239-253.

Moniaux N, Andrianifahanana M, Brand RE, Batra SK. (2004) Multiple roles of mucins in pancreatic cancer, a lethal and challenging malignancy. Br. J. Cancer 91: 1633-1638.

Moore AJ, Devine DA, Bibby MC. (1994) Preliminary experimental anticancer activity of cecropins. Peptide Res., 7: 265-269.

Moran EC, Kamiguti AS, Cawley JC, Pettitt AR. (2002) Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphphocytic leukemia. Br. J. Haematol., 116: 316-328.

Morgan M, Williams BA, Blay J, Hoskin DW. (2002) Chemosensitization of T-47D breast carcinoma cells to TRAIL and Fas receptor-induced killing. Anticancer Res., 22(2A): 673-676.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55-63.

Mukherjee S, Ghosh RN, Maxfield FR. (1997) Endocytosis. Physiol. Rev., 77: 759-803.

Naumov GN, Townson L, MacDonald IC, Wilson SM, Bramwell VH, Groom AC, Chambers AF. (2003) Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases.Breast Cancer Res Treat., 82: 199-206.

Nichols BL, McKee KS, Huebers HA. (1990) Iron is not required in the lactoferrin stimulation of thymidine incorporation into the DNA of rat crypt enterocytes. Pediatr. Res., 27: 525-528.

Nieminen AL, Dawson TL, Gores GJ, Kawanishi T, Herman B, Lemasters JJ. (1990) Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibitors, ionophores and oxidant chemicals. Biochem. Biophys. Res. Commun., 167: 600-606.

Niu Q, Perruzzi C, Voskas D, Lawler J, Dumont DJ, Benjamin LE. (2004) Inhibition of Tie-2 Signaling Induces Endothelial Cell Apoptosis, Decreases Akt Signaling, and Induces Endothelial Cell Expression of the Endogenous Anti-Angiogenic Molecule, Thrombospondin-1. Cancer Biol. Ther., 3: 402-405.

Nohl H, Jordan W. (1980) The metabolic fate of mitochondrial hydrogen peroxide. Eur. J. Biochem., 111: 203-210.

Norrby K, Mattsby-Baltzer I, Innocenti M, Tuneberg S. (2001) Orally administered bovine lactoferrin systemically inhibits VEGF(165)-mediated angiogenesis in the rat. Int. J. Cancer 91: 236-240.

Novgorodov SA, Szulc ZM, Luberto C, Jones JA, Bielawski J, Bielawski A, Hannun YA, Obeid LM. (2005) Positively charged ceramide is a potent inducer of mitochondrial permeabilization. J. Biol. Chem., 280: 16096-16105.

Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T. (2003) Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. Oncogene 22: 2034-2044.

O'Reilly M, Holmgren L, Shing Y, Cehn C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by Lewis lung carcinoma. Cell 79: 315-328.

O'Reilly M, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88: 277-285.

Papo N, Shai Y. (2003) Exploring peptide membrane interaction using surface plasma resonance: differentiation between pore formation versus membrane disruption by lytic peptides. Biochemistry 42: 458-466.

Parangi S, O'Reilly M, Christofori G, Holmgren L, Grosfeld J, Folkman J, Hanahan D. (1996) Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. Proc. Natl. Acad. Sci. USA 93: 2003-2007.

Park CB, Kim HS, Kim SC. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Comm., 244: 253-257.

Parrish J, Li L, Klotz K, Ledwich D, Wang X, XUe D. (2001) Mitochondrial endonuclease G is important for apoptosis in C. elegans. Nature 412: 90-94.

Pastorino JG, Tafani M, Rothman RJ, Marcineviciute A, Hoek JB, Farber JL. (1999) Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. J. Biol. Chem., 274: 31734-31739.

Pavlov EV, Priault M, Pietkiewicz D, Cheng EH, Antonsson B, Manon S, Korsmeyer SJ, Mannella CA, Kinlly KW. (2001) A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. J. Cell Biol., 155: 725-731.

Pejler P. (1996) Lactoferrin regulates the activity of heparin proteoglycan-bound mast cell chymase: characterization of the binding of heparin to lactoferrin. Biochem. J., 320: 897-903.

Perdigon G, de Moreno de LeBlanc A, Valdez J, Rachid M. (2002) Role of yoghurt in the prevention of colon cancer. Eur. J. Clin. Nutr., 56: S65-68.

Perticarari S, Presani G, Mangiarotti MA, Banfi E. (1991) Simultaneous flow cytometric method to measure phagocytosis and oxidative products by neutrophils. Cytometry 1: 687-693.

Pietenpol JA, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW, Vogelstein B. (1994) Paradoxical inhibition of solid tumor cell growth by bcl2. Cancer Res., 54: 3714–3717.

Pouny Y, Rapaport D, Mor A, Nicolas P, Shai Y. (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. Biochemistry 31: 12416-12423.

Rakha EA, Boyce RW, Abd El-Rehim D, Kurien T, Green AR, Paish EC, Robertson JF, Ellis IO. (2005) Expression of mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC and MUC6) and their prognostic significance in human breast cancer. Mod. Pathol., 18: 1295-1304.

Ran S, Downes A, Thorpe PE. (2002) Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res., 62: 6132-6140.

Ran S, Thorpe PE. (2002) Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy. Int. J. Radiat. Oncol. Biol. Phys., 54: 1479-1484.

Rapraeger AC, Krufka A, Olwin BB. (1991) Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science 252: 1705-1708.

Read SH, Baliga BC, Ekert PG Vaux DL, Kumar S. (2002) A novel Apaf-1-independent putative caspase-2 activation complex. J. Cell. Biol., 159: 739-45.

Regeimbal J, Bardwell JCA. (2002) DsbB Catalyzes Disulfide Bond Formation *de Novo*. J. Biol. Chem., 277: 32706-32713.

Ren J. (1991) Relationship between development of microvilli on tumor cells and growth or metastatic potential of tumor cells. Hokkaido Igaku Zasshi 66: 187-200.

Reynolds LP, Killilea SD, Redmer DA. (1992) Angiogenesis in the female reproductive cycle. FASEB J., 6: 886-892.

Ribatti D, Marimpietri D, Pastorino F, Brignole C, Nico B, Vacca A, Ponzoni M. (2004) Angiogenesis in neuroblastoma. Ann. NY Acad. Sci., 1028: 133-142.

Riedl SJ, Shi Y. (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat. Rev. Mol. Cell Biol., 5: 897-907.

Risso A, Braidot E, Sordano MC, Vianello A, Macri F, Skerlavaj B, Zanetti M, Gennaro R, Bernardi P. (2002) BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. Mol. Cell. Biol., 22: 1926-1935.

Roberts DL, Goping IS, Bleakley RC. (2003) Mitochondria at the heart of the cytotoxic attack. Biochem. Biophys. Res. Commun., 304: 513-518.

Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S. (2002) Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. J. Biol. Chem., 277: 29803-29809.

Robertson JD, Gogvadze V, Kropotov A, Vakifahmetoglu H, Zhivotovsky B, Orrenius S. (2004) Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. EMBO Rep., 5: 643-648.

Robinson CJ, Stringer SE. (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J. Cell Sci., 114: 853-865.

Rofstad EK, Henriksen K, Galappathi K, Mathiesen B. (2003) Antiangiogeneic treament with thrombospondin-1 enhances primary tumor radiation response and prevents growth of dormant pulmonary micrometastases after curative radiation therapy in human melanoma xenografts. Cancer Res., 63: 4055-4061.

Ross RW, Small EJ. (2002) Osteoporosis in men treated with androgen deprivation therapy for prostate cancer. J. Urol., 167: 1925-1956.

Roy MK, Kuwabara Y, Watanabe Y, Tamai Y. (2002) Peptides form the N-terminal end of bovine lactoferrin induce apoptosis in human leukemic (HL-60) cells. J. Dairy Sci., 85: 2065-2074.

Ruggeri B, Singh J, Gingrich D, Angeles T, Albom M, Yang S, Chang H, Robinson C, Hunter K, Dobrzanski P, Jones-Bolin S, Pritchard S, Aimone L, Klein-Szanto A, Herbert JM, Bono F, Schaeffer P, Casellas P, Bourie B, Pili R, Isaacs J, Ator M, Hudkins R, Vaught J, Mallamo J, Dionne C. (2003) CEP-7055: A novel, orally active pan inhibitor of vascular endothelial growth factor receptor tyrosine kinases with potent antiangiogenic activity and antitumor efficacy in preclinical models. Cancer Res., 63: 5978-5991.

Salvesen GS, Dixit VM. (1999) Caspase activation: the induced-proximity model. Proc. Natl. Acad. Sci. USA, 96: 10964-10967.

Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, Corfas G, Folkman. (2004) Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. Nat. Med., 10: 255-261.

Savarese DM, Savy G, Vahdat L, Wischmeyer PE, Corey B. (2003) Prevention of chemotherapy and radiation toxicity with glutamine. Cancer Treat. Rev., 29: 501-513.

Savino JA, Evans JF, Rabinowitz D, Auborn KJ, Carter TH. (2006) Multiple, disparate roles for calcium signaling in apoptosis of human prostate and cervical cancer cells exposed to diindolylmethane. Mol.Cancer Ther., 5: 556-563.

Schibli DJ, Hwang PM, Vogel HJ. (1999) The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. FEBS Lett., 446: 213-217.

Schibli DJ, Epand RF, Vogel HJ, Epand RM. (2002) Trytophan-rich antimicrobial peptides: comparative properties and membrane interactions. Biochem. Cell Biol., 80: 667-677.

Schimmer AD. (2004) Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res., 64: 7183-7190.

Schwartz AL. (1995) Receptor cell biology: receptor-mediated endocytosis. Pediatr. Res., 38: 835-843.

Scorrano L, Petronilli V, Di Lisa F, Bernardi P. (1999) Commitment to apoptosis by GD3 ganglioside depends on opening of the mitochondrial permeability transition pore. J. Biol. Chem., 274: 22581-22585.

Sehouli J, Stengel D, Elling D, Ortmann O, Blohmer J, Riess H, Lichtenegger W. (2002) First-line chemotherapy with weekly paclitaxel and carboplatin for advanced ovarian cancer: a phase I study. Gynecol. Oncol., 85: 321-326.

Sharp PS. (1995) The role of growth factors in the development of diabetic retinopathy. Metabolism 44: 72-75.

Shau H, Kim A, Golub SH. (1992) Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. J. Leukoc. Biol., 51: 343-349.

Shimamura M, Yamamoto Y, Ashino H, Oikawa T, Hazato T, Tsuda H, Iigo M. (2004) Bovine lactoferrin inhibits tumor-induced angiogenesis. Int. J. Cancer., 111: 111-116.

Shimazaki K, Tazume T, Uji K, Tanaka M, Kumura H, Mikawa K, Shimo-Oka T. (1998) Properties of a heparin-binding peptide derived from bovine lactoferrin. J. Dairy Sci., 81: 2841-2849.

Shimizu S, Narita M, Tsujimoto Y. (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399: 483-487.

Sid B, Sartelet H, Bellon G, El Btaouri H, Rath G, Delorme N, Haye B, Martiny L. (2004) Thrombospondin 1: a multifunctional protein implicated in the regulation of tumor growth. Crit. Rev. Oncol. Hematol., 49: 245-258.

Silvestri ME, Sundqvist V-A. (2001) An investigation into the heparin-binding properties of a synthetic peptide deduced from the antigenic domain 2 of human cytomegalovirus glycoprotein B. Scand. J. Immunol., 53: 282-289.

Silvestro L, Gupta K, Weiser JN, Axelson PH. (1997) The concentration-dependent membrane activity of cecropin A. Biochemistry 36: 11452-11460.

Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, and Fidler IJ. (1995) Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. Proc. Natl. Acad. Sci. USA, 92: 4562-4566.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J. Cell Biol., 144: 281-292.

Slee EA, Adrain C, Martin SJ. (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J. Biol. Chem., 276: 7320-7326.

Sorimachi K, Akimoto K, Hattori Y, Leiri T, Niwa A. (1997) Activation of macrophages by lactoferrin: secretion of TNF-α, IL-8 and NO. Biochem. Mol. Biol. Int., 43: 79-87.

Souza-Fagundes EM, Brumatti G, Martins-Filho OA, Correa-Oliveira R, Zani CL, Amarante-Mendes GP. (2003) Myriadenolide, a labdane diterpene isolated from Alomia myriadenia (asteraceae) induces depolarization of mitochondrial membranes and apoptosis associated with activation of caspases-8, -9, and -3 in Jurkat and THP-1 cells. Exp. Cell Res., 290: 420-426.

Strøm MB, Rekdel Ø, Svendsen JS. (2000) Antibacterial activity of 15-residue lactoferricin derivatives. J. Pept. Res., 56: 265-274.

Strøm MB, Rekdel Ø, Svendsen JS. (2002)The effects of charge and lipophilicity on the antibacterial activity of undecapeptides derived from bovine lactoferricin. J. Pept. Science 7: 36-43.

Strømhaug PE, Berg TO, Gjoen T, Seglen PO. (1997) Differences between fluid-phase endocytosis (pinocytosis) and receptor-mediated endocytosis in isolated rat hepatocytes. Eur. J. Cell. Biol., 73: 28-39.

Stuckey JA, St Charles R, Edwards BF. (1992) A model of the platelet factor 4 complex with heparin. Proteins 14: 277-287.

Subbalakshmi C, Sitaram N. (1998) Mechanism of antimicrobial action of indolicidin. FEMS Microbiol. Lett., 160: 91-96.

Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, Kalluri R. (2003) Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. Proc. Natl. Acad. Sci. USA 100: 4766-71.

Sugimachi K, Tanaka S, Taguchi K, Aishima S, Shimada M, Tsuneyoshi M (2003) Angiopoietin switching regulates angiogenesis and progression of human hepatocellular carcinoma. J. Clin. Pathol., 56: 854-860.

Sunnergren KP, Fairman RP, deBlois GG, Glauser FL. (1987) Effects of protamine, heparinase, and hyaluronidase on endothelial permeability and surface charge. J. Appl. Physiol., 63: 1987-1992.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397: 441-446.

Susuki Y, Ono Y, Hirabayashi Y. (1998) Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis. FEBS Lett., 425: 209-212.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol. Cell 8: 613-621.

Swenson S, Costa F, Minea R, Sherwin RP, Ernst W, Fujii G, Yang D, Markland FS Jr. (2004) Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression. Mol. Cancer Ther., 3: 499-511.

Takahashi A, Masuda A, Sun M, Centonze VE, Herman B. (2004) Oxidative stress-induced apoptosis is associated with alterations in mitochondrial caspase activity and Bcl-2-dependent alterations in mitochondrial pH (pHm). Brain Res. Bull., 62: 497-504.

Takeshima K, Chikushi A, Lee K-K, Yonehara S, Matsuzaki K. (2003) Translocation of analogues of the antimicrobial peptides magainin and buforin across human cell membranes. J. Biol. Chem., 278: 1310-1315.

Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW. (1997) Substrate specificities of caspase family proteases. J. Biol. Chem., 272: 9677-9682.

Tan S, Sagara Y, Liu Y, Maher P, Schubert D. (1998) The regulation of reactive oxygen species production during programmed cell death. J. Cell Biol., 141: 1423-1432.

Terry AH, Culp LA. (1974) Substrate-attached glycoproteins from normal and virustransformed cells. Biochemistry 13: 414-425.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, and McDonald DM. (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286: 2511-2514.

Tomita M, Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K. (1991) Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci., 74: 4137-4142.

Tomita M, Takase M, Bellamy W, Shimamura S. (1994) A review: the active peptide of lactoferrin. Acta. Paediatr. Jpn., 36: 585-591.

Top D, de Antueno R, Salsman J, Corcoran J, Mader J, Hoskin D, Touhami A, Jericho MH, Duncan R. (2005) Liposome reconstitution of a minimal protein-mediated membrane fusion machine. EMBO J., 24: 2980-2988.

Travis PA, Bouill EG, Hamil B, Tindle BH. (1988) Detection of factor VIII von Willebrand factor in endothelial cells in first-trimester fetuses. Arch. Pathol. Lab. Med. 112: 40-42.

Troppmair J, Bruder JT, Munoz H, Lloyd PA, Kyriakis J, Banerjee P, Avruch J, Rapp UR. (1994) Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J. Biol. Chem., 269: 7030-7035.

Tse V, Xu L, Yung YC, Santarelli JG, Juan D, Fabel K, Silverberg G, Harsh G. (2003) The temporal-spatial expression of VEGF, angiopoietins-1 and 2, and Tie-2 during tumor angiogenesis and their functional correlation with tumor neovascular architecture. Neurol. Res., 25: 729-738.

Tsuda H, Sekine K, Nakamura J, Ushida Y, Kuhara T, Takasuka N, Kim DJ, Asamoto M, Baba-Toriyama H, Moore MA, Nishino H, Kakizoe T. (1998) Inhibition of azoxymethane initiated colon tumor and aberrant crypt foci development by bovine lactoferrin administration in F344 rats. Adv. Exp. Med. Biol., 443: 273-284.

Tsujimoto Y, Shimizu S. (2000) Bcl-2 family: life-or-death switch. FEBS Lett., 466: 6-10.

Uhlmann EJ, Subramanian T, Vater CA, Lutz R, Chinnadurai G. (1998) A potent cell death activity associated with transient high level expression of BCL-2. J. Biol. Chem., 273: 17926-17932.

Ulvatne H, Haukland HH, Olsvik O, Vorland LH. (2001) Lactoferricin B causes depolarization of the cytoplasmic membrane of Escherichia coli ATCC 25922 and fusion of negatively charged liposomes. FEBS Lett., 492: 62-65.

Ulvatne H, Samuelson O, Haukland HH, Kramer M, Vorland LH. (2004) Lactoferricin B inhibits bacterial macromolecular synthesis in Escherichia coli and Bacillus subtilis. FEMS Microbiol. Lett., 237: 377-384.

Utsugi T, Schroit AJ, Conner J, Bucana CD, Fidler IJ. (1991) Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res., 51: 3062-3066.

Utsumi K, and Oda T. (1973) Ruthenium-red-induced cell agglutination and surface glycoprotein and mucopolysaccharide. J. Cell Sci., 13: 901—911.

Vailhe B, Feige JJ. (2003) Thrombospondins as anti-angiogenic therapeutic agents. Curr. Pharm. Des., 9: 583-588.

Van Berkel PH, Geerts ME, van Veen HA, Mericskay M, de Boer HA, Nuijens JH. (1997) N-terminal stretch Arg2, Arg3, Arg4 and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. Biochem. J., 328: 145-151.

Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 91: 627-637.

van der Kraan MI, van Marle J, Nazmi K, Groenink J, van't Hof W, Veerman EC, Bolscher JG, Amerongen AV. (2005) Ultrastructural effects of antimicrobial peptides from bovine lactoferrin on the membranes of *Candida albicans* and *Escherichia coli.* Peptides 26:1537-1542.

van Geelen AG, Slobbe-van Drunen ME, Muller AD, Bruggeman CA, Van Dam-Mieras MC. (1995) Membrane related effects in endothelial cells induced by human cytomegalovirus. Arch. Virol., 140: 1601-1612.

van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P. (2003) Mitochondrial intermembrane proteins in cell death. Biophys. Res. Commun., 304: 487-497.

Van Hofsten P, Faye I, Kockrum K, Lee JY, Xanthopoulos KG, Boman IA, Boman HG, Engstrom A, Andreu D, Merrifeild RB. (1985) Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from Hyalophora cecropia. Proc. Natl. Acad. Sci. USA 82: 2240-2243.

Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL. (2002) HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J. Biol. Chem., 277: 445-454.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380: 75-79.

Vlodavsky I, Fuks Z, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, Bar-Shavit R, Klagsbrun M. (1991) Extracellular matrix-resident basic fibroblast growth factor: implication for the control of angiogenesis. J. Cell. Biochem., 45: 167-176.

Vogel HJ, Schibli DJ, Jing W, Lohmeier-Vogel EM, Epand RF, Epand RF, Epand RM. (2002) Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. Biochem. Cell. Biol., 80: 49-63.

Vogt E, Ng AK, Rote NS. (1996) A model for the antiphospholipid antibody syndrome: monoclonal antiphosphatidylserine antibody induces intrauterine growth restriction in mice. Am. J. Obstet. Gynecol., 174: 700-707.

Volpert O, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP (2002) Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nature Med., 8: 349-357.

Volpert OV, Alani RM. (2003) Wiring the angiogenic switch: Ras, Myc, and Thrombospondin-1. Cancer Cell 3: 199-200.

Vorland LH, Ulvatne H, Rekdal O, Svendsen JS. (1999a) Initial binding sites of antimicrobial peptides in *Staphylococcus aureas* and *Escherichia coli*. Scand. J. Infect. Dis., 31: 467-473.

Vorland LH, Ulvatne H, Anderson J, Haukland HH, Rekdal O, Svendsen JS, Gutteberg TJ. (1999b) Antibacterial effects of lactoferricin B. Scand. J. Infect. Dis., 31: 179-184.

Vunnam S, Juvvadi P, Merrifield RB. (1997) Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. J. Pept. Res., 49: 59-66.

Wakabayashi H, Hiratani T, Uchida K, Yamaguchi H. (1996) Antifungal spectrum and fungicidal mechanism of an N-terminal peptide of bovine lactoferrin. J. Infect. Chemother., 1: 185-189.

Wakabayashi H , Kuwata H, Yamaguchi K, Teraguchi S, Tamura Y. (2004) No detectable transfer of dietary lactoferrin or its functional fragments to portal blood in healthy adult rats. Biosci. Biotechnol. Biochem., 68: 853-60

Walsh DA. (1999) Angiogenesis and arthritis. Rheumatology 38: 103-112.
Wang D, Pabst KM, Aida Y, Pabst MJ. (1995) Lipopolysaccharide-inactivating activity of neutrophils is due to lactoferrin. J. Leukoc. Biol., 57: 865-874.

Wang NS, Unkila MT, Reineks EZ, Distelhorst CW. (2001) Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. J. Biol. Chem., 276: 44117-44128.

Wang L, Wu WZ, Sun HC, Wu XF, Qin LX, Liu YK, Liu KD, Tang ZY. (2003) Mechanism of interferon alpha on inhibition of metastasis and angiogenesis of hepatocellular carcinoma after curative resection in nude mice. J. Gastrointest. Surg., 7: 587-594.

Ward PP, Uribe-Luna S, Conneely OM. (2002) Lactoferrin and host defense. Biochem. Cell. Biol., 80: 95-102.

Watt W, Koeplinger KA, Mildner AM, Heinrikson RL, Tomasselli , Watenpaugh KD. (1999) The atomic-resolution structure of human caspase-8, a key activator of apoptosis. Structure 7: 1135-1143.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292: 727-730.

Westermarck J, Kahari VM. (1999) Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J., 13: 781-792.

Williams BA, Makrigiannis AP, Blay J, Hoskin DW. (1997) Treatment of the P815 murine mastocytoma with cisplatin or etoposide up-regulates cell-surface Fas (CD95) expression and increases sensitivity to anti-Fas antibody-mediated cytotoxicity and to lysis by anti-CD3-activated killer-T cells. Int. J. Cancer 73: 416-423.

Williamson P, Schlegel RA. (1994) Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. Mol. Memb. Biol., 11: 199-216.

Whitley P, van Heijne G. (1993) The DsbA-DsbB system affects the formation of disulfide bonds in periplasmic but not in intramembraneous protein domains. FEBS Lett., 332: 49-51.

Woodle MC. (1993) Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation. Chem. Phys. Lipids 64: 249-262.

Wu HF, Monroe DM, Church FC. (1995) Characterization of the glycosaminoglycan-binding region of lactoferrin. Arch. Biochem. Biophys., 317: 85-92.

Wu HF, Church FC. (2003) Arginine 25 and Arginine 28 of lactoferrin are critical for effective heparin neutralization in blood. Arch. Biochem. Biophys., 412: 121-125.

Wyllie AH. (1980) Glucocorticoid induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555-556.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326-1331.

Yamauchi K, Tomita M, Giehl TJ, Ellison RT. (1993) Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect. Immun., 61: 719-728.

Yamada Y, Shinohara Y, Kakudo T, Chaki S, Futaki S, Kamiya H, Harashima H. (2005) Mitochondrial delivery of mastoparan with transferring liposomes equipped with a pH-sensitive fusogenic peptide for selective cancer therapy. Int. J. Pharm., 303: 1-7.

Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 288: 874-877.

Yang JC, Haworth L, Steinberg SM, Rosenberg SA, Novotny W. (2002) A randomized double blind placebo-controlled trial of bevacizumab (anti-VEGF antibody) demonstrating a prolongation in time to progression in patients with metastatic renal cancer. Proc. ASCO, 21, 4a.

Yang N, Strøm MB, Mekonnen SM, Svendsen JS, Rekdal Ø. (2004) The effects of shortening lactoferrin derived peptides against tumour cells, bacteria and normal human cells. J. Pept. Sci., 10: 37-46.

Yazidi-Belkoura I, Legrand D, Nuijens J, Slomianny M-C, van Berkel P, Spik G. (2001) The binding of lactoferrin to glycosaminoglycans on enterocyte-like HT29-18-C1 cells is mediated through basic residues located in the N-terminus. Biochim. Biophys. Acta., 1568: 197-204.

Ye J-S, Zheng X-J, Leung KW, Chen HM, Sheu F-S. (2004) Induction of transient ion channel-like pores in a cancer cell by antibiotic peptide. J. Biochem., 136: 255-259.

Yoshida M, Rybak RJ, Choi Y, Greenberg SJ, Barcos M, Kawata A, Matsuno F, Tsai H, Seon BK. (1997) Development of a severe combined immunodeficiency (SCID) mouse model consisting of highly disseminated human B-cell leukemia/lymphoma, cure of the tumors by systemic administration of immunotoxin, and development/application of a clonotype-specific polymerase chain reaction-based assay. Cancer Res., 57: 678-685.

Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. (1996) Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res., 56: 2013-2016.

Yoo Y-C, Watanabe S, Watanabe R, Hata K, Shimazaki K, Azuma I. (1997a) Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. Jpn. J. Cancer Res., 88: 184-190.

Yoo Y-C, Watanabe R, Koike Y, Mitobe M, Shimazaki K, Watanabe S, Azuma I. (1997b) Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk

protein-derived peptide: involvement of reactive oxygen species. Biochem. Biophys. Res. Commun., 237: 624-628.

Yuan F, Luenig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. (1994) Microvascular permeability and interstitial penetration of sterically stabilized (Stealth) liposomes in a human tumor xenograft. Cancer Res., 54: 3352-3356.

Yuan A, Yang PC, Yu CJ, Chen WJ, Lin FY, Kuo SH, Luh KT. (2000) Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am. J. Respir. Crit. Care Med., 162: 1957-1963.

Zadeh G, Qian B, Okhowat A, Sabha N, Kontos CD, GUha A. (2004) Targeting the Tie2/Tek receptor in astrocytomas. Am. J. Pathol., 164: 467-476.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med., 182: 367-377.

Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, Kroemer G. (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J. Exp. Med., 181: 1661-1672.

Zamzami N, Kroemer G. (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat. Rev. Cell Biol., 2: 67-71.

Zasloff M. Antimicrobial peptides of multicellular organisms. (2002) Nature 415: 389-395.

Zawydiwski R, Duncan R. (1978) Spontaneous ^{51}Cr release by isolated fat hepatocytes: an indicator of membrane damage. In Vitro 14: 707-714.

Ziere GJ, Bijsterbosch MK, van Berkel TJC. (1993) Removal of 14 N-terminal amino acids of lactoferrin enhances its affinity for parenchymal liver cells and potentiates the inhibition of beta-very low density lipoprotein binding. J. Biol. Chem., 268: 27069-27075.

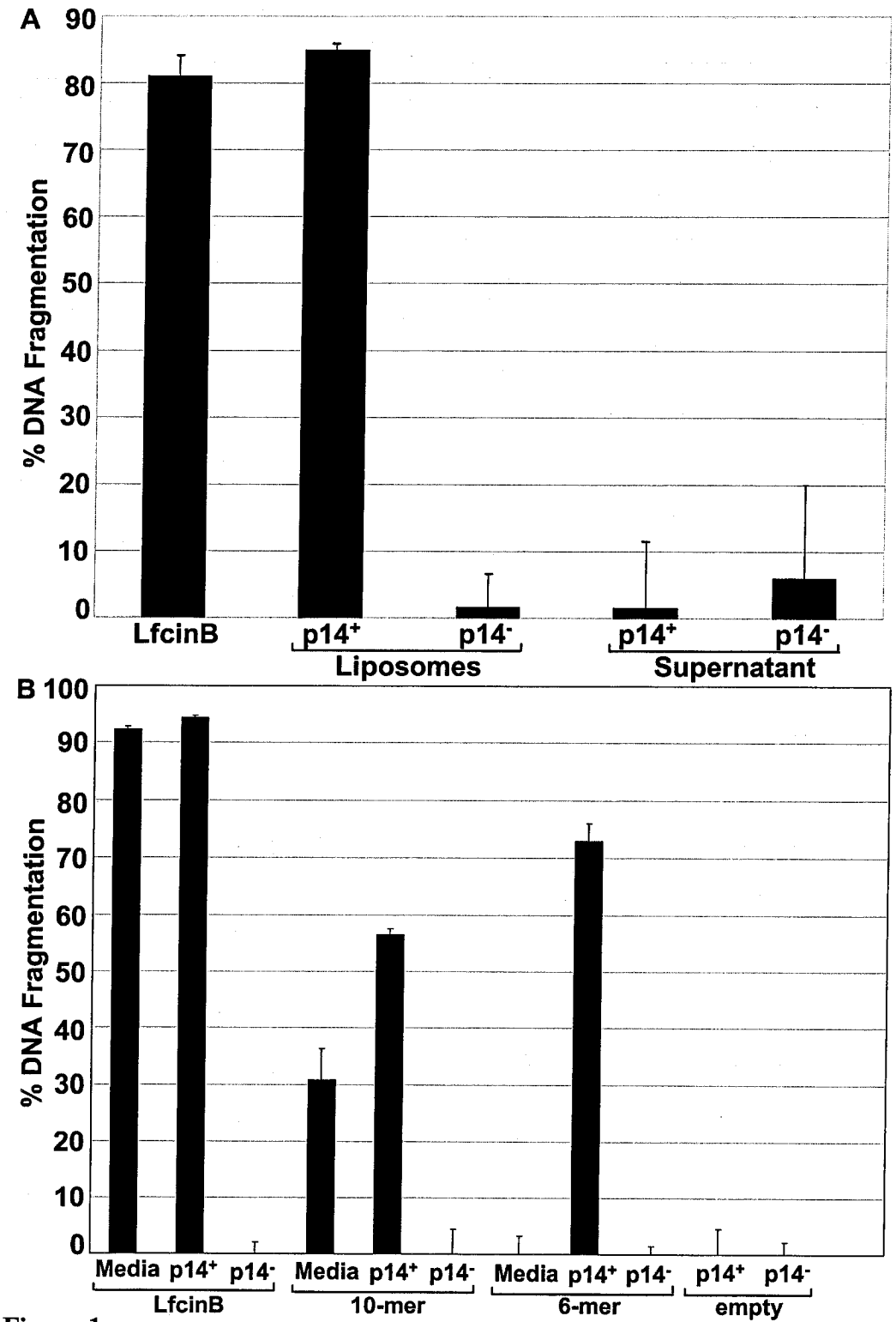
Zhivotovsky B, Samali A, Gahm A, Orrenius S. (1999) Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ., 6: 644-651.

Zoratti M, Szabo I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta., 1241: 139-176.

APPENDIX A

Additional Figures

Figure 1. *Peptide fragments of LfcinB, FKCRRWQWRM and RRWQWR, killed Jurkat T leukemia cells when encapsulated in fusogenic liposomes.* LfcinB, 10-mer, or 6-mer were entrapped in cationic p14-liposomes (60:30:8:2 molar ratio of DOPC:DOPE:cholesterol: DC-cholesterol) by ten freeze-thaw cycles using liquid nitrogen and a 37°C water bath. The resulting multilamellar liposomes were washed with HBS and resuspended in RPMI-1640 medium to give a final concentration of 33.3 μM lipid. **A.** Jurkat T leukemia cells were treated with free LfcinB (63 μM), or LfcinB contained within fusogenic ($p14^+$) liposomes (63 μM, 18 h). Control cells were treated with $p14^-$ liposome containing LfcinB (63 μM). Cells were also treated with the supernatants obtained from $p14^+$ and $p14^-$ liposomes containing LfcinB (63 μM) that were incubated with tissue culture medium for 18 h to detect leakage of peptide cargo from the liposomes. Cytotoxicity against Jurkat T leukemia cells was determined by JAM assay. **B.** Jurkat T leukemia cells were treated with free LfcinB, 10-mer, or 6-mer (all at 63 μM) or LfcinB, 10-mer, or 6-mer contained within fusogenic ($p14^+$) liposomes (63 μM, 18 h). Control cells were treated with $p14^-$ liposome containing LfcinB, 10-mer, or 6-mer (63 μM), or empty liposomes ($p14^+$, or $p14^-$). Cytotoxicity against Jurkat T leukemia cells was determined by JAM assay.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LfcinB
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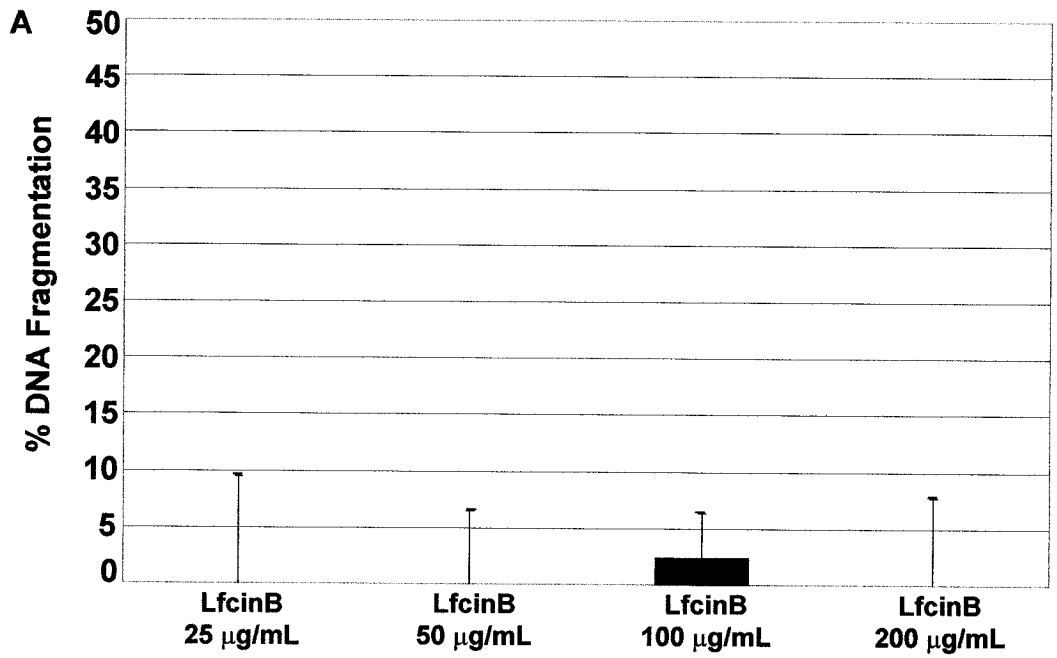
p14-
10-mer
Media
p14+
p14-
6-mer
p14+
p14-
empty

Figure 1

Figure 2. *LfcinB does not induce DNA fragmentation despite binding to PC3 prostate carcinoma cells.* **A.** PC3 prostate carcinoma cells were treated with increasing concentration of LfcinB for 18 h. Cytotoxicity was determined by JAM assay. **B.** PC3 prostate carcinoma cells were treated with biotin-LfcinB (200 μg/mL) for 1 h, and fluorescent microscopy was performed (200× magnification).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% DNA Fragmentation
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LfcinB 200 μg/mL

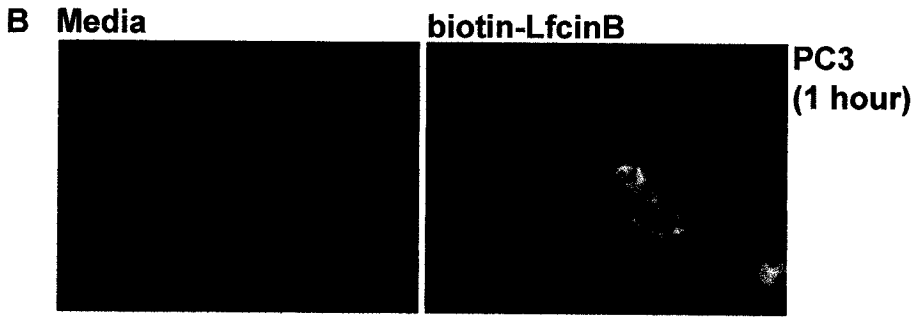
B
Media
biotin-LfcinB
PC3
(1 hour)

Figure 2

APPENDIX B

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