Anticancer activity of bovine lactoferricin and a cytolytic 9-mer peptide

– From milk to cancer vaccine?

Gerd Berge

A dissertation for the degree of Philosophiae Doctor

UNIVERSITY OF TROMSØ
Faculty of Health Sciences
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Gerd Berge, December 2009

Abbreviations

Apaf-1	Apoptosis protease activating factor-1
APC	Antigen presenting cell
CAP	Cationic antimicrobial peptide
DC	Dendritic cell
$\Delta\Psi m$	Mitochondrial membrane potential
Dip	β-diphenyl alalnine
GAG	Glycosaminoglycan
HMGB1	High mobility group box 1
HS	Heparan sulphate
HSC	Hematopoietic stem cell
IC_{50}	Inhibitory concentration 50%
i.p.	Intraperitoneal
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide
LFB	Bovine lactoferrin
LfcinB	Bovine lactoferricin
LfcinM	Murine Lactoferricin
PG	Proteoglycan
PS	Phosphatidylserine
SAR	Structure-activity relationship
s.c.	Subcutaneous
TAA	Tumor-associated antigens
TBI	Total body irradiation

List of publications

Paper I:

L.T. Eliassen, **G. Berge**, B. Sveinbjørnsson, L. Vorland, J.S. Svendsen, Ø. Rekdal.
Evidence for direct antitumor mechanism of action by bovine lactoferricin. *Anticancer Res. 22:2703-2710. 2002.*

Paper II:

L. T. Eliassen, **G. Berge**, A. Leknessund, M. Wikman, I. Lindin, C. Løkke, F. Ponthan, J. I. Johnsen, B. Sveinbjørnsson, P Kogner, T. Flægstad and Ø. Rekdal.
The antimicrobial peptide, Lactoferricin B, is cytotoxic to neuroblastoma cells *in vitro* and inhibits xenograft growth *in vivo. Int. J. of Cancer. 119. 493-500. 2006***.**

Paper III:

Berge, G., Eliassen, L. T., Sveinbjørnsson, Bartnes, K. Ø. Rekdal.
Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide. *Submitted to Cancer immunity, Immunotherapy, September 2009.*

Introduction

Cationic antimicrobial peptides

Cationic antimicrobial peptides (CAPs) are found in many diverse species playing a part in the innate immune system (*1, 2*). CAPs are important as antimicrobial agents in organisms ranging from insects to mammals, being able to kill a wide range of bacteria as well as fungi, enveloped viruses and protozoa (*3-6*). Additionally, there is increasing evidence that they have a diverse range of functions in modulating immunity, eliciting both pro- and anti-inflammatory activities by the host immune system (*7-9*).

In general, CAPs are short (< 40 amino acids), positively charged and are able to form amphipathic structures in nonpolar solvents, with clusters of hydrophobic and cationic amino acids spatially organized in discrete sectors of the molecule. There is little sequence conservation between these peptides and they are therefore categorized on the basis of their secondary structure (*10*). They are proposed to act by disrupting negatively charged microbial membrane because of electrostatic interaction, leading to membrane permabilization and disruption (*11, 12*).

Certain CAPs (e.g. bovine lactoferricin, cecropins, defensins and magainin 2) also exhibit direct cytotoxic activity against many different types of human cancer cells (*13-16*). Those CAPs possessing anticancer activity can be broadly divided into two categories based on their activity. The first group includes CAPs that are cytotoxic to microbes, cancer cells and normal mammalian cells, e.g. bee venom melittin (*17, 18*), tachyplesin isolated from horseshoe crab (*19, 20*), human neutrophil defensins (*15, 21*) and human LL-37 (*22, 23*). The second group includes CAPs that are highly potent to microbes and cancer cells, but *not* non-cancer cells exemplified by insect cecropins (*24, 25*), magainins isolated from the skin of frogs (*13, 26*), bovine lactoferricin (*14*) and others (*27*) (Table 1). However, at higher concentration most CAPs become cytotoxic against normal cells because of their fundamental amphipathic design (*6, 28*).

Table 1. Amino acid sequence of CAPs with anticancer activity

	Peptide	Amino acid sequence*	Charge	Ref.
Non-selecetive peptides	Melittin	GIGAVL**K**VLTTGLPALISWI**KRKR**QQ	+ 6	(*17*)
	Tachyplesin	**K**WCF**R**VCY**R**GICY**RR**C**R**	+ 6	(*19*)
	HNP-1 (defensin)	ACYC**R**IPACIAGE**RR**YGTCIYQG**R**LWAFCC	+ 3	(*21*)
	LL-37	LLGDFF**RKSK**E**K**IG**K**EF**KR**IVQ**R**I**K**DFL**R**NLVP**R**TES	+ 6	(*22*)
Selective peptides	Magainin 2	GIG**K**FLHSA**KK**FG**K**AFVGEIMNS	+ 3	(*29*)
	Cecropin A	**K**W**K**LF**KK**IE**K**VGQNI**R**DGII**K**AGPAVAVVGQATQIA**K**	+ 8	(*30*)
	LfcinB	F**K**C**RR**WQW**R**M**KK**LGAPSITCV**RR**AF	+ 8	(*31*)
	BMAP-28	GGL**R**SLG**R**KIL**R**AW**KK**YGPIIVPII**R**I	+ 7	(*32*)

*Amino acid sequences are given in one-letter code.
Bold text denotes amino acids that are positively charged at neutral pH.

Interaction of CAPs with cancer cells

Similar to bacteria, many cancer cells carry an increased net negative charge due to an elevated expression of anionic molecules, such as phosphatidylserine in the outermembrane leaflet (*33-35*) and terminal sialic acids on cell surface N-linked glycans or O-linked glycans (*36, 37*), compared to non-malignant cells. This negative charge enables the peptides to interact with and disrupt the cancer cells by electrostatic interaction. Other differences between the cellular membranes of the target cells that may have an influence on the interaction of CAPs with cancer cells is membrane fluidity which is typically increased in cancer cells relative to their healthy counterparts (*38, 39*). This may facilitate cancer cell membrane destabilisation by membrane-bound CAPs. Furthermore, the higher negative potential within cancer cells may also contribute to the selective membranolytic activity of CAPs (*40*). Another plausible explanation for the selective effect of the peptides is that cancer cells tend to have more abundant microvilli on their cell surface in comparison to non-transformed cells (*41*). This consequently increases the surface area of the tumorigenic cell membranes and may facilitate CAP-mediated cytotoxicity by allowing a greater number of CAP molecules to interact with the surface of the cancer cell (*42*). Taken together, these properties of the cancer cells may render them more susceptible to CAPs compared to non-malignant cells.

Mechanisms of cell killing by CAPs

The interaction between CAPs and cell membranes may result in cell death due to a membranolytic mode of action. The existence of such mechanism was first proved in a study of magainin and its synthetic analogues, in which irreversibly lysis of hematopoietic and solid tumor cells at concentrations having little effect on normal cells was shown (*40*). Further support for the membranolytic effect comes from the finding that CAPs act via a non-receptor-mediated pathway against the target cell membranes proved by using D-amino acid peptide analogous of different CAPs, displaying similar activities to the all L-amino acid parental peptides (*43, 44*). Several models have been proposed to describe the cytotoxic peptide-membrane interactions including the barrel-stave model, the carpet model and the toroidal model (Figure 1) (*10, 45*). In the barrel-stave model, monomers of α-helical CAPs (such as cecropin and melittin) bind to the cell membrane and aggregate into α-helical bundles to form transmembrane pores. In the carpet model the peptides attach perpendicularly to the membrane, cover the membrane in a carpet-like manner and interact with the lipid headgroups during membrane permeation without insertion into the hydrophobic core of the membrane. At some critical peptide concentration this results in membrane destabilisation and disintegration due to curvature stress and internal osmotic pressure. The toroidal model expands the barrel-stave and carpet model concepts by incorporating the lysis and the pore formation steps. In this model peptides are bound on the cell membrane like in the carpet model, followed by a thinning of the membrane. Aggregation of peptides to sufficient local concentrations increases the curvature strain on the membrane to an extent that transient toroidal-pores form. This is dependent on the peptide-lipid ratio.

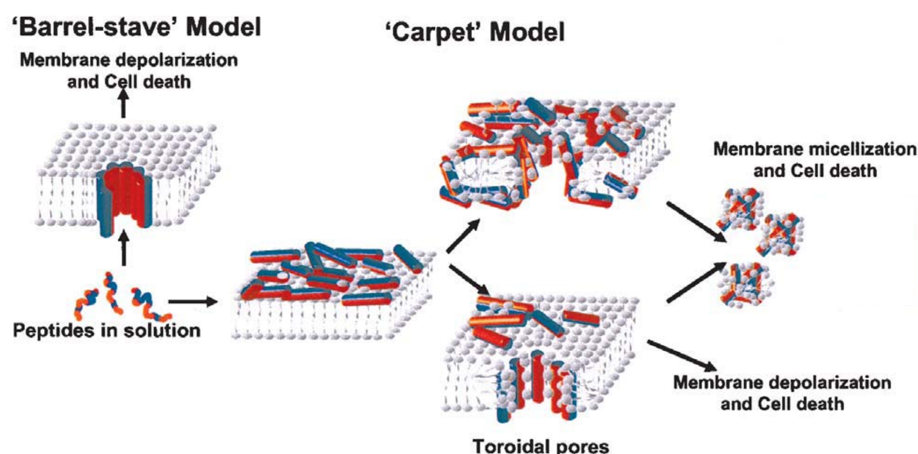

Figure 1. Proposed models for the interaction of membrane disrupting CAPs (modified from Papo (*46*)). Non-selective peptides interact with membranes by formation of transmembrane pores in the 'barrel-stave' model. More selective anticancer peptides bind in the first step mainly by electrostatic interactions and attach perpendicular to the outer membrane and cover it in 'carpet-like' manner. The destruction of the membrane occurs only after a critical concentration of peptides. Toroidal pores are an intermediate step for the carpet-model in which transient pores are formed. This has been described for peptides that were long enough to span the membrane (*47, 48*).

The different models of membrane interactions between CAPs and cancer cells explain how the cells are killed by membrane destabilisation. Nevertheless, it is likely that some CAPs gain access to the cytosolic compartment of cancer cells via cell membrane destabilisation or toroidal pore formation. The mitochondria of eukaryotic cells are negatively charged and have a highly negative transmembrane potential. Once inside the cell, these CAPs may induce permeation and swelling of mitochondria, resulting in release of cytochrome *c*, which leads to apoptosis (*49*). Some studies have supported this mechanism in which CAPs kill cancer cells by apoptosis rather than necrosis. Ellerby *et al*. (*50*) reported on a synthetic cationic membrane-active peptide conjugated to a homing domain that exhibited antitumor activity by targeting mitochondria and triggering apoptosis. BMAP-28 has also shown to trigger apoptosis in cancer cells via depolarisation of mitochondrial membranes (*51*).

***In vivo* anticancer effects of CAPs**

A number of *in vitro* studies have documented that some CAPs are more active against cancer cells compared to normal cells, they are active against multidrug-resistant tumor cells due to their lytic effect on the plasma membrane (*52-54*), and some CAPs even show synergy with classical chemotherapy (*24, 55, 56*). These promising results have motivated further studies *in vivo* to find out whether CAPs could be used as antitumor agents. Mice bearing tumors administered intraperitoneally were injected i.p. with magainin 2 (both L- and D-analogues) (*13*). Both peptides were active although the *in vivo* and *in vitro* results did not correlate. The D-analogue was ~10-fold more active *in vitro* whereas it was only ~2-fold more active *in vivo* against murine tumors. This study illustrates the major obstacle of *in vivo* treatment; to keep similar activity in animals as is found in cell cultures. Treatment of tumors with CAPs *in vivo* is challenging since the peptides can be enzymatically degraded or inactivated by negatively charged serum components, and additionally since CAPs act by a non-receptor pathway and therefore can be toxic to normal cells at certain concentrations.

Different strategies have been used to overcome these problems. Avoiding toxicity against normal cells led several researchers to target the peptides to specific sites using homing domains. A cationic antimicrobial peptide $(KLAKLAK)_2$ conjugated with a CNGRC homing domain exhibited antitumor activity (*50*). Here the homing domain selectively targeted tumor blood vessels (*57*), and after receptor-mediated internalisation of the conjugate, the cationic peptide targeted mitochondria and triggered apoptosis. Another example is that RGD-tachyplesin inhibited s.c. prostate cancer by intraperitonal injection of the peptide (*58*). RGD corresponds to a homing domain that allows it to bind integrins on both tumor cells and endothelial cells and thereby facilitates internalization of the peptide (*57*). Other examples are the linking of peptides to ligand hormones in order to target cancers like prostate, breast, ovarian and testicular cancer which upregulate gonadotropin receptors (*59, 60*). However, these strategies do not protect the peptides against proteolysis or serum inactivation.

Serum inactivation is also an important factor for reduction of activity of CAPs when used *in vivo*. For example, the antitumor activity of human defensins is completely abolished by low levels of serum (*15*). In order to overcome both serum inactivation and toxicity against normal cells, diastereomers were designed (*61*). These are synthetic antimicrobial peptides composed of both D and L amino acids, and they were inhibiting melanoma and lung metastasis in mice (*62*). Intratumor or systemic inoculation of a 15-mer diastereomer caused growth inhibition of prostate carcinoma xenografts, but the peptide had significant systemic toxicity at concentrations slightly higher than the therapeutic one. The diastereomeric peptide were

therefore further synthesized into a pH-dependent lytic peptide by substitution of lysins for histidines, thereby making the peptides positively charged and active only at acidic pH values (*63*). Solid tumors create an acidic microenvironment (*64, 65*) in which these peptides will be activated. Now diastereomeric peptide possesses four functions: targeting due to negatively charged cancer cells, activation at low pH and thereby less toxicity, cytotoxic activity against cancer, and avoiding proteolysis due to D-amino acids.

To avoid proteolysis the vector-mediated delivery of genes encoding antimicrobial peptides into cancer cells was used (*66*). Expression constructs carrying cecropin or melittin were introduced into a human bladder carcinoma-derived cell line, and the resulting cell clones were analyzed for tumorigenicity in nude mice, resulting in either complete loss or reduced tumorigenicity. Newly, Soman *et al*. has demonstrated targeted nanocarriers that delivered melittin specifically to tumor cells in mice, and reduced tumor growth (*67*).

Local treatment of tumors by CAPs can be performed and this can avoid the challenges of toxicity, degradation and serum inactivation. This has not been widely studied since the type of cancers possible to reach by local treatment is restricted and because most medical agents are administered by oral or systemic routes. Examples of local treatment with CAPs are D-magainin which were injected intratumorally into subcutaneous human melanoma in nude mice and inhibited tumor growth (*68*). Diastereomers has also been injected directly in tumors and shown inhibition of tumor growth (*62, 63*). Another example of local treatment of cancer has been shown by the use of Gomesin, a cationic 18-mer derived from spider hemocytes. This peptide inhibited the growth of subcutaneous murine melanoma after topical administration of the Gomesin-containing cream (*69*).

Lactoferricin

Lactoferricin is a CAP produced by acid-pepsin hydrolysis of lactoferrin (LF) (*70*), an iron-binding glycoprotein found in exocrine secretions such as saliva and milk, as well as in the secretory granules of neutrophils (*71-73*). LF is a multifunctional protein involved in several biological activities related to host innate defense mechanisms (*2*). Bovine lactoferricin (LfcinB) has attracted considerable interest since lactoferricin of bovine origin exhibits greater antimicrobial activity than lactoferricins of human, murine or caprine origin (*74*). Substantial levels of LfcinB are found in human stomach following ingestion of bovine lactoferrin (*75*), implying that LfcinB is a natural breakdown product from the digestion of cow's milk. LfcinB isolated from cow's milk consists of 25 amino acid residues (Table 1), and forms an amphipathic twisted β-sheet configuration in aqueous solution that contains

nearly all of the hydrophobic residues on one face and the cationic residues on the other face (*76*).

LfcinB exhibits *in vitro* cytotoxic activities against different types of mouse and human cancer cell lines, including leukemia cells, fibrosarcoma cells, various carcinomas, and neuroblastoma cells (*14, 54, 77, 78*). LfcinB does not harm untransformed human cells, including fibroblasts, resting and activated T lymphocytes, epithelial cells and human umbilical vein endothelial cells (HUVEC), at peptide concentrations that are lethal to cancer cells (*14, 54, 79*). LfcinB is also an effective anticancer peptide *in vivo* because it prevents tumor growth and metastasis in several different mouse models of cancer (*54, 77, 80*). Moreover, LfcinB also possesses antiangiogenic effects, which may contribute to the cytotoxic effects of the peptide by inhibition of tumor progression *in vivo* (*80, 81*). Another possible role of LfcinB is as an immunomodulatory agent. LfcinB appears to have an anti-inflammatory effect as it has affinity for endotoxin (*82*) and binds unmethylated CpG-containing oligonucleotides (*83*). By neutralizing these molecules, LfcinB prevents inflammation. In addition, LfcinB appears to inhibit the inflammatory response through the classical complement pathway (*84*).

Lactoferricin derivatives

The cytotoxic activity of LfcinB against cancer cells is very much dependent on the amphipathic structure and high net positive charge of the peptide since cytotoxic activity is increased in LfcinB derivatives with clear cationic and hydrophobic sectors (*85-87*).

Structural parameters that are important for the antitumor activity and selectivity of synthetic peptides designed from the N-terminal region of LfcinB have been described by performing structure-activity relationship (SAR) studies. Helicity and amphipatic conformation were shown to be critical for antitumor activity, as peptide derivatives with cationic and lipophilic sectors have increased activity (*87, 88*). A relatively high positive charge of the peptide seemed to be a prerequisite for antitumor activity. The length of peptide correlated strongly with antitumor activity in which the minimum length of a peptide sequence with antitumor activity and selectivity were not less than ten residues (*87*). Moreover, the importance of hydrophobic residues was highlighted by the increased activity of LfcinB derivatives containing large and rigid Trp analogs, or an N-terminus modified by an acyl group or another bulky moiety (*85*). Based on these studies it was concluded that it is possible to construct highly active, specific antitumor peptides having less than ten amino acids by carefully

modulating these structural parameters and by introducing non-coded hydrophobic aromatic amino acids.

Based on these parameters the 9-mer LTX-302 was constructed, being cationic and amphipatic, containing five cationic Lys residues, three bulky Trp-residues and one non-coded aromatic residue *β*-diphenyl alalnine (Dip) and with an amidated C-terminal. The non-coded residue in the short LTX-302-peptide may increase the destabilization of tumor cell membrane and additionally it may increase the half life *in vivo*.

Aims of the study

Bovine lactoferricin (LfcinB) derived from the protein bovine lactoferrin found in cow milk has been the starting point for this thesis. LfcinB is a cationic antimicrobial peptide which exhibits antimicrobial and antitumor activities. Our group has studied several approaches regarding the antitumor effects of LfcinB, such as the peptide structure, the mechanism of action on cancer cells, the interaction with cancer cell membrane and the *in vivo* effects of LfcinB and its derivatives. In this thesis the overall aim was to study the mode of action underlying the anticancer effect of LfcinB *in vitro* and the effects of LfcinB and the shorter derivative LTX-302 in animal tumor models.

Following aims were defined:

1. What are the antitumor effects of LfcinB *in vivo* and *in vitro*?
2. What is the mechanism by which LfcinB perform its cytotoxic effect on cancer cells?
3. What underlies the immune therapeutic effects observed by intratumoral administration of LTX-302 in a murine lymphoma model?

Summary of papers

Paper I: **Evidence for a direct antitumor mechanism of action of bovine lactoferricin**

In this paper we demonstrated that LfcinB elicits an antitumor effect mediated through a direct mechanism of action on the tumor cells. The antitumor activities of bovine lactoferrin protein (LFB), murine lactoferricin (LfcinM) and LfcinB on murine tumor cell lines (Meth A fibrosarcoma, B16F10 melanoma and C26 colon carcinoma) were investigated in cell cultures as well as in established tumors in syngeneic mice. Both cyclic and linear forms of LfcinB and LfcinM were included in this study. The results demonstrated that LfcinB was cytotoxic against the three cancer cell lines, and cyclic LfcinB was more effective than the linear form. *In vivo* studies demonstrated a significant inhibition of tumor growth when Meth A tumors were treated locally with cyclic LfcinB. No significant antitumor effects were seen in the more aggressive B16F10 and C26 cancer cell lines. Additionally, antitumor effects of LfcinM were not seen neither *in vitro* nor *in vivo*, indicating that the antitumor activity of LfcinB in Meth A tumors is non-receptor mediated. Histological examinations of Meth A tumors treated by intratumoral injection of LfcinB demonstrated extensive inflammation and necrosis one day after treatment, clearly indicating a direct effect of the peptide on the tumor cells. Scanning electron microscopy proved that Meth A cells was disrupted by a direct action of LfcinB in which pore formation were followed by cell lysis. Based on these results we conclude that the antitumor activity of LfcinB was not receptor-mediated and a stable secondary structure of LfcinB was crucial for the antitumor effect. Local treatment of LfcinB induced necrosis *in situ* and the tumor cells were killed by a direct lytic effect on the tumor cells.

Paper II: The antimicrobial peptide, Lactoferricin B, is cytotoxic to neuroblastoma cells *in vitro* and inhibits xenograft growth *in vivo*

In paper II antitumor effects of LfcinB were investigated in more detail. LfcinB displayed selective cytotoxicity to all human neuroblastoma cell lines investigated compared to non-transformed fibroblasts. Additionally, the multidrug-resistant cell lines were more sensitive to LfcinB compared to the non-resistant cancer cell lines. *In vivo* studies also verified that intratumoral injection with LfcinB displayed significant inhibitory effects on the SH-SY-5Y neuroblastoma established in nude rats.

Among the cell lines tested, the MYCN-amplified Kelly cell line was shown to be most susceptible to the effects of LfcinB. This cell line was therefore chosen for further studies to characterize the effects of LfcinB on the membrane level. Treatment of Kelly cells with a sub-inhibitory concentration of LfcinB demonstrated decrease in mitochondrial potential by using the fluorescent probe JC-1, supporting that the mitochondria are a target for LfcinB. Activation of caspase-9, -6 and -7 indicated an activation of mitochondria-dependent apoptosis. However, specific caspase-inhibitors did not affect the cytotoxic activity of LfcinB, showing that apoptosis via the mitochondrial pathway of cell death is not the major contributor of cytotoxic activity. Morphological changes in Kelly cells treated with LfcinB were studied in more detail by transmission electron microscopy. These studies revealed an irreversible damage on the inner and outer mitochondrial membrane after 3 h of treatment. After 12 h a severe swelling and bursting of the whole cell was evident, a typical sign of necrosis. No apoptotic bodies which is a hallmark of the apoptotic process were seen. These observations suggest that the neuroblastoma cells were killed by necrosis. To identify the targets for LfcinB in the Kelly cells, localization of the peptide were investigated. LfcinB was found in blebs on the plasma membrane of Kelly cells and internalised inside the cells in the mitochondria. In conclusion, although the apoptotic machinery was activated, the cells were killed by necrosis due to a dual mechanism of the peptide on both mitochondria and the cell membrane.

Paper III: Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide

Here we revealed that LTX-302, a 9-mer peptide optimalized from parental LfcinB, can induce therapeutic vaccination against a murine lymphoma by intratumoral administration of the peptide. LTX-302 showed selectivity towards several cancer cell lines. On A20 B-cell lymphoma the cytotoxic effect was due to lysis of the plasma membrane. Established A20 B-cell lymphomas of BALB/c origin in syngeneic mice were completely regressed in the majority of the animals upon LTX-302 treatment. Cured mice were found to be protected against rechallenge with A20 cells, but not against Meth A sarcoma cells, indicating a specific immune response. Complete regression was T-cell dependent since it was not observed in nude mice having defect T-cells. Adoptive transfer of spleen cells from cured animals to irradiated naïve animals demonstrated that the protection was transferable. Depletion of T-cells from the splenocytes before adoptive transfer abrogated the protection. Both CD4 and CD8 T-cells were required for the protection.

These studies show that local treatment of cancer by a CAP can induce active immunization against the cancer, conferring a novel strategy for therapeutic vaccination against cancer

Discussion

Effect of LfcinB and its shorter derivative, LTX-302, on cancer cells

As shown in paper I and II, LfcinB exhibited cytotoxic effects against both murine and human cancer cell lines. These effects were selective against the tumor cell lines compared to the non-cancerous cells. The 9-mer cationic peptide LTX-302 derived from LfcinB also showed selectivity against cancer cells. Comparison of the activity of LfcinB and LTX-302 against Meth A fibrosarcoma (from Paper I and III) demonstrated that LTX-302 was more than twice as effective as LfcinB. This shows that the synthetic peptide was successfully designed to display an increased activity and keeping the selectivity towards cancer.

The selective cytotoxic activity against tumor cells compared to normal cells is believed to be due to a more negatively charged cell surface of the tumor cells. The plasma membranes of normal cells on the other hand consist primarily of zwitterionic and neutral phospholipids (*89, 90*). A higher than normal expression of anionic molecules such as phosphatidylserine (PS) (*34, 35, 91*) and O-glycosylated mucins (*92, 93*) has been found on cancer cells. Alteration in the carbohydrate portion of glycoproteins and glycolipids, including increased sialylation, also contribute to a more negatively charged tumor cell surface (*37, 94*). Moreover, the highly negatively charged glycosaminoglycan (GAG) side chains attached to a core protein, proteoglycan (PG), may interfere with the peptide-membrane interaction. It has been shown that several cancer cells have a different expression of cell surface PGs, compared to their normal counterparts (*95-97*). These differences on the plasma membrane between cancer and non-cancer cells can explain why LfcinB and LTX-302 are toxic to cancer cells at lower concentrations than required to kill normal erythrocytes and fibroblasts.

The various cancer cell lines also show different sensitivity towards LfcinB or LTX-302 treatment. In Paper II, LfcinB was most active against the MYCN-amplified neuroblastoma cell lines. The non-MYCN-amplified neuroblastom cell lines were less sensitive, showing that the cytotoxic effects of LfcinB were not influenced by the multidrug-resistant mechanisms in the MYCN-amplified cell lines. This probably reflects differences on the cancer cell membranes. Recent studies show that heparan sulphate (HS) on the surface of tumor cells inhibit the cytotoxic activity of longer CAPs such as LfcinB (*98*), while shorter CAPs such as the 9-mer LTX-302 show increased cytotoxic activity (*personal communication*). Since the amount of HS expressed on different cancer cell lines can vary, this may affect the sensitivity toward CAPs. Moreover, it has also been shown that there is a correlation between surface PS

density and sensitivity of various cancer cell lines to a 9-mer synthetic CAPs (*99*). This study show that increased amount of PS on cancer cells increase the sensitivity of these cells towards CAPs. In addition, a study by Risso *et al.* demonstrated that removal of sialyl moieties on cancer cells by neuramidase-treatment made these cells less susceptible for BMAP peptides (*100*). Hence, differences on the cell membranes among different cancer cell lines, and between cancer cells and non-malignant cells, influence the susceptibility of CAPs to act upon the cells. Therefore, more large-scale screening of various cancer cell lines is necessary to identify which type of cells being most sensitive for LfcinB and LfcinB-derivatives.

Peptide structure of importance for the antitumor activity

In Paper I anticancer activities was demonstrated by LfcinB, but not the murine analogue LfcinM, indicating that the activity of LfcinB is not receptor-mediated. LfcinB has higher net positive charge than LfcinM (*101*), which probably makes LfcinM less able to interact electrostatically with anionic cancer cell membranes and hence is not able to induce irreversible lysis of the target cells. In addition there appears to be a requirement for a stabilized secondary structure as cyclic LfcinB was more active than its linear analogue (Paper I). It has been demonstrated that cyclic LfcinB attains a highly amphipathic structure in solution (*76*), which probably make the peptide capable of interacting with negatively charged membrane surfaces. The linear LfcinB peptide on the other hand may adopt a more unordered secondary structure (*102*). Based on the results described in Paper I a series of SAR studies on LfcinB-derivatives were undertaken by our group, concluding that it was possible to construct highly active, specific antitumor peptides having less than ten amino acids. This was obtained by carefully modulating the structural parameters of helicity, cationic sector, charge, lipophilic sector, and by introducing non-coded hydrophobic aromatic residues in synthetic LfcinB-derivatives (*87, 88, 103*). A 9-mer peptide, LTX-302 (WKKWDipKKWK-NH_2), was synthesized, with an optimized cationic and hydrophobic sector and containing the large bulky aromatic amino acid Dip. This peptide indeed proved to be selective and highly active against cancer cell lines (Paper III).

Mode of action underlying the anticancer effect of LfcinB *in vitro*

In Paper I we demonstrated that LfcinB killed the murine Meth A tumor cells by disrupting the cell membrane resulting in necrosis. This was performed by a relatively high concentration of peptide. In Paper II the anticancer mode of action of LfcinB was studied in

more detail, using human neuroblastoma cells (Kelly cells) and a sub-inhibitory concentration of LfcinB.

Morphological changes in the cells treated with LfcinB were studied by transmission electron microscopy. These studies revealed an irreversible damage on the inner and outer mitochondrial membrane after 3 h of treatment. After 12 h a severe swelling and bursting of the whole cell was evident, and no apoptotic bodies were seen. To identify the targets for LfcinB in the Kelly cells, localization of the peptide were investigated. Both immunolabelled and fluorescent-labelled LfcinB were applied. Immunolabelling revealed that LfcinB was located close to the plasma membrane and in small membrane blebs after a few minutes exposure. Such blebs has been reported as a result of external disturbance ((*104*), and these results indicate a direct effect on the plasma membrane. The fluorescent-labelling showed that LfcinB co-localized with mitochondria in addition to the plasma membrane and in blebs on the plasma membrane. Hence, the mitochondria seemed to be a specific target for LfcinB after internalisation, and this is probably due to their negatively charged membrane and highly negative membrane potential (*50*). Dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) was induced after only 20 minutes treatment with LfcinB on Kelly cells. Loss of mitochondrial potential is commonly associated with destabilization of the outer mitochondrial membrane, which leads to release of cytochrome *c* (*105*). Released cytochrome *c* creates a high-molecular weight complex together with Apaf-1 and pro-caspase-9 which then activate pro-caspase-9 (*106*). Caspases play critical roles in the initiation and execution of apoptotic pathway (*107*). Caspase-9 is an initiator caspase for the mitochondria-dependent pathway of apoptosis (*108*) and activation of caspase-9 leads to activation of downstream executioner caspases, including caspase-3, caspase-6 and caspase-7 (*109*). Once activated, the executioner caspases exert their action and cleave cell proteins that are important for maintaining cell structure and function (*110, 111*), thereby resulting in the morphological and biochemical changes that are hallmark of apoptosis (*110, 112*). In Paper II the effect of LfcinB on the caspase-machinery was examined by studying activation of caspase-3, -6, -7, -8 and -9 by western blot analysis. Activation of caspase-9, -6 and -7 was evident, but not caspase-8 and -3. Activation of caspase-9 indicated an LfcinB-induced activation of mitochondria-dependent apoptosis. Pro-caspase-3 was only weakly upregulated but not activated, whereas pro-caspase-8 was not detected. This is in agreement with a study demonstrating that caspase-3 and -8 are deficient in a larger percentage of the MYCN-amplified neuroblastoma cell lines (*113*). Use of specific caspase-inhibitors or pan-caspase inhibitor did not affect the LfcinB-induced cell death, demonstrating that the induced

apoptosis was not responsible for the cytotoxic activity of LfcinB against the neuroblastoma. Taken together, the results suggest that LfcinB may have a dual mechanism of action against neuroblastoma cells in which both mitochondria and plasma membrane was directly affected by the treatment, superimposed with necrosis.

The mechanism by which LfcinB mediates anticancer activity has been studied by other groups. It has been shown that LfcinB causes human leukemia and carcinoma cells to die by apoptosis through a mitochondria- and caspase-dependent pathway that involves an increased formation of reactive oxygen species, loss of mitochondrial transmembrane potential and the activation of the caspase cascade culminating in apoptosis (*14, 78*). The process by which LfcinB triggers mitochondria-dependent apoptosis was suggested to be a result of cell membrane damage allowing LfcinB to enter the cytoplasm via toroidal pore formation (*114*). Hence, the level of irreversible damage to the cytoplasmic membrane relative to the extent of mitochondrial membrane damage caused by LfcinB may ultimately determine whether the cancer cells die by membrane damage or apoptosis. Therefore, depending on the cancer cell type, exposure to LfcinB may result in cell death by either apoptosis or a membranolytic mechanism (Paper I and II).

Complete regression of tumors obtained in mice treated by peptide

Paper I and II demonstrated that LfcinB inhibited tumor growth of both murine sarcoma and human neuroblastoma in wild-type mice and nude rats, respectively. These studies revealed that tumor growth was inhibited after local treatment with LfcinB, and complete regression was achieved in some occasions in the syngeneic mouse model (data not shown). The fact that LfcinM did not exhibit anticancer effects *in vivo* indicated that the inhibitory effect of LfcinB was non-receptor mediated. This inspired further investigation on the design of more active and selective peptides, from which the 9-mer peptide LTX-302 was selected. In Paper III the main objective was to study *in vivo* anticancer effects of LTX-302. A screening for murine cancer cell lines on BALB/c background was initiated, with superior effects seen in the A20 B-cell lymphoma. Immunocompetent BALB/c wild-type mice bearing subcutaneously established A20 tumors were treated with LTX-302 by intratumoral administration. Complete regressions of tumors were observed in a majority of the animals. In immunodeficient BALB/c nude mice, tumor growth inhibition was induced while complete regressions were not obtained. Complete regression of tumors in immunocompetent animals has also been obtained with LfcinB (data not shown). T-cells therefore seem to be critical to obtain complete regressions of tumors. Histological examinations of LTX-302-treated A20 tumors in

wild-type mice showed massive necrosis, inflammation and great infiltration of lymphocytes. From the literature it is known that there is a great correlation between tumors infiltrated of lymphocytes and regression of tumors (*115, 116*).

In vivo studies of anticancer effects of CAPs have mainly focused on systemic administration of peptide, in which CAPs are coupled to homing domains in order to obtain high specificity for the target cells. These peptide conjugates were shown to kill cancer cells by apoptosis (*20, 50*). Another strategy has been to avoid serum inactivation and proteolysis during systemic administration by designing diastereomeric peptides which kill cancer cells selectively via necrosis (*61*). Most of these studies have been performed in xenograft models in which human cancer cell lines were inoculated in nude mice. This is a standard preliminary cancer model to study whether any anticancer effect is conceivable. Some studies have investigated anticancer effects of CAPs by intratumoral administration, and a study by Soballe *et al*. demonstrated complete regression of tumors in nude mice subsequent to single injections with very high dose of magainin-derivative (*68*). In another study, the effect of a proapoptotic peptide was investigated by treating fibrosarcoma in immunocompetent mice with intratumoral injections of the peptide (*20*). Complete regression was attained, although minimal lymphocyte infiltration of the treated tumors was observed, indicating that the peptide treatment did not incite a significant inflammatory response. Common for both these experiments that attained complete regression is that they were not able to reject a rechallenge of the same tumor. Since LTX-302 induced a complete regression of tumors, rechallenge studies were performed to investigate whether an immune response against the A20 lymphoma was induced.

LTX-302 induced therapeutic vaccination against A20 lymphoma

When cured mice were rechallenged with A20 four weeks after they became tumor-free, A20 cells were rejected, showing that a secondary immune response was evident after the LTX-302 treatment. To our knowledge, Paper III is the first report in which a CAP is able to induce a vaccination effect against a certain type of tumor.

The immune response against A20 seemed to be tumor-specific since there was no inhibition of tumor growth when Meth A cells were injected into mice cured of A20 tumor. Further studies to characterize the immune response directed against A20 revealed that the protection could be transferred to untreated naïve mice via splenocytes from donors previously treated with LTX-302. Depletion of specific T-cell subsets in the adoptively transferred splenocytes indicated that both $CD4^+$ and $CD8^+$ T-cells were required for the protection. The tumor growth pattern of mice receiving CD4 or CD8-depleted splenocytes from cured donors

displayed somewhat different growth curves (data not shown). Depletion of CD4 resulted in transient complete regression of challenged A20 tumor, although all mice succumbed to re-growth of tumor within short time. Depletion of $CD8^+$ T-cells resulted in almost no tumor inhibition, indicating that the $CD8^+$ T-cells were important for the direct killing effect against the tumor cells, while $CD4^+$ T-cells were important for the long-term memory against A20 tumor. This is in accordance with published data showing that to obtain long-lived, robust memory cells both $CD8^+$ and $CD4^+$ T-cells are needed to be stimulated (*117, 118*).
In conclusion, we demonstrated that an active immune response was induced against A20 lymphoma by injection of LTX-302 intratumorally in subcutaneous tumors in immunocompetent mice.

Hypothetic scenario for induction of secondary immunity against A20 lymphoma

Paper III has demonstrated sensitization of T-cells following intratumoral administration of LTX-302 in established A20 tumors. The induction of antitumor immune response requires the recruitment of antigen presenting cells (APC) into tumor lesions followed by effective uptake of tumor associated antigens (TAA). The APCs transport internalized TAA to draining lymph nodes where they present TAA peptides for the activation of tumor-specific cytotoxic and helper T-cells (*119*). Dendritic cells (DC) are the most potent antigen-presenting cells in the immune system (*120*), and they play a pivotal role in the induction of immunity. Necrosis may provide appropriate environment for maturation and activation of DC by two ways; a) release of large amounts of tumor debris that can serve as antigen source for dendritic cells (DC) (*121*) and b) danger signals for maturation and activation of DC (*122-124*).

In support of these two mechanisms of activating DCs are the SEM-studies showing necrosis of A20 cells and histology showing necrosis, inflammation and infiltration of cells into treated tumors. Our lab has also newly demonstrated that the danger molecule, nuclear protein high mobility group box 1 (HMGB1) was released after LTX-302 treatment of A20 cells (*personal communication*). HMGB1 is released upon necrotic cell death and act as a danger signal by activating DCs (*125*). In the study of proapoptotic peptide mentioned earlier complete regression did not induce protection probably because the tumor cells were killed by apoptosis which did not stimulate lymphocyte infiltration (*20*). In sum, the cytolytic effect of LTX-302 is probably important for the induced immune response by acting as a source of TAA and danger signals.

Concluding remarks

In the present thesis the main focus has been to explore the anticancer activity of LfcinB and the *de novo* designed peptide, LTX-302. In Paper I and II the mechanisms of action of LfcinB on cancer cells were investigated, including the anticancer effect of LfcinB *in vivo.* LfcinB was shown to act both on the plasma membranes and on the mitochondrial membranes, resulting in necrotic cell death. *In vivo* studies of the more active peptide LTX-302, demonstrated complete regression of murine B-cell lymphomas in the majority of the immunocompetent mice. Further studies *in vivo* revealed that protection against the lymphoma was induced in the cured animals, and this protection was T-cell dependent. We have for the first time shown that a CAP can be used as a cancer vaccine when the peptide is administered intratumorally.

A further study on this vaccination effect induced by a CAP is needed to explore in more detail what happens inside the tumor. Does the peptide induce chemotaxis when injected in the tumor? Does the peptide induce antigenicity? What is the identity of the cells that infiltrate the treated tumors? What will be the effect of combining LTX-302 with other immunotherapeutic agents?

The historical emphasis in tumor immunotherapy has been on the identification of and vaccination with defined TAA (*126*). The idea of turning the tumor to be its own cellular vaccine represents a paradigm shift (*127*). Local destruction of tumor by cryoablation or electrochemotherapy in combination with Cpg administration have shown promising results (*128, 129*), indicating that a proper local treatment of the tumor could induce immune responses, and could represent a new challenge for the next years. Use of chemotherapy to increase the level at which tumor antigens are cross-presented supplied by proinflammatory adjuvans that are directed at the APC has also been suggested (*130*). The results from Paper III is in accordance to this view that the tumor can act as its own vaccine after treatment with an agent that creates an environment at the tumor site that favours both TAA available for loading and activation of DCs.

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