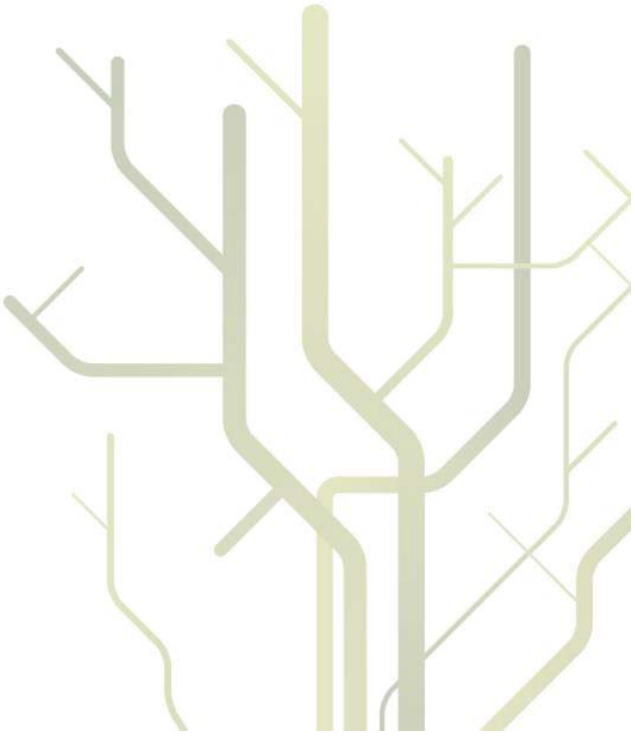
FACULTY OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL BIOLOGY
MOLECULAR INFLAMMATION RESEARCH GROUP

Short Lytic Anticancer Peptides as a Novel Therapy against Cancer

Ketil André Camilio

A dissertation for the degree of Philosophiae Doctor
June 2013



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Tromsø 2013

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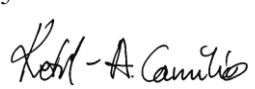

Ketil André Camilio

ABSTRACT

Cancer is the leading cause of deaths worldwide, with a significant increase in the number of annual incidents. Concurrently, cancer-related therapy has been met with a number of challenges, such as toxic side effects and an increase in multi-drug resistant cancer cells, thereby spawning a need for new and improved therapies. Cationic antimicrobial peptides (CAPs) are naturally occurring molecules found in most species, often as an integral part of the first line of defense against pathogens. Although CAPs vary extensively in amino acid sequence and structural motifs, most of them share common features such as cationicity and amphipathicity, which enable them to interact with and disrupt cellular membranes. Several CAPs have shown promising potential as novel anticancer agents with an ability to selectively kill cancer cells. Additionally, anticancer peptides (ACPs) are able to interact with cancer cell membranes through electrostatic interactions due to their higher than normal expression of anionic molecules. ACPs kill cancer cells through a membranolytic mode of action or through an interaction with intracellular targets, further supporting their potential as novel anticancer drugs.

Recent progress within cancer therapy has primarily been in the areas of targeted therapy and immunotherapy, with this thesis demonstrating that short lytic ACPs may have a potential as novel anticancer and immunotherapeutic agents. Structure-activity relationship studies on bovine lactoferricin allowed us to *de novo* design short chemically modified lytic ACPs (LTX-302 and LTX-315) with an improved therapeutic potential compared to bovine lactoferricin. The intratumoral (i.t.) administration of LTX-302 induced a complete regression of- and a long-term and transferrable tumor-specific immune protection against syngeneic A20 B cell lymphomas (Paper I). A more active nonapeptide, LTX-315, was able to induce complete regression and a long-term tumor immune protection against highly aggressive and low immunogenic syngeneic B16 melanomas (Paper II). By inducing rapid necrosis and local inflammation due to the release of Danger-Associated Molecular Pattern molecules, i.t. administration of the ACPs stimulated the infiltration of immune cells into the tumor parenchyma, thus creating a synergistic relationship between the direct disruptive effects and the indirect immunomodulatory effects of the peptides.

This thesis also demonstrates that ACPs with a high cell membrane disruptive potential such as LTX-315 can be used in an immune augmenting adjuvant setting due to its potential to stimulate immune responses (Paper III). The low-dose intradermal administration of LTX-315 induced reversible tissue damage, leading to local inflammation and the infiltration of immune cells at the injection site. When used together with a tumor cell lysate, the combination was able to mount a long-term tumor immune protection against syngeneic B16 melanomas.

Taken together, the data presented in this thesis demonstrates that i.t. treatment with short lytic LTX-ACPs can have potential as new immunotherapeutic agents by enlisting local tumor control, followed by protective immune responses. Moreover, LTX-315 has an immune augmenting adjuvant potential when used in combination with a whole cell cancer vaccine.

LIST OF PUBLICATIONS

Paper I

Gerd Berge, Liv Tone Eliassen, **Ketil Andre Camilio**, Kristian Bartnes, Baldur Sveinbjørnsson and Øystein Rekdal. Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide. *Cancer Immunol Immunother*. (2010) 59:1285-94.

Paper II

Ketil André Camilio, Gerd Berge, Chandra Sekhar Ravuri, Øystein Rekdal and Baldur Sveinbjørnsson. Complete Regression and Protective Immune Responses obtained in B16 Melanomas after Treatment with LTX-315. *Manuscript*

Paper III

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Long-term Protection against B16 Melanomas upon Vaccination with Tumor Cell Lysate in Combination with LTX-315 as a Novel Adjuvant. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ACP	Anticancer Peptide
APC	Antigen Presenting Cell
APD	Antimicrobial Peptide Database
ATP	Adenosine Triphosphate
BCG	Bacille Calmette Guérin
BIP	*β*-(4,4'-biphenyl)alanine
CAP	Cationic Antimicrobial Peptide
CRT	Calreticulin
CTL	Cytotoxic T Lymphocyte
DAMP	Damage/Danger-Associated Molecular Pattern
DC	Dendritic Cell
DIP	*β*-diphenylalanine
DNA	Deoxyribonucleic Acid
DTIC	Dimethyl-Triazone-Imidazol Carboxamide
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HMGB-1	High Mobility Group Box-1
HSP	Heat Shock Protein
ICD	Immunogenic Cell Death
IFN-γ	Interferon Gamma
IL	Interleukin
I.T.	Intratumoral
LfcinB	Bovine Lactoferricin
LPS	Lipopolysaccharide
LTX-ACP	Lytix-Anticancer Peptide
MHC	Major Histocompatibility Complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazodium bromide
PAMP	Pathogen-Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
RAGE	Receptor for Advanced Glycation End products
RGP	Radial Growth Phase
TAA	Tumor Associated Antigen
Tbt	*β*-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine
TCL	Tumor Cell Lysate
TCR	T Cell Receptor
T_H	T Helper
TIL	Tumor Infiltrating Lymphocyte
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-Alpha
Tpc	*β*-[2-(Pmc)-indol-3-yl]alanine
Treg	Regulatory T Cell
VGP	Vertical Growth Phase
SAR	Structure Activity Relationship
UTP	Uridine Triphosphate
α-ACP	α-helical Anticancer Peptide

1 MELANOMA

Melanoma is a disease that dates back many centuries, and was first described as "fatal black tumors with metastases and black fluid in the body" in the middle of the 1600s, although historic annals show that melanoma was probably first mentioned by Hippocrates as early as the 5th century B.C.[1]. John Hunter is reported to be the first to operate on metastatic melanoma in 1787, describing it as "cancerous fungous excrescence"[2]. The French physician René Laennac was the first to describe melanoma as a disease entity, "la mélanose," which was presented in a lecture at the Faculty of Medicine in Paris in 1804 and subsequently published in 1806[3]. Later, in 1838, Robert Carswell introduced the term melanoma to define these same pigmented malignant tumors[1], while, in 1840, Samuel Cooper stated that advanced melanoma was untreatable and the only chance of a therapeutic benefit would come from early removal of the disease[4]. Close to two centuries later, this situation remains largely unchanged.

1.1 THE BIOLOGY OF MELANOMA

Malignant melanoma, which develops from a neoplastic transformation of melanocytes following genetic mutations, is the most aggressive and deadliest form of skin cancer, causing the majority (75%) of skin cancer-related deaths[5]. The incidence of melanoma is related to skin pigmentation and is therefore highest in races with less pigmented skin, such as with Caucasians, particularly if one is living in sunny climates such as Oceania, Northern America, Europe, Southern Africa and Latin America[6]. Melanoma starts in the melanocytes located between the outer layer of the skin (the epidermis) and second layer (the dermis), and develops due to unregulated apoptosis and uncontrolled cellular proliferation (Figure 1). This is primarily caused by genetic mutations to melanocytes following UV radiation from the sun, although UV light from sunbeds may also contribute to the disease[7]. Genetic mutations linked to melanoma and known to increase one's susceptibility to melanoma include *BRAF* V600 mutations (present in 50% of melanomas), *NRAS*, *CDNK2A* and *PTEN* deletions, *MITF, TERT* and *CCND1* amplification/alteration, as well as *TP53* and *MAP2K1*, *PPP6C*, *RAC1*, *SNX31*, *TACC1*, *STK19* and *PREX2* mutations among others[8, 9]. From 1970 to 2009, the incidence of melanoma has increased by 800% among young women and 400% among young men[10], with an annual increase of 2.8% since 1981 in the US[11]. It is estimated that a total of 76,690 new cases of invasive melanoma will be diagnosed in the US in 2013, with 9,480 estimated to result in death[12].

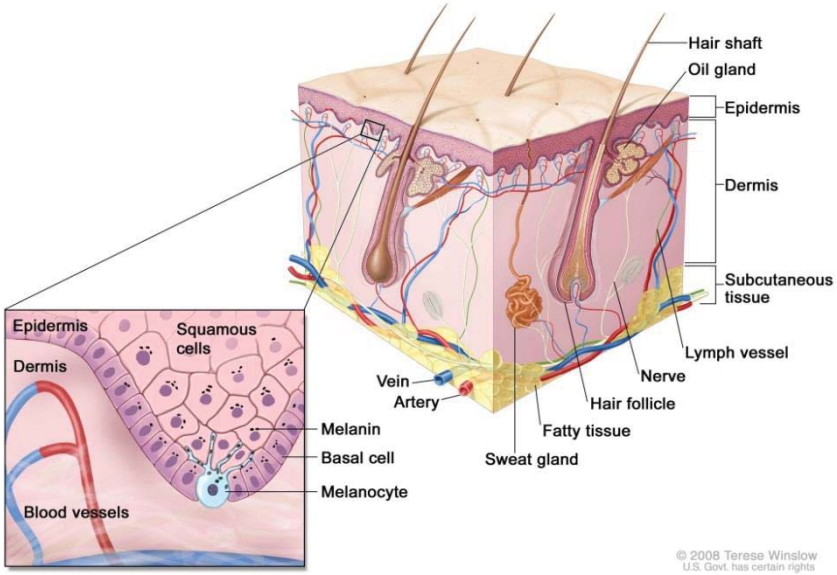

Figure 1: Anatomy of the skin - Cross section of the skin, including the epidermis, the dermis, the subcutaneous tissue (hypodermis) and the location of melanocytes in the skin. Illustration used with permission, copyright 2008 by Terese Winslow[13].

1.2 MELANOMA STAGING

Melanoma is divided into four different stages[14] with the early stages (Stages I/II) being highly curable, though as the disease progresses the survival rate drops dramatically (Table 1). Early stage melanoma starts with uncontrolled growth of the melanocytes in the skin, and at this stage the tumor is less than 1 mm thick (in the epidermis) and easily curable by surgery (radial growth phase). As the melanoma cells start to acquire invasive potential they can penetrate deeper into the skin, radically changing their behavior and reaching the vasculature necessary for metastasis to new tissues (vertical growth phase). At this stage, the invasive melanoma can spread through blood or lymph vessels, is more than 1 mm in thickness and has reached the dermis in the skin (Figure 2).

Table 1: Overview of the different stages of malignant melanoma[14]

Stage	Characteristics	Survival
0	Melanoma *in situ*	99.9%
I/II	Invasive melanoma (1-2 mm thick primary tumor)	89-95%
II	High risk melanoma (1-4mm thick primary tumor)	45-79%
III	Regional metastasis to lymph node(s)	24-70%
IV	Distant metastasis (e.g. skin or lung)	7-19%

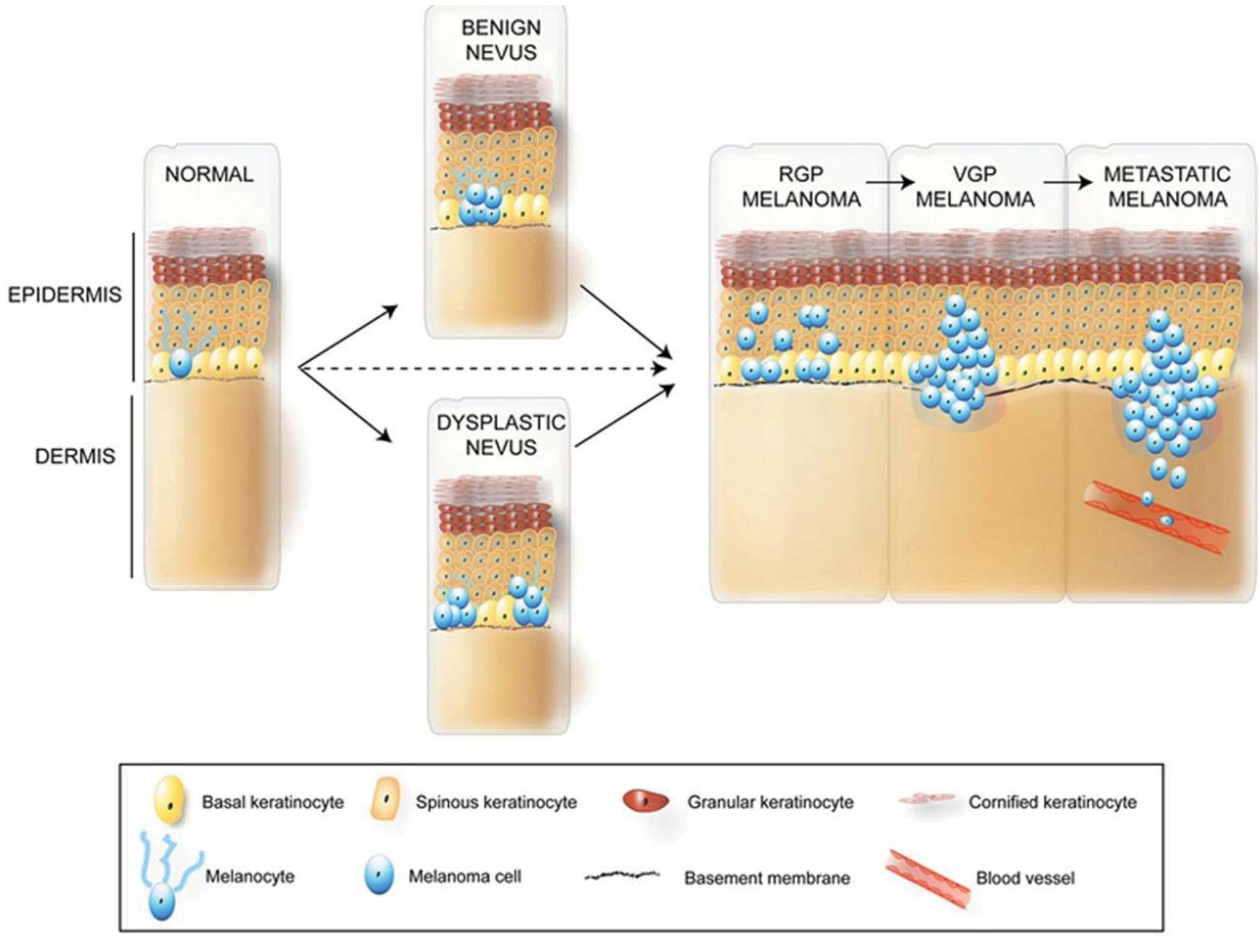

Figure 2: Development of malignant melanoma - Malignant melanoma arises in the epidermis and develops into metastatic melanoma through a multi-step process involving precursor lesions such as benign nevus and dysplastic nevus. RGP, Radial Growth Phase; VGP, Vertical Growth Phase. Illustration used with permission, copyright 2008 by Frontiers in Bioscience[15].

1.3 TREATMENT

Prior to any treatment decision, melanoma is clinically diagnosed with a skin biopsy. Depending on the stage of the melanoma, the skin biopsy may also be accompanied with a sentinel lymph node biopsy. Advanced melanoma has a poor prognosis and treatment is done using a multidisciplinary approach.

1.3.1 Surgery

In a treatment setting, surgical excision of the tumor will be the first option evaluated. Although surgical excision may remove the tumor, more surgery is often needed later to minimize the risk of recurrence. As illustrated in a recent article by Kunishige et al.[16], melanoma *in situ* should be treated with surgical margins of no less than 0.9 cm although many surgeons consider a margin of 0.5 cm as the standard for *in situ* melanoma[17]. Using 0.9 cm surgical margins gave a survival rate of 98.9%[16]. As the disease progresses and metastasizes, metastatic tumors and lymph nodes may be surgically removed as well. Even if surgery represents the cure in the early phase of the disease, the prognosis in the metastatic phase remains very poor.

1.3.2 Radiation Therapy

Radiation therapy is often applied succeeding the surgical resection of local or regional advanced melanomas or for unresectable distant metastasis. Moreover, it has been shown to reduce local occurrence but does not prolong survival[18].

1.3.3 Chemotherapy

A selection of chemotherapeutic agents is used against metastatic melanoma, including dacarbazine (dimethyl-triazeno-imidazol carboxamide (DTIC)), temozolomide (a dacarbazine analog) and cisplatin, with limited success as monochemotherapy, but an improvement has been shown when used as polychemotherapy[19].

1.3.4 Immunotherapy

Adjuvant treatment, such as a high-dose interferon (e.g. interferon alpha) treatment, may be applied for high-risk melanomas, although the increase in overall survival is relatively small and often linked to unpleasant side effects[20]. IL-2 (a cytokine produced by human T-helper lymphocytes) is also used against metastatic melanoma, thereby offering the possibility of a complete and long-lasting tumor regression in a small percentage of patients[21]. Improvements within malignant melanoma treatment have mainly been within immunotherapy, as demonstrated by the approval of Ipilimumab (Yervoy; Bristol-Myers Squibb, USA) in March of 2011, a monoclonal antibody targeting the inhibitory CTLA-4 ligand to treat unresectable or late-stage metastatic melanoma. For now, ongoing research and improvement in therapy is increasing its focus towards targeted therapy and immunotherapy, e.g. the targeting of important genes (*BRAF*, *MEK* and *KIT*) with inhibitors, as well as the targeting of receptors with specific monoclonal antibodies (anti-PD-1, anti-OX44 and anti-4-1BB[22, 23].

2 CATIONIC ANTIMICROBIAL PEPTIDES

The field of antimicrobial peptides started in the 1960s with Spitznagel and Zeya, who discovered that basic proteins and peptides in polymorphonuclear leukocytes had antimicrobial properties[24, 25], which were later named antimicrobial peptides[26, 27]. Antimicrobial peptides, often named cationic antimicrobial peptides (CAPs), are small molecules found in a large diversity of species, such as bacteria, fungi, plants and animals[28]. CAPs vary extensively in the amino acid sequence and encompass a wide variety of structural motifs (Table 2). However, recurrent structural and functional aspects are observed among peptides from different species, particularly in relation to their cationicity and amphipathicity (i.e., the net charge at neutral pH varies from +2 to +9), as these qualities enable them to interact with and disrupt lipid membranes. In 1999, Epand and Vogel divided CAPs into several groups: linear peptides, which form amphipathic and hydrophobic helices; cyclic peptides and small proteins, which form β-sheet structures; peptides with unique amino acid compositions, cyclic peptides with thio-ether groups in the ring, lipopeptides terminating in an amino alcohol and macrocyclic knotted peptides[29]. Furthermore, numerous CAPs have been isolated from nature in addition to many synthetic variants. As of June 2013, the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.php) contains 2,233 entries of CAPs with a range of activities (Figure 3). Due to their diverse activities and direct cytotoxic effect, they are often an integrated part of the immune system of eukaryotic organisms by mounting a first line defense against pathogens. Furthermore, some CAPs have been found to have lipopolysaccharide (LPS) neutralizing abilities[30] and immune modulatory effects[31-33]. In addition to their recognized antibacterial activities, several CAPs show promising anticancer activities[34-37], including CAPs with the aptitude to kill cancer cells, which are referred to as anticancer peptides (ACPs).

Table 2: Primary structure of naturally occurring CAPs with anticancer activity

	Peptide	Amino acid sequence	References
α-helical anticancer peptides	BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHL	38
	BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI	38
	Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	39
	Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAILS	39
	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	40, 41
	Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	42
	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	43
β-sheet anticancer peptides	HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	27
	HNP-2	CYCRIPACIAGERRYGTCIYQGRLWAFCC	27
	HNP-3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC	27
	LfcinB	FKCRRWQWRMKKLGAPSITCVRRAF	44
	Tachyplesin I	KWCFRVCYRGICYRRCR	45
Linear anticancer peptides	PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	46

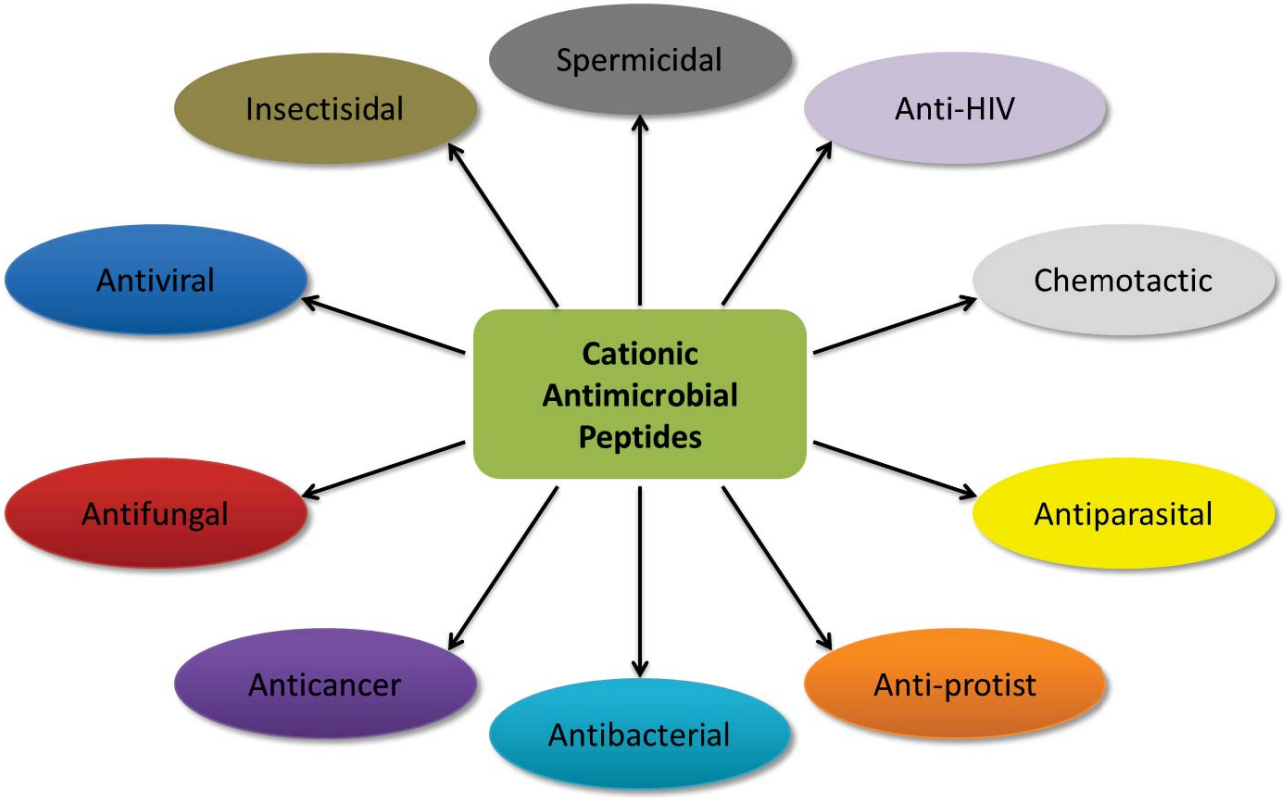

Figure 3: Multiple functions of CAPs in nature - Simplified diagram representing the diversity of functions that cationic antimicrobial peptides have in nature. A specific CAP can possess one or several of these functions, for example having antibacterial, antifungal and anticancer characteristics. The characteristics were adapted from the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.php).

2.1 α-HELICAL ANTICANCER PEPTIDES

Several α-helical ACPs (α-ACPs) found in nature have been found to display anticancer activities (e.g. cecropins, cathelicidins, magainins and melittin), thereby giving them a therapeutic potential against cancer. α-ACPs are recognized by their secondary structure, which resemble an amphipathic or hydrophobic α-helix (Figure 4A).

2.1.1 Cecropins

Cecropin A and B are ACPs derived from insect sources (first discovered in the giant silk moth *Hyalophora cecropia*)[39], which are able to lyse different types of human cancer cells at peptide concentrations that are not harmful to normal eukaryotic cells *in vitro*[47-49] and *in vivo*[48, 49].

2.1.2 Cathelicidins

Cathelicidins (e.g. Human Cationic AMP of 18 kDa, hCAP-18) are ACPs derived from a variety of cell types such as neutrophils[39] and squamous epithelial cells[50]. $hCAP18_{109-135}$ induces apoptosis in a human squamous carcinoma cell line while displaying no cytotoxic effects on normal gingival fibroblasts or keratinocytes[51]. Other cathelecidins studied are the bovine-derived cathelecidins, BMAP-27 and BMAP-28[38], and the linear cathelecidin PR-39 isolated from porcine small intestines and neutrophils[46, 52].

2.1.3 Magainins

In 1987, Zasloff reported on magainins, a group of ACPs isolated from the skin of the African clawed frog *Xenopus laevis*[42]. Magainin 2 displayed selective cytotoxic activity against several tumor cell lines *in vitro* through a membrane-associated mechanism of action[53-56], with analogues designed from magainin 2 revealing a potent antitumor effect in several mice models[57, 58].

2.1.4 Melittin

Melittin is an alkaline polypeptide isolated from European honeybee (*Apis mellifera*) venom[43], which showed the non-selective killing of cancer cells[59, 60] through a membrane-associated mechanism leading to cell lysis[61]. In addition, both the targeting of a melittin-avidin conjugate[62] and an immunoconjugate containing a melittin peptide analogue[63], to the tumor microenvironment, demonstrated antitumor effects *in vivo*. Local intratumoral administration of the melittin-avidin conjugate caused a significant anticancer response against murine B16 melanomas in syngeneic mice[62].

2.2 β-SHEET ANTICANCER PEPTIDES

ACPs such as defensins, lactoferricin and tachyplesin are recognized by their β-sheet secondary structure. Such peptides are folded into β-sheets (Figure 4B and 4C) due to conserved Cys residues, thus creating intramolecular disulfide bridges between the NH_2-terminal and COOH-terminal regions of the peptide[64, 65].

2.2.1 Defensins

Defensins have been isolated from a number of species, but the most studied are the α- and β-defensins of human origin[28, 66, 67]. Usually found as part of the innate immune system, e.g. in neutrophils[26, 27], these peptides have exhibited cytotoxic activity against several tumor cells of both human and murine origin[68-70]. As for several other CAPs the mechanism of action is a membrane-related mechanism[69, 71, 72].

2.2.2 Bovine Lactoferricin

Bovine lactoferricin (LfcinB) is an ACP isolated from cow's milk after acid-pepsin hydrolysis of the lactoferrin protein[73, 74]. In addition to others, our group showed that LfcinB exhibited *in vitro* cytotoxic activity against a selection of murine and human cancer cell lines such as leukemia cells, fibrosarcoma cells, various carcinomas and neuroblastoma cells[75-78]. LfcinB binds to cancer cell membranes, thereby leading to cell membrane pore formation and destabilization, and either cell death by lysis or through effects on the mitochondria[78, 79]. Furthermore, the peptide inhibited the *in vivo* growth and/or metastasis of several different tumor types in mice[76, 78, 80].

2.2.3 Tachyplesin

Tachyplesin I was isolated from the horseshoe crab (*Tachypleus tridentatus*) by Nakamura et al. in 1988[45]. The peptide was shown to kill cancer cells through a cytolytic mechanism of action by binding to hyaluronan, which is often over-expressed on tumor cells compared to normal cells[81, 82].

When Tachyplesin I was coupled to an integrin homing domain and injected intraperitoneally, it was able to inhibit the *in vivo* growth of B16 melanomas in syngeneic mice[83].

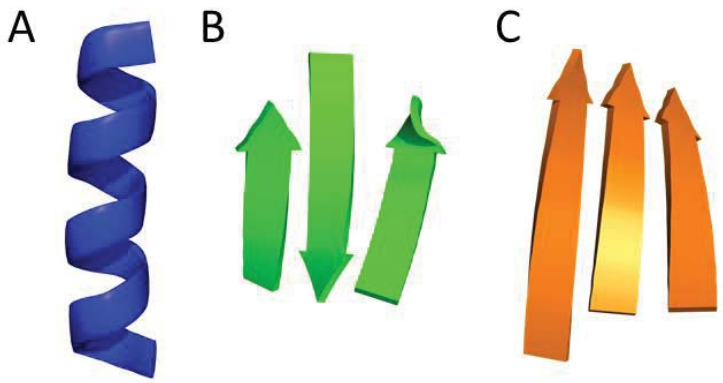

Figure 4: CAP secondary structures - CAPs are typically recognized by an α-helical (A) or β-sheet secondary structure (B and C). β-sheet secondary structures are commonly divided into anti-parallel β-sheets (B) and parallel β-sheets (C). Figures were generated using PyMOL 1.3.

2.3 STRUCTURAL PARAMETERS IMPORTANT FOR ANTICANCER ACTIVITY

Structure activity relationship (SAR) studies performed on LfcinB and derivatives have indicated that structural parameters such as charge of the cationic sector and overall charge, the angle subtended by the total cationic sector, the overall secondary structure, the number, size and position of the aromatic residues in the lipophilic sector, as well as the length of the peptide sequence, are all important parameters for antitumor activity[84-89].

2.3.1 Importance of the cationic- and aromatic sector

A study performed with 15 different peptide derivatives from the α-helical region of LfcinB revealed that the angle subtended by the total cationic sector, along with the number and position of positively charged residues in the cationic region, were important for the cytotoxic effect of α-helical peptides against tumor cells[85]. A net charge of +7 was required to achieve a high anticancer activity and tumor cell specificity[86], with most ACPs having a net charge of +2 to +7[89]. Moreover, the number and position of the aromatic residues in the lipophilic sector were all central to the antitumor activity[85]. Modifications made to the aromatic sector of LfcinB derivatives by Eliassen et al. showed that by replacing tryptophan residues in the aromatic sector with large bulky non-coded aromatic amino acids such as *β*-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine (Tbt), *β*-[2-(Pmc)-indol-3-yl]alanine (Tpc), *β*-(4,4'-biphenyl)alanine (Bip) or *β*-diphenylalanine (Dip), the anticancer activity of the peptides could be increased[87]. SAR studies performed on 10 different analogues of the idealized amphipathic helical 21-mer peptide $(KAAKKAA)_3$ revealed that the cytotoxic activity of an idealized α-helix is largely dependent on the position of the tryptophan residues within the hydrophobic sector. Tryptophan residues located adjacent to the cationic sector were more important for antitumor activity than tryptophan residues located opposite to the cationic sector in an α-helix[88], hence indicating that both the amino acid type and the location of the aromatic residues in the aromatic sector are important for ACP cytotoxic activity against cancer cells.

2.3.2 Importance of peptide sequence and length

In addition to peptide charge and the location of amino acids within the cationic and aromatic region, the length of the peptide sequence has shown to be of importance for antitumor activity. A peptide as short as 6 amino acids (M6) displayed cytotoxicity against three different cancer cell lines, including both murine and human. However, the shortest peptide that exhibited selective activity against tumor cell lines contained 10 amino acid residues (M2), and there was a strong correlation between antitumor activity and peptide sequence length and net positive charge of the peptides[86]. The majority of ACPs have a primary structure consisting of 10-40 amino acids[89]; therefore, the order and type of the amino acids within ACPs and the conformation (secondary structure) of ACPs seem to be essential for the cytotoxic effect against cancer cells.

2.4 SYNTHETIC ANTICANCER PEPTIDES

In recent years, several groups have attempted to create novel and more efficient synthetic ACPs based on structural parameters important for anticancer activity. Synthetic lytic peptides are of interest in a therapeutic setting due to their ability to selectively permeabilize negatively charged phospholipid membranes, including those of cancer cells[85-87, 90-93]. Common to these synthetic peptides is their cationic and amphipathic secondary structure, thus optimizing them for cancer cell phospholipid membrane interaction. Several D-analogues (D-$K_4R_2L_9$ and D-K_6L_9) synthesized by Papo et al. displayed a selective killing of cancer cells compared to normal cells, both *in vitro* and *in vivo*[91, 92, 94, 95]. Additionally, intratumoral injections of the DP-1 peptide (ACP peptide linked to the PTD-5 protein transduction domain) induced a partial or complete regression of MCA205 fibrosarcoma tumors in mice without any apparent side effects[96]. Furthermore, a single intratumoral injection of r7-kla induced significant tumor tissue loss and extensive necrosis in HT1080 human fibrosarcoma xenografts grown in immune-deficient mice[97].

2.4.1 The LTX-300 series

By chemically modifying LfcinB derivatives and using an array of SAR studies, our group was able to synthesize shorter and more effective ACPs compared to LfcinB[84-87]. A series of nonapeptides (The LTX-300 series) with anticancer activities were created and screened for antitumor activity. One such peptide, LTX-302 (W-K-K-W-Dip-K-K-W-K-NH_2) has a potential to adopt a helical coil structure (predicted by the Garnier-Osguthorpe-Robson V method[98] (Figure 5 top). LTX-302 was very active against the murine A20 B-cell lymphoma cell line, both *in vitro* and *in vivo*, while retaining high cancer cell selectivity. LTX-302 induced phospholipid membrane damage and cell death by necrosis[93]. Moreover, when A20 cells were treated *in vitro* with LTX-302, the danger signal cytokine High Mobility Group Box-1 (HMGB-1) was released from the cells. HMGB-1 has been shown to be important in responses important in infection, injury and inflammation, acting as a Damage-Associated Molecular Pattern molecule (DAMP)[99, 100]. When LTX-302 was administered intratumorally into intradermally established A20 tumors *in vivo*, tumor tissue samples demonstrated that LTX-302 induced necrotic cell death and inflammation. This was seen by an infiltration of immune cells into both primary tumors and cured animals rechallenged with A20 tumors, indicating an immunogenic cancer cell death and a long-term protective effect against A20 cells in

the cured animals[93]. A peptide analogue of LTX-302, LTX-315 (K-K-W-W-K-K-W-Dip-K-NH_2), has a potential to adopt a helical coil structure (predicted by the Garnier-Osguthorpe-Robson V method[98] (Figure 5 bottom). LTX-315, although less selective than LTX-302, was chosen as a new lead compound because of its superior anticancer activity against a panel of tumor cells. LTX-315 is more active and less selective due to its larger aromatic/lipophilic sector, with about a 50/50 relationship between the aromatic and cationic sector for LTX-315 and 40/60 for LTX-302, respectively (see helical projections in Figure 5). Both peptides were designed for local treatment (intratumoral administration) of transdermally accessible tumors.

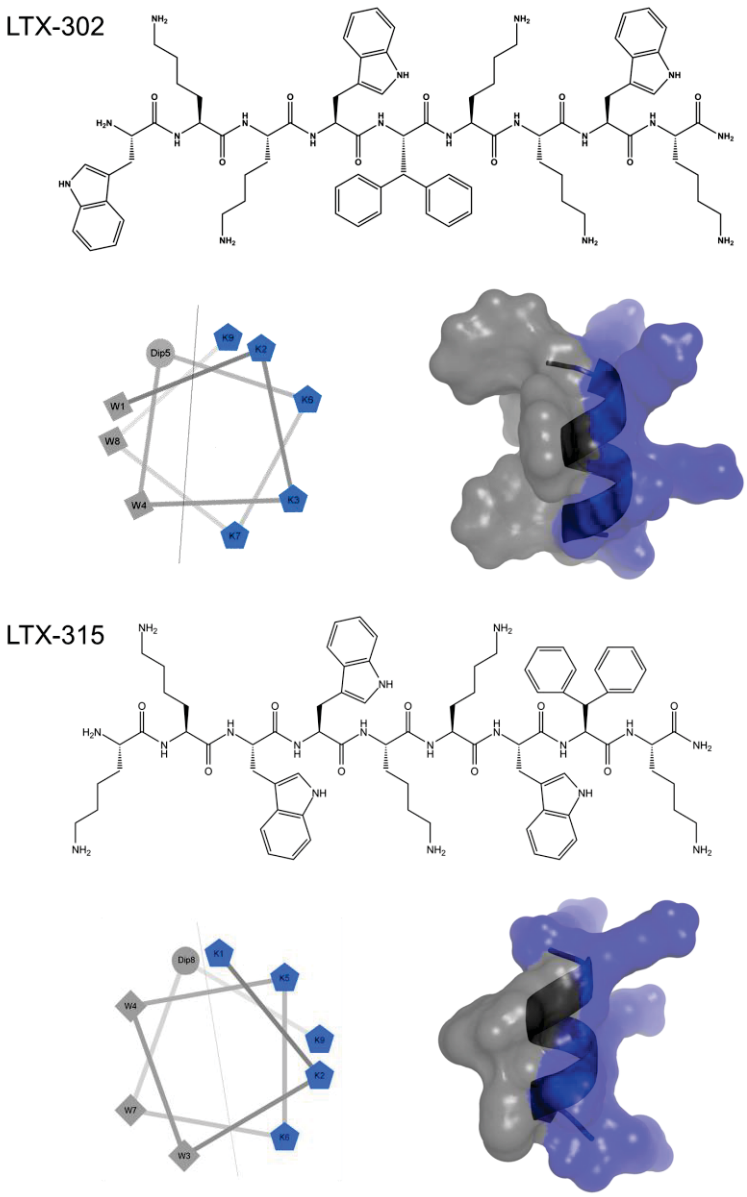

Figure 5: Structural representations of LTX-302 and LTX-315 - Chemical structure of LTX-302 **(top)** and LTX-315 **(bottom)**. Helical wheel representations of LTX-302 and LTX-315, designed as an idealized amphipathic α-helix containing cationic lysine residues (in blue) and lipophilic aromatic residues (in grey). A line separates the cationic and aromatic sector of the peptides. The secondary structure models of LTX-302 and LTX-315 illustrate the surface charge and the amphipathic conformation of the peptides with cationic residues on one side (in blue) and aromatic residues on the opposite side (in grey). Chemical structures were generated using ChemDraw 11. Helical wheels were created using Helical Wheel Projections Version: Id: wheel.pl,v 1.4. Secondary structures were generated using PyMOL 1.3.

3 CAPs AND THEIR INTERACTION WITH CELLULAR MEMBRANES

There are fundamental differences between the membrane composition of malignant cells and normal cells, as the specificity exhibited by some ACPs relies on differences in the lipid composition between cancer cells and non-malignant cells. Similar to bacteria, several cancer cells carry a net negative charge due to a higher than normal expression of anionic molecules such as phosphatidylserine[101, 102], sialic acid on glycoproteins (e.g. mucins)[103, 104] and heparan sulfate on proteoglycans[105-107]. By contrast, untransformed cells have an overall neutral charge because of the zwitterionic nature of their major membrane components, e.g. sphingomyelin and phosphatidylcholine[108]. The overall negative charge of transformed cancer cell membranes provides the potential to electrostatic interactions between cationic ACPs and anionic cell membrane components, thus allowing for the selective killing of cancer cells by ACPs. Additional factors that may contribute to the selective killing of cancer cells by ACPs include membrane fluidity and cell-surface area. Compared to non-malignant cells, cancer cells often have a greater membrane fluidity[109, 110], and cell-surface area (additional microvilli)[37, 111, 112] leading to an improved anticancer activity of ACPs due to an increased membrane destabilization and the ability to bind more ACP molecules. Therefore, the membrane seems to be the key determinant of their action, either as the main target of the peptide action or by forming a barrier that must be crossed by peptides to target intracellular targets[113].

3.1 CAP MECHANISMS OF ACTION

Several ACPs can kill cancer cells through a membranolytic mode of action first displayed by the selective lysis of tumor cells by magainin and its synthetic analogues[53]. This was revealed to be a direct membrane lytic effect and not a receptor-mediated pathway, as illustrated by showing that all D-amino acid analogues of different ACPs had the same cytotoxic effect as the all L-amino acid parental peptides[114, 115]. The mechanisms by which CAPs insert into membranes and promote their biological activity have mainly been studied in bacterial membranes using α-helical peptides, and it is unknown whether the mechanisms of action will be the same for cancer cell membranes. Following the initial binding to the cellular membrane through electrostatic interactions between the positively charged CAP and the negatively charged membrane, peptides must be locally concentrated to exert their anticancer activity. After reaching the threshold concentration, the membranolytic action can initiate[116]. The threshold concentration is described as the minimum peptide concentration necessary at the membrane surface to promote its biological effects[117]. Parameters influencing the threshold concentration include the propensity of peptide assembly, peptide charge, amphipathicity and hydrophobicity, in addition to membrane fluidity and composition[118, 119]. After initial membrane binding of the anticancer peptide and the threshold concentration has been acquired, a conformational transition takes place, in which the conformation of the peptide at the lipid-water interface is rearranged, thereby leading to peptide insertion and membrane permeability.

3.1.1 Membranolytic and non-membranolytic activity

Depending on both the kind of CAP and the peptide concentration, several mechanisms of action for CAPs have been reported. Some CAPs cause stable membrane pores (barrel stave or toroidal pore models), membrane thinning (molecular electroporation or sinking rafts models) or micellization in a detergent-like way (carpet/detergent model)[120, 121]. The molecular mechanism(s) of membrane damage depends on the nature of both peptides and membrane lipids[122, 123]. In the "barrel-stave model"[124], amphipathic peptide helices form a pore in the membrane with the lipophilic side of the peptide facing the hydrophobic core of the membrane bilayer and the hydrophilic portion lining the pore, which is similar to a barrel composed of helical peptides as the staves (Figure 6A). This mechanism of action is primarily displayed by highly hydrophobic peptides[125, 126]. The "barrel-stave model" cannot account for cytolytic activity by CAPs that are <23 amino acids since they are not sufficiently long enough to span the cell membrane[36], thus demanding quite specific peptide properties that only occurs for a few peptides[127]. In the "toroidal pore model"[128], CAPs are inserted into the cellular membrane, inducing a continuous bend in the membrane leaflet through the pore so that the central lumen of the pore is lined by both the inserted peptides and the lipid head groups[116] (Figure 6B). The model can be formed by a much greater variety of peptides compared to the "barrel stave model"[127], and it has been suggested that some CAPs acting by this mechanism of action may cross through the membrane and act on intracellular targets[129]. In the "carpet/detergent model"[130], CAPs will bind to anionic cell membrane components and become aligned in parallel to the cells' surface, thereby creating a carpet-like appearance[131] (Figure 6C). After reaching the threshold concentration of the peptide, the membrane will start to destabilize and collapse due to the curvature stress and internal osmotic pressure, ultimately leading to cellular lysis. In this model the peptides never enter into the hydrophobic core of the cellular membrane[132-134]. As previously mentioned, in addition to the membranolytic mechanisms of action, there are also several non-membranolytic mechanisms of action exerted by CAPs[113, 135, 136], e.g. the "molecular electroporation model"[137], which forms pores in membranes under the influence of an external electric field (Figure 6D), and the "sinking raft model"[138], which produces a mass disproportion that directs the peptide translocation through an increase in membrane curvature due to an imbalance of a mass ratio for the preference of binding to a particular lipid domain (Figure 6E).

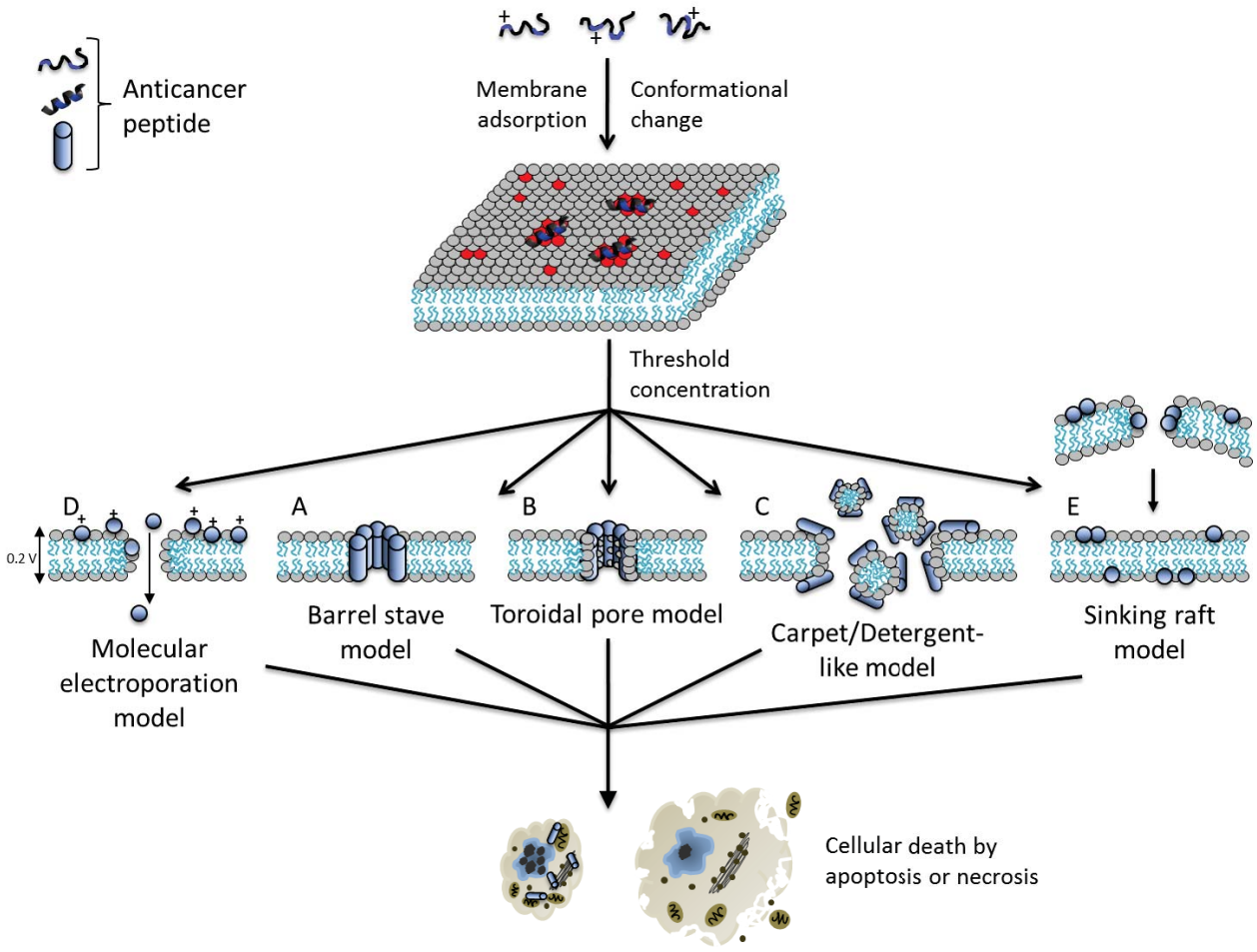

Figure 6: Cationic antimicrobial peptide mechanisms of action - Overview of some of the mechanisms of action exerted by CAPs on cellular membranes after reaching the necessary threshold concentration, as discussed in the literature. Positively charged CAPs interact with negatively charged cell membranes through electrostatic interactions and undergo membrane adsorption and conformational change. Following binding of the peptide to the cell membrane, the peptide can complete its activity through a variation of mechanisms, e.g. the "barrel stave model"[124] **(A)**, the "toroidal pore model"[128] **(B)**, the "carpet model"[130] **(C)**, the "molecular electroporation model"[137] **(D)**, and the "sinking raft model"[138] **(E)**. Depending on the mechanism of action, cells will either die by apoptosis or necrosis. The models were generated using Microsoft PowerPoint 2010.

3.1.2 Intracellular targets

Suggested as an alternative mechanism of action to the membranolytic one, an increased focus has been put towards looking into the possibility of CAPs causing cell death by non-permeabilizing mechanisms or by targeting intracellular components[139]. Several studies have revealed that with certain CAPs, membrane permeabilization or membrane disruption alone is insufficient to cause cellular death. Thus, other complementary- or novel mechanisms of action have been suggested (Table 3). First, the active peptide has to cross the membrane and translocate to the cytoplasm to gain access to intracellular targets. Once in the cytoplasm, peptides usually concentrate and unleash several different processes. Buforin II, a linear CAP, has been shown to alter the septum formation in cytokinesis, inhibit the cell wall, nucleic acid and protein synthesis and suppress essential protein functions[140]. CAPs have also been shown to interact with eukaryotic organelles such as mitochondria, which are important in the immunological resolution of fungal infections and cancer cases[79, 141].

Table 3: Intracellular components targeted by CAPs

Intracellular target/mode of action	References
DNA and cell division	
DNA binding	142-144
DNA repair enzymes	145
Inhibition of nucleic-acid synthesis	146-148
Septum formation	52, 148
Enzymatic activity and protein synthesis	
Ribonucleotide reductase	149
Inhibition of DnaK chaperone	150, 151
Inhibition of protein synthesis	146-148
Cell wall	
Cell wall precursor Lipid II	152, 153
Chitin-binding activity	154
Eukaryotic organelles	
Energetic metabolism impairment (mitochondria)	141
Mitochondria	78, 79, 155
Energetic metabolism failure (autophagic-like cell death)	156

4 ANTICANCER PEPTIDES AS DRUGS

Cancer treatment by conventional chemotherapy is limited by factors such as toxic side effects and the development of multi-drug resistance by cancer cells. Consequently, there is an increasing need for the development of new anticancer therapies with a higher selectivity for neoplastic cells compared to chemotherapy, leading to less cytotoxic side effects during treatment, as well as avoiding the problem of chemoresistance. As previously shown, several ACPs are able to kill cancer cells at concentrations that are harmless to untransformed cells[39, 77, 157], thereby displaying a selectivity not achievable with chemotherapeutic drugs. ACPs are also predicted to be able to kill dormant or slowly growing malignancies due to their membranolytic effect, leading to cell membrane lysis independent of the proliferative status, which unlike chemotherapy normally kills rapidly dividing cells and is unable to kill dormant or slowly proliferating cells[158]. In addition to their direct membrane effects, ACPs have been shown to exert anticancer activities through more indirect approaches such as interfering with tumor-associated angiogenesis[80, 159] or via their ability to modulate the host immune response as both pro- and anti-inflammatory agents[32, 160]. Hence, ACPs may offer several important advantages over chemotherapeutic drugs.

4.1 ANTICANCER PEPTIDES IN DEVELOPMENT

ACPs and their therapeutic potential are intensively investigated, and several promising peptides have been discovered[89]. However, current clinical trials looking into the therapeutic potential of ACPs are scarce[161]. One example is Oncopore™ (also known as LTX-315), a 9-residue lactoferricin-derived lytic peptide designed and developed by Lytix Biopharma AS[162]. Oncopore™ is designed for the local treatment of tumors by intratumoral administration and is currently in a clinical Phase I trial. The peptide induces rapid cancer cell necrosis and anticancer immune responses following treatment. Another example is Talactoferrin Alfa, a recombinant naturally occurring human protein developed by Agennix AG[163], which is designed for oral administration against non-small cell lung cancer and is currently waiting to enter a clinical phase III trial. The protein works by stimulating the body's immune system, maturing and activating dendritic cells (DCs) in the gut-associated lymphoid tissue, thus leading to an enhanced anti-tumor immune response initiated by dendritic-cell mediated immunotherapies[127]. The reason for the few clinical trials on ACPs might be linked to the numerous challenges associated with developing peptides as drugs, such as the cost of peptide synthesis, which is an expensive and time-consuming process, although less expensive than conventional immunotherapies such as the use of monoclonal antibodies and (whole cell) cancer vaccines. Other challenges are the potential proteolytic degradation of peptides *in vivo*, the lack of specificity and the lack of knowledge concerning the toxicology and pharmacokinetics of ACPs[89, 127, 164, 165].

5 THE DANGER SIGNAL THEORY

In 1994, Polly Matzinger published an essay in Annual Reviews Immunology in which she postulated a new theory about the complexity of the immune system. For many years, immunologists held the view that the immune system's primary goal was to discriminate between the self (non-foreign substances) and the non-self (foreign molecules). However, Matzinger discussed the possibility that the immune system does not care about the self and non-self, but more about detection and protection against danger caused by danger signals[166]. Danger signals are molecules or molecular structures released or produced by cells undergoing stress or abnormal cell death, also known as immunogenic cell death. These signals are later processed by resting antigen presenting cells (APCs), primarily DCs, which become activated and produce co-stimulatory signals to initiate immune responses[167]. Therefore, danger signals can be seen as immunomodulatory molecules usually divided into two large subclasses, endogenous danger signals that are produced by the organism itself (also known as Damage/Danger-Associated Molecular Pattern molecules (DAMPs) or alarmins) and exogenous danger signals, which are produced by different organisms (also known as Pathogen-Associated Molecular Pattern molecules (PAMPs)). DAMPs can initiate and preserve immune responses in the noninfectious inflammatory response, while PAMPs can initiate and preserve the infectious inflammatory response[168]. The work in this thesis focuses on DAMPs, thus the following sections are meant to give an introduction into this field.

5.1 DAMPs

DAMPs usually apply their mechanism of action through the maturation and stimulation of DCs, leading to an augmentation of responses from $CD8^+$ T cells. They are structurally diverse endogenous molecules passively released from necrotic cells upon infection or tissue injury or secreted by stimulated leukocytes and epithelia (Figure 7). Once released extracellularly, DAMPs promote their activity by binding to pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs), and their effects can be either beneficial or detrimental based on several factors such as timing of the release, dose and context. DAMPs employ detrimental effects when the presence is excessive and chronic, which leads to injury, while when expressed in a transient and self-limited manner such as during injury and acute inflammation, they mediate repair[169, 170]. Examples include heat shock proteins (HSPs) such as HSP70, grp96 and HSP90, which have been shown to have immunogenic potential such as DC maturation *in vitro*[171, 172] as well as effects on DCs *in vivo*[173]. Even so, it is not clear whether HSPs truly contain endogenous adjuvant activity or if they are due to LPS contamination[174, 175]. Other DAMPs are IL-1β, induced in keratinocytes by inflammatory stimuli[176]; hyaluronic acid which has been shown to stimulate DCs[177]; nucleotides such as ATP and UTP, which are also able to activate DCs[178, 179]; uric acid, which is capable of maturing DCs and inducing the expression of co-stimulatory molecules[180], S100 proteins (e.g. S100A8 and S100A9), which when released during the activation of phagocytes lead to the production of cytokines such as tumor necrosis factor-alpha (TNF-α)[181] and High Mobility Group Box protein 1 (HMGB-1), which is able to induce immune activity such as cytokine stimulation[182].

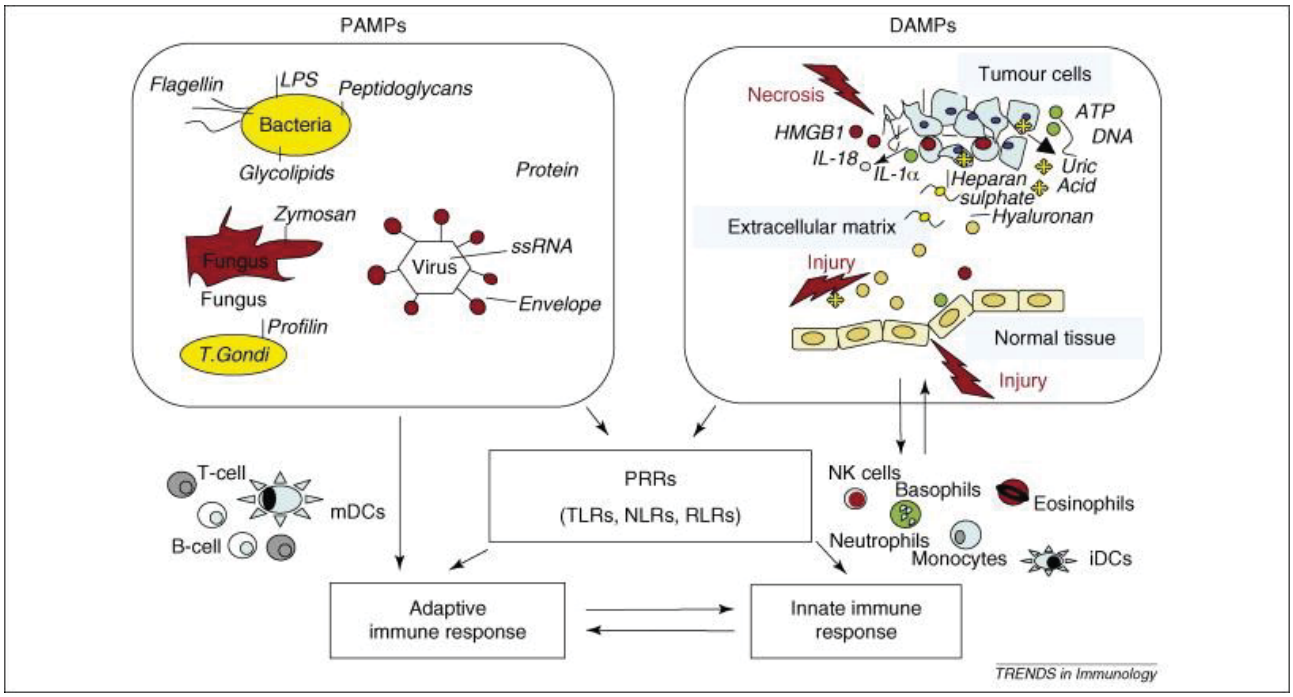

Figure 7: PAMPs and DAMPs that initiate innate and adaptive immune responses - Outline of events and molecules that can induce immune responses due to the stimulation by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Proteins such as HMGB-1 or non-proteins such as ATP and uric acid normally reside inside cells, but upon cellular stress or damage (e.g. necrosis) are released outside the cell. DAMP expression is usually elevated in neoplastic tissues compared to normal tissues. Injury to normal tissue or extracellular matrix can release DAMPs such as heparin sulfate or hyaluronan. The receptors that recognize DAMPs are often pattern recognition receptors (PRRs). LPS, lipopolysaccharide; ssRNA, single strand RNA; T. gondii, Trypanasoma gondii (protozoan parasite); HMGB-1, high mobility group box 1 protein; PRRs, pattern recognition receptors; TLRs, Toll-like receptors; NLRs, NOD1-like receptors; RLRs, RIG-I-like receptors. Illustration used with permission, copyright 2007 by Elsevier Limited[168].

5.1.1 HMGB-1

HMGB-1 is a nuclear non-histone chromatin-binding protein comprised of two DNA-binding domains, which regulates chromatin structure and transcription through altering the conformation of DNA[183-185]. HMGB-1 occurs as a nuclear component, but also in the cytoplasm of nucleated cells and it is prominently expressed in platelets[186]. Once released extracellularly, HMGB-1 is able to induce powerful immune responses such as cytokine stimulation[182]. The translocation of HMGB-1 from intracellular to extracellular can occur in macrophages and DCs by active secretion, and often involves post-translational modification[187, 188], or through passive release following cell death such as necrosis[99, 189]. Necrosis, whether prompted by physical or chemical trauma, is considered to be pro-inflammatory due to the release of DAMPs such as HMGB-1, while HMGB-1 often remains intracellular during apoptosis (unless secondary necrosis occurs), thus differentiating between immunogenic and non-immunogenic cell death, respectively. The immune activity of HMGB-1 may not depend entirely on the location of HMGB-1 (outside versus inside the cell), but also on the redox state of the protein. Reduced HMGB-1 (released from necrotic cells) induces inflammation, while HMGB-1 released from apoptotic cells is oxidized by reactive oxygen species and does not induce an immune response[189-194] (Figure 8). After being released extracellularly, HMGB-1 can stimulate immune responses via receptors such as Receptor for

Advanced Glycation End products (RAGE), TLR2, TLR4, TLR9 or via cytokines. Studies have shown that HMGB-1 is involved in the activation of macrophages and DCs[195, 196] and burst neutrophil recruitment[197]. Nonetheless, much is still unknown about how HMGB-1 exerts its activity. However, it is thought that the protein frequently (or always) acts in concert with another molecule, e.g. DNA, LPS, IL1β, and IFN-γ, to induce inflammation[191, 194, 198, 199].

5.2 IMMUNOGENIC CELL DEATH

In recent years, a new concept of immunogenic cell death (ICD) has emerged. Initiated by DAMPs, ICD has the potency to stimulate an immune response against antigens derived from dead cells, particularly cancer cells[200]. Depending on the therapy given, cancer cell death can be immunogenic or non-immunogenic. Moreover, cancer therapies capable of inducing ICD can lead to a tumor-specific immune response, ultimately having a role in determining the efficacy of the specific anticancer therapy[201]. ICD stimulates an increase in tumor infiltrating lymphocytes and may even change the ratio between cytotoxic $CD8^+$ T lymphocytes (CTLs) over $FOXP3^+$ regulatory T cells (Tregs) within the tumor parenchyma[202-204]. This means that by inducing cancer ICD through usage of the appropriate anticancer therapy, the patient's own dying cancer cells can operate as an *in situ* vaccine capable of stimulating tumor-specific immune responses, which in turn can control (and sometimes even eradicate) residual cancer (stem) cells[201, 205-207] (Figure 9).

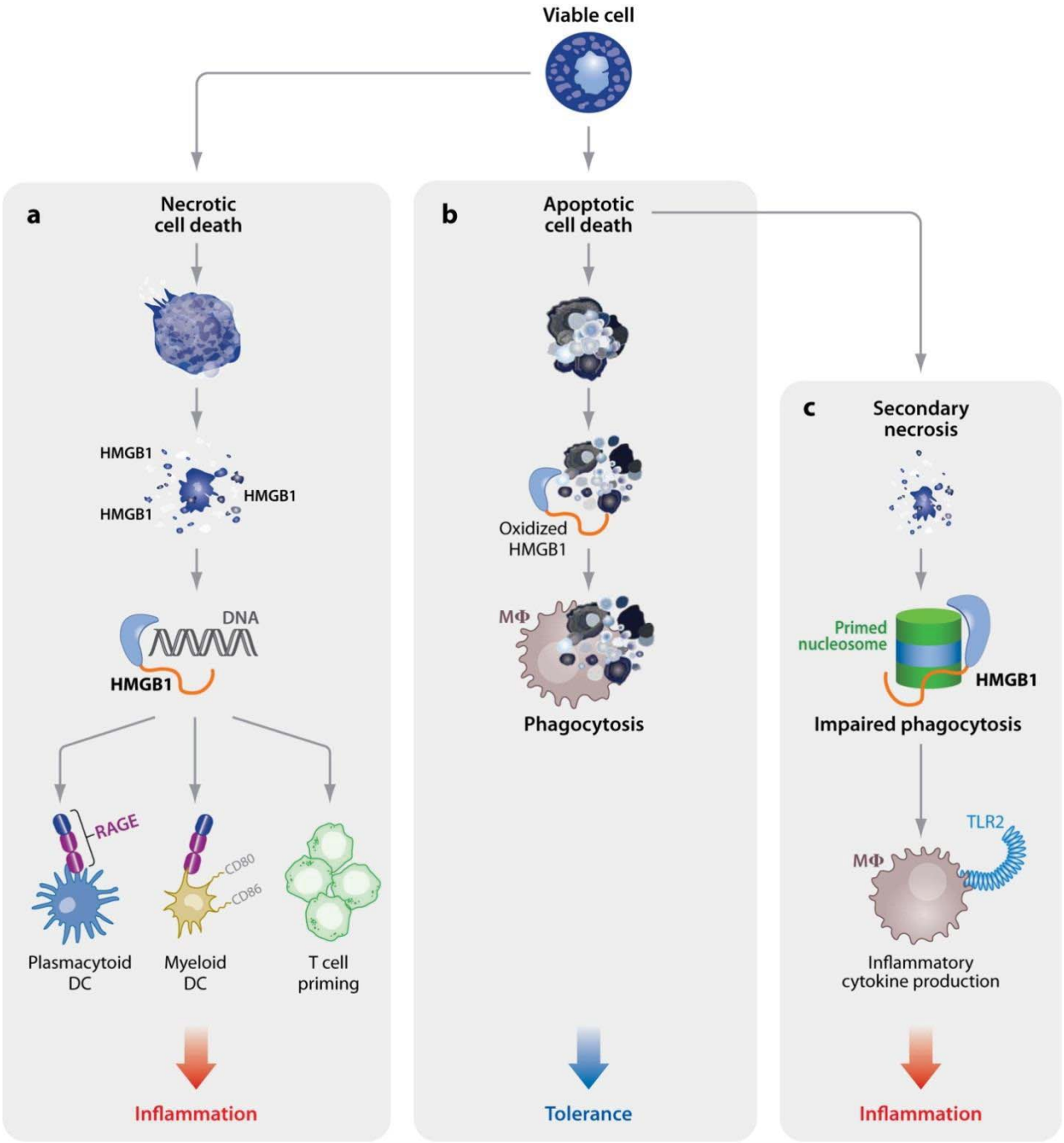

Figure 8: HMGB-1 in immunogenic versus non-immunogenic cell death - How cells die may determine the subsequent effects on the immune system. Cell death through necrosis is highly proinflammatory **(a)**, while cell death by apoptosis often is tolerogenic **(b)**. In cases where apoptotic cells are not recognized and removed by phagocytosis, secondary necrosis will occur, thereby leading to inflammation **(c)**. Necrotic cell death will lead to the release of DAMPs such as HMGB-1, which in association with other molecules (DNA or immune complexes) can activate plasmacytoid DCs, myeloid DCs and macrophages, leading to a T cell priming and expansion of effector T cells **(a)**. In apoptotic cell death, HMGB-1 will be oxidized by reactive oxygen species and deliver tolerogenic signals to dampen immune activation **(b)**. During secondary necrosis, HMGB-1 can form complexes with released nucleosomes which can induce inflammatory cytokine production from macrophages, thus giving an alternative route to an immune response **(c)**. Illustration used with permission, copyright 2010 by Annual Reviews Inc.[191].

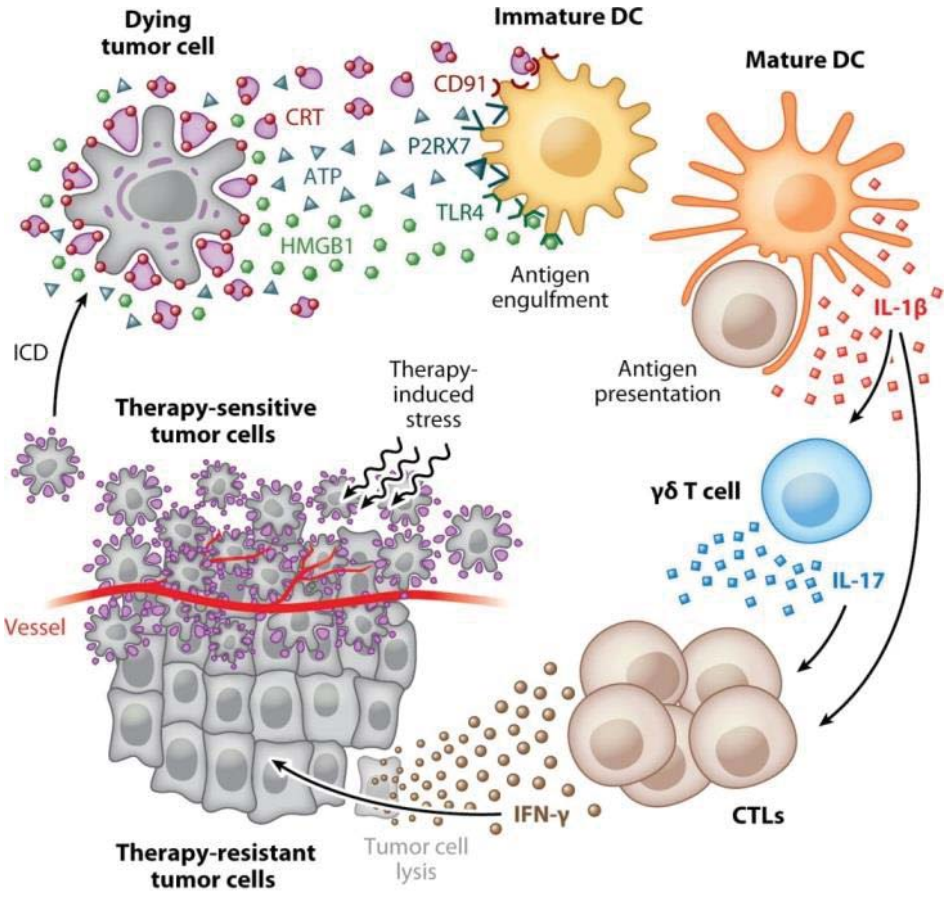

Figure 9: Properties of immunogenic cell death (ICD) - Abnormal cellular stress or death may lead to a cascade of events inducing ICD. As a result of endoplasmic reticulum stress and autophagy, CRT is exposed on the outer leaflet of the plasma membrane of cells undergoing ICD. ATP is secreted during apoptosis; in addition, cells undergoing ICD release HMGB-1 as the cellular membrane becomes permeabilized during secondary necrosis. CRT, ATP and HMGB-1 bind to receptors on immature DCs, initiating the maturation and recruitment of DCs into the tumor bed (stimulated by ATP), the engulfment of tumor antigens by DCs (stimulated by CRT), and optimal antigen presentation to T cells (stimulated by HMGB-1). Altogether, these processes result in a potent immune response which can lead to the eradication of chemotherapy-resistant tumor cells. ATP, adenosine triphosphate; CRT, calreticulin; CTL, cytotoxic $CD8^+$ T lymphocyte; DC, dendritic cell; HMGB-1, high mobility group box 1; IFN, interferon; IL, interleukin; TLR, Toll-like receptor. Illustration used with permission, copyright 2013 by Annual Reviews[207].

6 CANCER VACCINES

Advances in immunotherapy and a need for new and improved cancer therapies have set the stage for cancer vaccine products[208]. By enlisting the patient's own immune system, primarily through the activation of T cells, one can induce a specific immune response that recognizes and destroys tumors. This principle was demonstrated as early as in the 1890s when Dr. William Coley administered bacterial toxins (Coley's toxins) into patients with inoperable tumors, which induced tumor regression and laid the foundation for cancer immunotherapy[209, 210]. A cancer vaccine strategy should consider several critical factors such as selecting immunogenic, tumor-specific antigen targets, which platform to use for antigen delivery and augmenting the immunostimulatory setting in which the vaccine is delivered through an adjuvant[211]. The cancer vaccine antigen source and platform for delivery varies, and several strategies have been previously implemented. Some examples include peptide/small epitope vaccines delivered together with an adjuvant, plasmid DNA vaccines (similar to peptide vaccines), recombinant viral or bacterial vaccines and cellular vaccine approaches that use whole cells or cell lysates as the source of antigen or as the delivery platform[212-214].

6.1 WHOLE CELL CANCER VACCINES

Whole tumor cell vaccines can either be autologous (from the same organism) or allogeneic (from donor(s) within the same species, so genetically different). Autologous or allogeneic whole tumor cell vaccines include tumor cells either transduced with potential immune stimulating genes such as interleukin 2 (IL-2), granolucyte macrophage colony stimulating factor (GM-CSF) or interferon gamma (IFN-γ)[215, 216], or through the injection of irradiated whole tumors cells or tumor cell lysate (TCL). Although autologous tumor cell-based vaccine strategies are linked to some drawbacks, e.g. the limited access to clinical tumor cell line samples, the low number of tumor cells for vaccination, the release of immune-suppressive molecules by tumor cells and the cost and time necessary to establish a vaccine strategy, several tumor vaccines are already under clinical development[217]. An advantage to the whole tumor cell approach is that the entire tumor cell is the source of immunogens expressing all the tumor-associated antigens (TAAs) necessary to induce an antitumor immune response, and the first US Food and Drug Administration approval for a therapeutic cancer vaccine was recently granted. Sipuleucel-T (Provenge; Dendreon, Inc.) is an autologous peptide pulsed dendritic cell vaccine against advanced castrate-resistant prostate cancer. Other whole cell vaccines in Phase III testing are Melacine (Corixa Corp.), an allogeneic tumor cell lysate vaccine consisting of two melanoma cell lines delivered with Detox adjuvant against Stage IV melanoma (approved in Canada and awaiting approval in the USA)[218, 219] and OncoVax (Vaccinogen), an autologous cancer vaccine against Stage II colon cancer administered with bacillus Calmette-Guérin (BCG) adjuvant, which has now been authorized for commercial use in Switzerland and The Netherlands[211, 220, 221]. TCL as the antigen source in a cancer vaccine setting is a promising alternative to the use of individual TAAs (e.g. tumor antigen derived peptides)[222]. Clinical studies have demonstrated that autologous and allogeneic whole tumor cells (undefined TAAs) gave significantly better results on objective clinical responses compared to molecularly defined synthetic antigens (peptide antigens)[223]. Moreover, TCL will allow for presentation of

antigens to both CTLs and $CD4^+$ T helper cells, hence generating a strong overall anti-tumor immune response and a long-term $CD8^+$ T cell memory via $CD4^+$ T cell help. Thus, TCLs prepared from autologous or allogeneic tumor cells contain an extensive repertoire of TAAs. Clinical studies have however shown disappointing results with whole cell vaccines that have been administered without adjuvants[224].

6.2 CANCER VACCINES AND THE NEED FOR NEW ADJUVANTS

Current therapeutic cancer vaccines in clinical trials are often poorly immunogenic, exhibiting a lack of efficacy sometimes combined with safety concerns (high toxicity). Thus, there is a demand for safe novel adjuvants to increase the immunogenicity of cancer vaccine antigens. Existing adjuvants are either defined as antigen delivery systems or immune potentiators, hence acting as delivery systems that present antigens for extended periods of time or acting as danger signals with a direct effect on immune cells, respectively[221, 225]. The limited success of therapeutic cancer vaccines is related to the inability of present adjuvants to induce potent T cell immune responses of a T_H1 type, which is particularly important in providing protective immunity[226]. Most currently licensed adjuvants such as alum[227] and water-in-oil emulsions (e.g. incomplete Freund´s adjuvant)[228], stimulate T_H2 responses, making them unsuitable for a large number of applications requiring CTL-mediated immune responses, including cancer[221]. Consequently, there is a demand for strong and safe novel adjuvants that induce tumor-specific immune responses of a T_H1 type. Immunogenic cell death is defined by the exposure of several intracellular factors including DAMPs[207]. DAMPs released from stressed or dying cells are often considered natural endogenous adjuvants due to their ability to induce immune responses[175]. DAMPs can initiate and preserve immune responses in the noninfectious inflammatory response[168], thus giving them a potential to be used in an adjuvant setting.

6.3 LTX-315 AS A NOVEL ADJUVANT

Adjuvants (from the Latin word "adjuvare", to help), are molecules capable of augmenting the immune response to an antigen. DAMPs released from stressed or dying cells are often considered natural endogenous adjuvants[175]. To induce immune responses and T cell activation; two signals are required from an APC. One of the signals required is an antigen, presented by the major histocompatibility complex (MHC) Class I (found on all nucleated cells) or Class II (found on APCs) to the T cell antigen receptor (TCR) on T cells. However, the antigen signal alone will only induce tolerance and not an immune response. The second signal required is an antigen-independent signal, which binds to receptors on APCs such as the TLRs, prompting APC maturity. The mature APC can then express co-stimulatory molecules and stimulate T cell responses through the binding of CD80/CD86 on APCs to CD28 on T cells[175, 229]. LTX-315 is a membranolytic peptide shown to induce rapid cellular death through necrosis, both *in vitro* and *in vivo* (Paper II). This means that the peptide may have a potential as a novel adjuvant when used locally due to the cellular lysis imposed and the following release of DAMPs. It is unknown whether LTX-315 has a direct adjuvant effect on its own and/or if it is just a precursor to cellular necrosis and the extracellular release of DAMPs (indirect effect), which are needed to induce an immune response.

AIMS OF THE THESIS

The overall aim of this thesis was to elucidate the antitumor activity and mechanism of action of two different lytic anticancer nonapeptide analogs, LTX-302 and LTX-315, by studying the *in vitro* and *in vivo* effects against a murine A20 B cell lymphoma and B16F1 melanoma model, respectively.

The specific aims of this thesis were:

- To evaluate the therapeutic potential of LTX-302 in a syngeneic B cell lymphoma mouse model;
- To investigate the therapeutic potential of LTX-315 in a highly aggressive and low immunogenic syngeneic melanoma mouse model;
- To investigate the anticancer mode of action for LTX-315 following intratumoral administration;
- To evaluate the potential of LTX-315 as a novel adjuvant in combination with a tumor cell lysate vaccine.

SUMMARY OF PAPERS

Paper I

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

In Paper I we investigated the anticancer properties and therapeutic potential of LTX-302, a short lytic anticancer peptide, against syngeneic B cell lymphomas following intratumoral administration. LTX-302 is a membranolytic peptide designed to adopt an α-helical coil structure subsequent of interaction with cancer cell membranes. LTX-302 was shown to rapidly kill A20 B lymphoma cells through membrane-induced lysis *in vitro* and to incite immunogenic cell death *in vivo*. Intratumoral administration resulted in tumor necrosis and infiltration of inflammatory cells into the tumor parenchyma followed by a complete regression of the tumors in the majority of the animals. Moreover, animals achieving LTX-302-induced complete tumor regressions were protected against a re-challenge with live A20 cells, but not against Meth A sarcoma cells. The tumor resistance could be adoptively transferred via spleen cells from LTX-302-treated mice, and the resistance was abrogated by depletion of T lymphocytes. The study shows the therapeutic potential of LTX-302 and its ability to induce a long-term, specific cellular immunity against A20 lymphomas following local treatment. Thus, LTX-302 created an important foundation for the further design and optimization of short lytic anticancer peptides.

Paper II

Title: Complete Regression and Protective Immune Responses obtained in B16 Melanomas after Treatment with LTX-315

In Paper II we studied the anticancer properties and therapeutic potential of LTX-315, a new lead compound, against highly aggressive and low immunogenic B16F1 melanomas in syngeneic mice. Although less selective than LTX-302, LTX-315 (also an α-helical coil) is a more potent membranolytic anticancer peptide due to its slightly larger aromatic/lipophilic sector. LTX-315 induced rapid necrosis of both murine and human melanoma cell lines, as well as the extracellular release of Danger-Associated Molecular Pattern molecules such as the High Mobility Group Box-1 protein *in vitro* and immunogenic cell death *in vivo*. Intratumoral administration of LTX-315 resulted in tumor necrosis and the infiltration of T lymphocytes into the tumor parenchyma followed by complete regression of the tumor in the majority of the animals. Peptide treatment mounted inflammatory responses, demonstrated by the up-regulation of proinflammatory cytokines such as interleukin (IL) 1β, IL6 and IL18 *in vivo*. Finally, animals cured by LTX-315 treatment were protected against a re-challenge with live B16 tumor cells, indicating a protective immune response against the cancer. Consequently, LTX-315 is a very potent anticancer peptide capable of eradicating highly aggressive and notoriously difficult to treat B16 melanomas, demonstrating the peptide's potential to be used as a novel immunotherapeutic agent.

Paper III

Title: Long-term Protection against B16 Melanomas upon Vaccination with Tumor Cell Lysate in Combination with LTX-315 as a Novel Adjuvant

Paper III examined the adjuvant potential of LTX-315 in combination with a B16F1 tumor cell lysate against B16 melanomas. The membranolytic mechanism of action of LTX-315 and its ability to induce immunogenic cell death led us to believe that LTX-315 may have potent immune stimulating properties through the release of Danger-Associated Molecular Pattern molecules. Intradermal injections of LTX-315 induced inflammation in the skin, observed as a massive infiltration of $CD45^+$ immune cells at the injection site. Animals immunized with tumor cell lysate in combination with LTX-315 demonstrated a long-term immune protection against repeated challenges with live B16F1 tumor cells, as well as an improved disease-free survival compared to controls. Thus, LTX-315 may represent a next generation novel adjuvant that may be combined with current (cancer) vaccines.

RESULTS AND DISCUSSION

THERAPEUTIC POTENTIAL OF SHORT SYNTHETIC LTX ANTICANCER PEPTIDES (LTX-ACPs) IN LOCAL TREATMENT OF TUMORS (PAPER I AND PAPER II)

In Papers I and II, the anticancer effect and mode of action for two synthetic anticancer peptides (ACPs) was investigated. Specifically, Paper I aimed at investigating the anticancer effect, mode of action and indirect immunomodulatory properties of LTX-302 against syngeneic murine A20 B cell lymphomas, whereas Paper II aimed at investigating the anticancer effect, mode of action and indirect immunomodulatory properties of a more potent peptide, LTX-315, against highly aggressive and low immunogenic syngeneic murine B16 melanomas.

LTX-ACPs can induce the release of Damage-Associated Molecular Pattern molecules (DAMPs) *in vitro*

Naturally occurring ACPs have been shown to exhibit anticancer effects through the lysis of cancer cells at concentrations not harmful to untransformed cells[47, 48, 51, 56, 76, 77] through interactions with the cellular membrane[53, 55, 59, 69, 71, 78, 79, 113] or intracellular targets[139, 140] such as mitochondria[79, 141]. Following membrane destabilization and disintegration the intracellular content will be released, thus discharging Damage-Associated Molecular Pattern molecules (DAMPs), e.g. High Mobility Group Box-1 protein (HMGB-1), into the extracellular environment. HMGB-1 has been shown to stimulate and mature dendritic cells (DCs), leading to an activation of the immune system and the consequential augmentation of responses from $CD8^+$ T cells[195-197].

LTX-302 and LTX-315 have displayed cytotoxic activity against both human and murine cancer cell lines, with a selectivity of approximately eightfold and twofold, respectively, compared to non-malignant human cells. The selective cytotoxicity against cancer cell compared to non-malignant cells is believed to be due to a more negatively charged cell surface on cancer cells, thereby helping to facilitate electrostatic interactions between the cationic peptide and the negatively charged cancer cell membrane. Several cancer cells carry a net negative charge due to a higher than normal expression of anionic molecules such as phosphatidylserine[101, 102], sialic acid on glycoproteins (e.g. mucins)[103, 104] and heparan sulfate on proteoglycans[105-107]. By contrast, untransformed cells have an overall neutral charge because of the zwitterionic nature of their major membrane components, e.g. sphingomyelin and phosphatidylcholine[108]. Additional factors that may contribute to the selective killing of cancer cells by ACPs include membrane fluidity[109, 110] and cell-surface area[37, 111, 112]. Since LTX-302 and LTX-315 were designed for the local treatment of solid tumors, the anticancer selectivity is less important compared to drugs designed for systemic use, while neither peptide displayed IC_{50} hemolytic activity against human red blood cells within the concentration range tested. Red blood cell membranes are neutral due to their high content of phospholipids such as sphingomyelin and phosphatidylcholine[230], demonstrating that both peptides are membrane specific. In addition, LTX-302 and LTX-315 induced the release of HMGB-1 *in vitro* from A20 B

cell lymphoma and B16F1 melanoma cells, respectively. An acute release of HMGB-1 and other inflammatory molecules can promote innate and adaptive anti-tumor immune responses[184, 191], along with subsequent tumor regression[100]. HMGB-1 released by dying tumor cells is mandatory for licensing host DCs to process and present tumor antigens[231], and is related to clinical outcome after chemoradiation[232]. Together with calreticulin (CRT) and adenosine triphosphate (ATP), HMGB-1 is also linked to immunogenic cell death (ICD), and is even a prerequisite for ICD[207, 233]. Therefore, LTX-302 and LTX-315 may induce the ICD of cancer cells (and normal cells) through the release of DAMPs such as HMGB-1, as demonstrated by the translocation of HMGB-1 from the intracellular to the extracellular compartment following peptide treatment *in vitro*. However, this should be further studied by investigating the release of CRT and ATP from cancer cells *in vitro*, as well as exploring the role of DAMPs *in vivo*.

Local treatment with LTX-ACPs induces tumor necrosis followed by inflammation and tumor regression

Many studies have demonstrated *in vivo* anticancer effects with naturally occurring ACPs following systemic or local administration[48, 49, 57, 58, 62, 76], as well as pro-apoptotic targeting ACPs[96, 234-237]. In some cases, treatment with ACPs was also able to inhibit tumor metastasis[80], hence indicating an involvement of the immune system in addition to the direct membrane effect exhibited by ACPs. However, the mechanism behind the tumor regression induced by ACPs is largely unknown and the effect is sometimes temporary, with the cessation of peptide treatment causing tumor relapse and growth in syngeneic models[237]. Our group has previously shown that the intratumoral (i.t.) injection of bovine lactoferricin is able to induce tumor necrosis and regression in syngeneic mouse models[76], in addition to inhibiting tumor xenograft growth in nude rats[78].

Histological examinations revealed that LTX-302 induced necrosis and inflammation in A20 lymphomas established in syngeneic mice. LTX-302 acts through a membranolytic mechanism of action, as illustrated by electron microscopy. The electron micrographs demonstrated that LTX-302 interacts with the cellular membrane of A20 cancer cells and kills them through peptide-induced lysis. The same was observed for LTX-315 against B16 melanomas, as shown by an extensive hemorrhagic necrosis of the tumor tissue followed by inflammation. The inflammatory response was further validated by the up-regulation of pro-inflammatory cytokines such as IL1β, IL6 and IL18 in the tumor tissue following injection with LTX-315. Similar to LTX-302, LTX-315 has been shown to act through a membranolytic mode of action, which is illustrated by both electron microscopy and confocal microscopy (data not shown). Cellular lysis, or necrosis, is an immunogenic form of cell death since it induces the release of DAMPs such as HMGB-1[191]. Together with other DAMPs, HMGB-1 will stimulate an inflammatory response and a subsequent activation of the innate and possibly adaptive immune system[168, 207]. Thus, the injection of short synthetic ACPs such as LTX-302 and LTX-315 appears to induce rapid tumor necrosis by a direct lytic effect on the cellular membrane of cancer cells. The induced cellular lysis leads to the release of DAMPs, such as HMGB-1, stimulating an inflammatory response and activation of the immune

system, thereby creating a synergistic effect between the direct killing mechanism of ACPs and the organism's own immune system.

The inflammatory response following intratumoral treatment with LTX-ACPs stimulates the infiltration of immune cells into the tumor parenchyma

As a result of tumor cells necrosis, the release of DAMPs and the induction of ICD, local treatment with LTX-302 and LTX-315 all help to stimulate an immune response and the consequential infiltration of immune cells into the tumor parenchyma. A histological examination demonstrated that both peptides induced the infiltration of immune cells 24 hours post-treatment, with an augmentation in the number of infiltrating cells 5 days post-treatment for LTX-315. The immunolabeling of B16 tumors with anti-CD3 revealed that the majority of the infiltrating cells were $CD3^+$ T cells, and a similar response has been seen with the i.t. administration of oncolytic viruses. Following injection of the virus, tumor-specific immune responses against the cancer have been observed. Similar to LTX-302 and LTX-315, oncolytic viruses are designed to directly lyse tumor cells, which then lead to the release of pro-inflammatory cytokines that activates innate and adaptive immune responses against the tumor[238]. Studies have shown that treatment with oncolytic viruses induced the release of HMGB-1 and other inflammatory signals from cancer cells infected with the virus[239]. Furthermore, several clinical studies using oncolytic viruses have demonstrated that virus-mediated destruction or the damage of tumors can lead to an antitumor immune response and the infiltration of T cells[240-242], which is also observed in intralesional clinical models[243]. Thus, oncolytic virus treatment exerts antitumor effects through multiple mechanisms, including direct cancer cell death (necrosis or apoptosis), the release of HMGB-1 and inflammatory cytokines, and last the induction of innate immunity and anti-tumor T cell responses[244]. Local treatment with ACPs may induce a similar response and inflammation due to rapid cancer cell lysis and the subsequent release of DAMPs and tumor-associated antigens (TAAs). Tumor necrosis and the release of endogenous danger signals will induce the inflammation and maturation of DCs, hence leading to TAA availability and presentation[191, 245]. Immunogenic cell death may be crucial for the success of a therapy, as it opens up for the possibility of anticancer immune responses to control the neoplastic disease by eliminating residual cancer cells or maintaining micrometastases in a stage of dormancy, thereby creating the necessary synergic interaction between immune cells and dying/dead cancer cells[233].

The quantity of tumor-infiltrating lymphocytes (TILs) with cytotoxic and memory phenotypes has been shown to be related to the clinical outcome of cancer[246]. A strong lymphocytic infiltration is associated with a good clinical outcome and has been demonstrated in several different tumors, including colorectal cancers[247], melanoma[248], breast cancer[249], ovarian cancer[250] and lung carcinoma[251]. This has been further validated by the fact that immune deficiencies are associated with the growth and aggressiveness of tumors in mouse models[252, 253] and by the higher incidences of cancer in immunosuppressed humans after transplantation[254]. Although solid tumors are commonly infiltrated by immune cells (e.g. T and B lymphocytes, natural killer cells, DCs, macrophages, neutrophils, eosinophils and mast cells), in contrast to immune cells responsible for

chronic inflammation and a pro-tumorigenic environment, the presence of immune cells linked to acute inflammation such as a high numbers of lymphocytes, particularly T cells, is linked to a good prognosis in many cancers with a longer disease-free and/or overall survival[246, 255]. This is further demonstrated by the fact that certain chemotherapeutic drugs known to induce ICD (e.g. anthracyclines and oxaliplatin) exhibits a better therapeutic outcome and show an increase in TILs compared to others that may induce a "silent" non-immunogenic cell death[207]. Thus, ACP-induced inflammation, as seen with LTX-302 and LTX-315, and the subsequent infiltration of TILs into the tumor parenchyma, may account for their therapeutic potential and the demonstrated increase in disease-free survival observed in syngeneic models.

LTX-ACPs and their potential as *in situ* vaccination agents

Although initial cancer therapies might be successful in eradicating the bulk of the tumor, recurrence of the cancer is always a risk. Hence, the success of an anticancer therapy is limited as long as it cannot prevent a reappearance of the cancer. To prevent a relapse it is important to activate the patient's own immune system, primarily through the activation of T cells, leading to an immune response that recognizes and destroys any potential residual cancer. Cancer therapies able to incite ICD through the release of DAMPs typically have a better therapeutic outcome compared to therapies that do not incite ICD[207]. Additionally, a successful therapy should be able to overcome cell-autonomous challenges such as the evolution of therapy-resistant tumor cells (from the selection pressure imposed by the therapy)[256]. ICD and the subsequent activation and augmentation of cytotoxic T cells specific to the tumor cells will prompt a long-term immune protection against the cancer. ACPs typically act through a membranolytic mechanism of action, which will secure ICD through the release of DAMPs. Therefore, the local treatment of solid tumors with ACPs may have a potential as a novel therapy against cancer due to their dual ability to both kill cancer cells directly, but also to stimulate immune responses from the release of DAMPs.

LTX-302[93] and LTX-315 (Paper II) have both been shown to induce ICD, as defined by Kroemer et al[207]. We have demonstrated a long-term protective immune response (*in situ* vaccination) against several different tumors upon re-challenge with live cancer cells. In contrast, recent studies using pro-apoptotic anticancer peptides delivered either intravenously[234-237] or through i.t.[96, 237] did not exhibit a similar immune protection after treatment. These peptides induce apoptotic cell death in tumors primarily by the disruption of mitochondrial membranes, whereas in some cases a cessation of peptide treatment led to rapid tumor growth and death in the animals[237], indicating that ICD was not induced and that ICD is crucial to achieving a long-term immune protection against cancer. LTX-302 induced complete regression and subsequent specific immune protection against A20 lymphomas in immune-competent mice (as demonstrated by re-challenge) while inducing a significant growth inhibition, but not a long-lasting complete regression in immune-deficient mice, thus demonstrating that an intact immune system is critical for the antitumor response *in vivo*. Moreover, the immune response was shown to be transferrable by adoptive transfer of spleen cells from LTX-302-cured animals to naïve animals. The protection was T cell-dependent, as shown by depletion of T lymphocytes ($CD4^+$ and $CD8^+$)[93]. Taken together, these observations strongly

suggest that LTX-302-induced protection against tumor regrowth reflects a specific, cell-mediated secondary immune response and that both $CD4^+$ and $CD8^+$ T cells are crucially involved. LTX-315 has induced complete regression and subsequent immune protection in several different intradermally established tumors such as A20 lymphomas, CT26WT colon carcinomas and highly aggressive and low immunogenic B16 melanomas (data not published). Immune protection was demonstrated by a re-challenge with live tumor cells. Since LTX-302 and LTX-315 are closely related peptide analogs, it is plausible to believe that the immune protection induced by LTX-315 is also specific and T cell-dependent, although this must be validated by future studies. Thus, i.t. treatment with synthetic ACPs such as LTX-302 and LTX-315 might present a novel *in situ* cancer vaccination strategy by mediating local tumor control via direct tumor cell lysis and subsequent protection against recurrence and metastasis by inducing tumor specific immunity. Figure 10 is a hypothetical model based on Kroemer et al.'s model for ICD[207] for what occurs *in vivo* following i.t. administration with LTX-peptides.

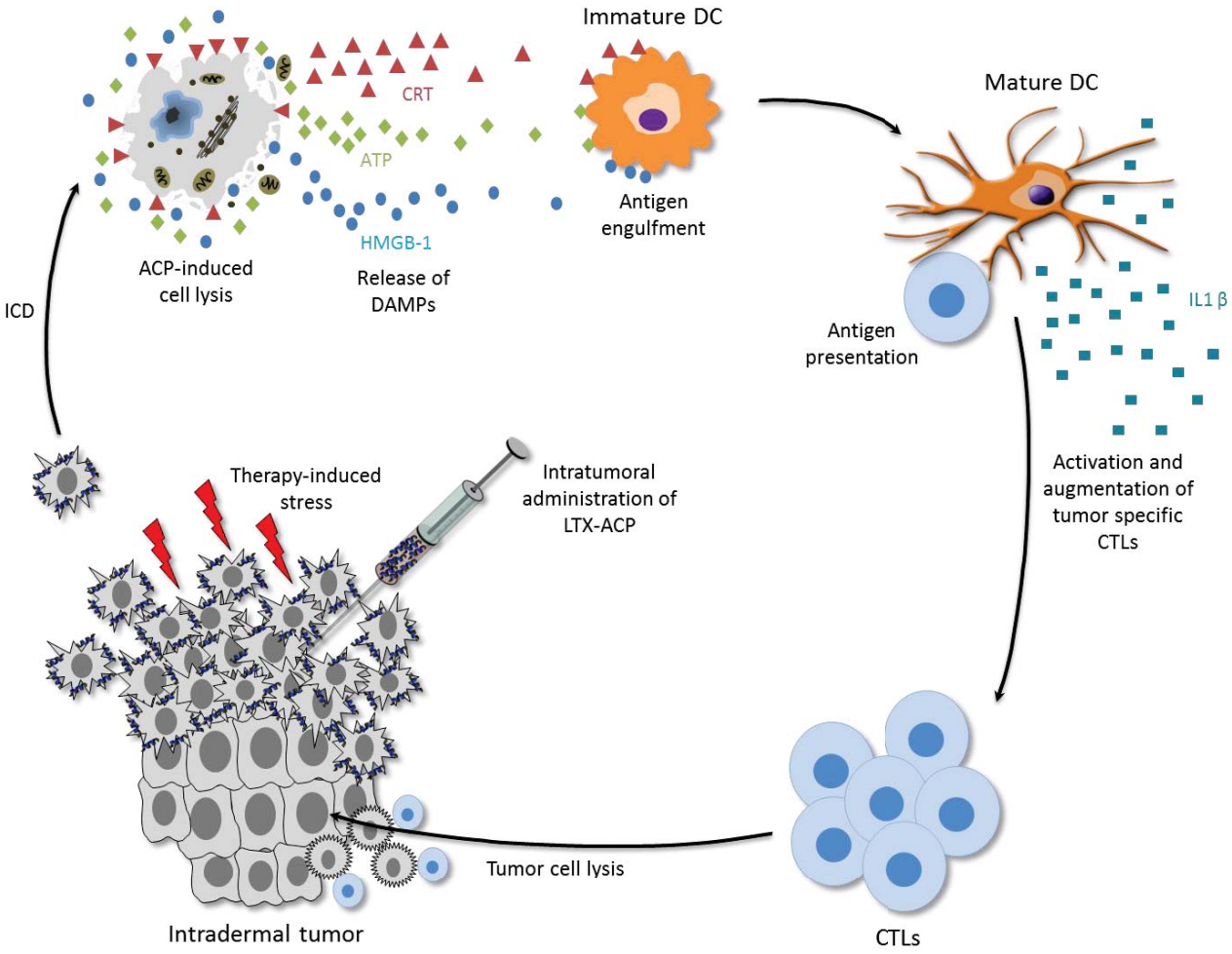

Figure 10: A proposed model for *in situ* vaccination with LTX-peptides – LTX-ACP-induced cellular stress and ICD may lead to a cascade of events stimulating the immune system. LTX-ACPs bind to the cancer cell membrane and kill the cells by membrane lysis (necrosis). Consequently, DAMPs such as CRT, ATP and HMGB-1 are released and bind to receptors (e.g. PRRs) on immature DCs, initiating the maturation and recruitment of DCs into the tumor bed (stimulated by ATP), the engulfment of tumor antigens by DCs (stimulated by CRT) and optimal antigen presentation to T cells (stimulated by HMGB-1). Overall, these processes result in a potent T cell-driven immune response, which leads to the eradication of the residual viable tumor cells. ACP, anticancer peptide; ATP, adenosine triphosphate; CRT, calreticulin; CTL, cytotoxic $CD8^+$ T lymphocyte; DAMPs, danger-associated molecular pattern molecules; DC, dendritic cell; HMGB-1, high mobility group box 1; ICD, immunogenic cell death; IL, interleukin; PRR, pattern recognition receptor. The illustration was modified from Kroemer et al.[207] using Microsoft PowerPoint 2010.

THE POTENTIAL OF SHORT SYNTHETIC ANTICANCER PEPTIDES (LTX-ACPs) AS A NOVEL ADJUVANT IN COMBINATION WITH WHOLE CELL VACCINES (PAPER III)

In Paper III, the potential of LTX-315 as a novel adjuvant in a whole cell cancer vaccine setting was investigated. Specifically, Paper III aimed at investigating the adjuvant and anticancer effects of LTX-315 following intradermal injection of the peptide, in combination with B16F1 tumor cell lysate (TCL), against highly aggressive and low immunogenic syngeneic B16 melanomas.

Intradermal injection of LTX-315 induces infiltration of immune cells

LTX-315 shows cytotoxic activity towards normal human fibroblasts (MRC-5) and endothelial cells (HUV-EC-C) at concentrations harmless to red blood cells. In addition, peptide treatment with LTX-315 demonstrates the release of HMGB-1 from cancer cells *in vitro* due to a rapid lytic mode of action (Paper II). DAMPs are considered strong endogenous adjuvants[175], while HMGB-1 has previously been shown to initiate and preserve immune responses in the noninfectious inflammatory response[168]. A histological examination following the i.t. administration of LTX-315 demonstrates that LTX-315 induces a massive infiltration of immune cells such as $CD3^+$ T cells into the injection site, hence leading to a long-term immune protection against the cancer. These results led us to believe that LTX-315 may have a potential as a novel adjuvant due to its indirect immunomodulatory properties.

A low-dose (compared to i.t.) intradermal injection of LTX-315 in mice exhibited inflammation and immune cell infiltration at the injection site. A histological examination of normal murine skin following a local injection of LTX-315 showed an increase in infiltrating $CD45^+$ immune cells 2 hours post-injection, with a peak in cell infiltration seen at 72 hours post injection. The membranolytic properties of LTX-315 induced local inflammation, probably due to the release of DAMPs (strong endogenous adjuvants), thus making it a candidate to be used as a novel adjuvant. The induced local inflammation will direct neutrophils and macrophages, as well as other immune cells involved in wound healing, into the injection site[257]. After seven days (168 h), the skin and smooth muscle layer showed signs of a full recovery, demonstrating that the tissue damage was temporary and that a complete regeneration of the area was attained.

LTX-315 and its potential as a novel adjuvant

In a cancer vaccine setting, adjuvants are crucial to induce a long-lasting antitumor immune response[211], which is due to their ability to induce inflammation and the subsequent activation and infiltration of immune cells into the vaccination site[258]. If professional APCs such as DCs are faced with an antigen signal alone, a tolerance to the antigens will be induced. Adjuvants serve to promote and enhance immune responses to vaccine components; therefore, by the help of an adjuvant, a second critical antigen-independent signal can be evoked. The second signal will bind to specific receptors on APCs, and induce APC maturity and activation. At this stage, the mature APC

will be prepared for antigen uptake and can express co-stimulatory molecules and facilitate T cell responses, hence stimulating a specific immune response against the presented antigens[175, 229]. Furthermore, DAMPs released from stressed or dying cells are often considered natural endogenous adjuvants due to their ability to activate the immune system[175].

In combination with LTX-315, immunizations (prophylactic vaccinations) of animals with an intradermal injection of TCL led to an augmented immune protection against B16 melanomas compared to LTX-315 and TCL alone (no protection), thereby demonstrating that LTX-315 has adjuvant properties. Moreover, the injection of LTX-315 2 h prior to injection with TCL induced a superior immune protection compared to LTX-315 being injected simultaneously or 2 h after TCL, thus indicating that a pre-activation of the immune system is optimal before antigen delivery. Additionally, immunizations of syngeneic mice with a TCL containing *in vitro*-cultured B16F1 melanoma cells, in combination with LTX-315 as an adjuvant, induced a long-term protection against a challenge with live B16 melanoma cells. When animals were intradermally re-challenged with live B16F1 cells 12 weeks after the final immunization, 14 out of 18 animals had developed long-lasting protection and were tumor free 45 weeks after the primary tumor challenge. To investigate whether the long-term protection still persisted one year after the primary tumor challenge, a second tumor re-challenge was performed with live B16F1 cells 45 weeks post the primary tumor challenge. Twelve animals were re-challenged, and nine out of the 12 animals were tumor free 80 days post the second re-challenge (56 weeks post-primary challenge), demonstrating that the induced immune protection was long-term for the majority of the animals. Moreover, the immune protection was unaffected by a boosting of the immune system through an immunization given one week prior to the re-challenge.

The adjuvant property of LTX-315 comes from its ability to stimulate local immune cell infiltration in the skin subsequent to peptide-induced necrosis/tissue damage, which is probably due to the release of DAMPs. The local stimulation of the immune system and the infiltration of immune cells create an optimal environment for the introduction and uptake of TAAs by mature APCs (Figure 11 is a hypothetical model based on Kroemer et al.´s model for ICD[207] for what occurs *in vivo* following low-dose intradermal administration with LTX-315 in combination with TCL). The use of whole tumor cells (e.g. TCL) as the antigen source in a cancer vaccine setting is a promising alternative to the use of individual TAAs (e.g. tumor antigen derived peptides)[222]. In whole cell vaccines, there is no need to define, test or select for immunodominant epitopes. Furthermore, TCL will allow for the presentation of antigens to both cytotoxic $CD8^+$ T lymphocytes and $CD4^+$ T helper cells, hence generating a stronger overall anti-tumor response and a long-term $CD8^+$ T cell memory via $CD4^+$ T cell help. For this reason, TCL prepared from autologous or allogeneic tumor cells contains an extensive repertoire of TAAs, but requires suitable adjuvants to enhance its immunogenicity if it is to be used as an efficient cancer vaccine. Whole cell vaccines alone fail to induce an efficient immune response to eradicate tumors, showing a poor immunogenicity[224]. It has also been shown that the clinical outcome of immunotherapy trials using autologous/allogeneic whole tumor cells (undefined TAAs) gave significantly better results on objective clinical responses compared to molecularly defined synthetic antigens (tumor antigen derived peptides)[223].

The novelty of LTX-315 as an adjuvant lays in its formulation and immune-stimulating properties, as LTX-315 was originally developed for the local treatment of solid tumors. However, due to the indirect immunomodulatory properties that LTX-315 demonstrated through the obtained *in situ* vaccination, and a subsequent long-term immune protection against the cancer following i.t. treatment, we postulated that the peptide had potential as an immune augmenting adjuvant. The direct disruptive effect of the peptide on the plasma membrane of cells allows for the release of endogenous danger signals and a stimulation of the immune system, thereby illustrating a mechanism of action different from other adjuvants. LTX-315 is also unique in its formulation, insofar as being formulated in a sterile saline, while most current adjuvants are water-in-oil or oil-in-water formulations. Many clinical trials have used incomplete Freund's adjuvant (IFA)-based vaccines to induce tumor-specific T cell responses in patients with cancer. Although antigen-specific T cell responses were induced, objective antitumor responses have been rare[259]. Instead of promoting an effective T cell response at the tumor site, a study in mice showed that a peptide vaccine in water-in-oil adjuvant led to a trapping of tumor-specific $CD8^+$ T cells at the vaccination site[260]. IFA-based vaccines are water-in-oil emulsions of antigen in mineral oil with mannide mono-oleate as a surfactant[261], which may help explain the lack of objective antitumor responses in clinical studies using IFA-based vaccines. It seems as if non-persistent and rapidly biodegradable adjuvants (such as LTX-315) may be a better choice for future vaccine regimens[262]. Consequently, LTX-315 should have a significant potential as a novel adjuvant, but nevertheless, further studies need to be performed to assess the potential of LTX-315 to induce tumor-specific immune responses.

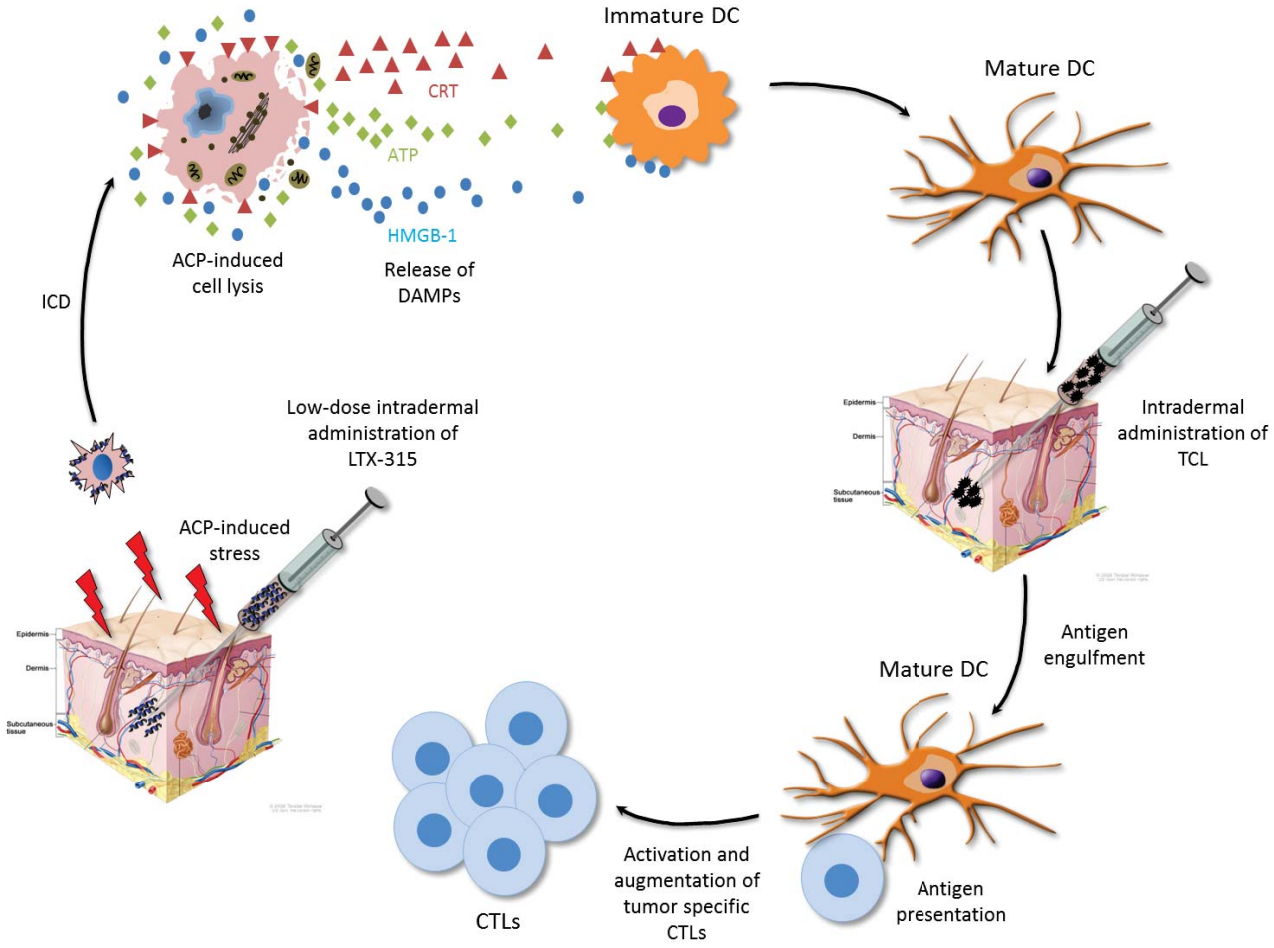

Figure 11: A proposed model for *in vivo* prophylactic vaccination with TCL in combination with LTX-315 as an adjuvant – LTX-ACP-induced cellular stress and ICD may lead to a cascade of events stimulating the immune system. LTX-ACPs bind to the membrane of normal skin cells and kill the cell by membrane lysis (necrosis). Consequently, DAMPs such as CRT, ATP and HMGB-1 can be released and bind to receptors (e.g. PRRs) on immature DCs, initiating the maturation and recruitment of DCs into the injection site (stimulated by ATP). Mature DCs will be introduced to cancer antigens through the injection of TCL, hence initiating the engulfment of tumor antigens by DCs (stimulated by CRT) and optimal antigen presentation to T cells (stimulated by HMGB-1). Overall, these processes result in a potent T cell-driven immune response that can give a long-term protection against cancer. ACP, anticancer peptide; ATP, adenosine triphosphate; CRT, calreticulin; CTL, cytotoxic $CD8^+$ T lymphocyte; DAMPs, danger-associated molecular pattern molecules; DC, dendritic cell; HMGB-1, high mobility group box 1; PRR, pattern recognition receptor; TCL, tumor cell lysate. The illustration was modified from Kroemer et al.[207] using Microsoft PowerPoint 2010.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis addresses the potential of LTX-ACPs as a novel therapy within cancer by investigating the local treatment of solid tumors and the subsequent immune modulating properties.

Our results demonstrate that short lytic ACPs may have a potential as novel anticancer immunotherapeutic agents, and we show that LTX-ACPs have a selective cytotoxicity towards cancer cells compared to non-malignant cells and human red blood cells. In addition, the intratumoral treatment of transdermally accessible tumors with LTX-ACPs induces a complete regression of the tumors, as well as a secondary immune protection against the relapse of the cancer. This argues strongly in favor of a dual effect in which LTX-ACPs induce immunogenic cell death through a direct disruptive effect on the cellular membrane (direct effect), as well as mounting tumor-specific immune responses against the cancer (indirect effect). Moreover, we show that treatment with LTX-ACPs incites local inflammation, possibly through the release of DAMPs such as HMGB-1, and that the immune protection against the cancer is T cell-dependent.

This thesis also provides evidence that LTX-315 has a potential as a novel adjuvant in a cancer vaccine setting. The lysis of non-malignant cells induces inflammation, thereby stimulating and maturing APCs for the antigen uptake and presentation of TAAs to mount a tumor-specific immune response by activating and augmenting $CD8^+$ cytotoxic T lymphocytes.

In conclusion, the work presented in this thesis demonstrates that LTX-ACPs may have a promising potential to be implemented as a novel therapy against cancer, both due to their direct killing mechanism and indirect immunomodulatory effects.

Future studies should consist of mechanistic studies to further investigate the mode of action for LTX-ACPs *in vivo*. The involvement of DAMPs such as CRT, ATP and HMGB-1 should be clarified through histological examinations on tumor tissue or by using Western Blot on cells treated with LTX-ACPs *in vitro*, which will strengthen the hypothesis that LTX-ACPs induce immunogenic cell death. Additionally, the involvement of immune cells such as T cells in the immune protection achieved in B16 melanomas can be studied through adaptive transfer studies in syngeneic C57BL/6 mice, while immune cell phenotyping can be performed using flow cytometry on ACP-treated tumors. Additionally, the adjuvant potential of LTX-315 is still largely uncharted and should be examined in a peptide vaccine (specific epitope) setting so that the potential antigen-specific immune response can be assessed using immune response monitoring assays such as ELISPOT. Moreover, LTX-315 has great potential to be used in a combination therapy setting due to its dual mechanism of action (direct killing and adjuvant properties), e.g. together with anticancer agents to overcome tumor-related immune suppression such as low-dose cyclophosphamide and Ipilimumab, or together with immune stimulators such as IFN-γ and cancer vaccines.

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Paper I

Paper II

Paper III

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