Sabrina Riedl

DESIGN OF NEW ANTICANCER DRUGS DERIVED FROM THE HUMAN HOST DEFENSE PEPTIDE LACTOFERRICIN

DISSERTATION

zur Erlangung des akademischen Grades einer

Doktorin der Naturwissenschaften

eingereicht an der

Technischen Universität Graz

durchgeführt am

Institut für Biophysik und Nanosystemforschung

an der

Österreichische Akademie der Wissenschaften

bei

Dipl.-Ing. Dr. Dagmar Zweytick

Univ. Doz. Dipl.-Ing. Dr. Karl Lohner

Graz, Juli 2011

Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources /resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

………………………………. ……………………………………………….

Date Signature

Acknowledgments

Zusammenfassung

Auch im 21. Jahrhundert ist Krebs noch immer ein großes Problem und für 13% aller Todesfälle weltweit verantwortlich. Therapien wurden zwar erfolgreich weiterentwickelt und verbessert, aber in mehr als 50% der Fälle ist Krebs noch immer nicht heilbar. Die größten Probleme dabei sind Wiederauftreten von Rezidiven, Resistenzen, Metastasierung und schwere Nebenwirkungen durch herkömmliche Therapien aufgrund fehlender Spezifität.

Immunabwehrpeptide wie z.B. menschliches Lactoferricin (hLFcin) und deren Derivate sind eine neue Strategie im Kampf gegen Krebs, indem sie spezifisch die Krebszellmembran angreifen. Diese Immunabwehrpeptide sind Teil des angeborenen Immunsystems vieler verschiedener Spezies und weisen einige gemeinsame Charakteristika auf, sie besitzen eine kurze Aminosäuresequenz, sind kationisch und amphipathisch. Aufgrund ihrer positiven Ladung können sie mit dem negativ geladenen Phospholipid Phosphatidylserin (PS) interagieren, das im Zuge der malignen Umwandlung an der Oberfläche der Krebszelle exponiert wird. Gesunde Zellen weisen hingegen die neutralen Phospholipide Phosphatidylcholin (PC) und Sphingomyelin (SM) in der äußeren Schicht der Zellmembran auf.

In der beschriebenen Studie konnten wir beweisen, dass PS signifikant an der Oberfläche von verschiedenen adhärenten Krebszelltypen exponiert wird. Des Weiteren zeigte sich, dass nicht nur Krebszellen von Primärtumoren sondern auch von Metastasen und Krebszellen von Zelllinien als auch primäre Krebszellen aus frischem Tumorgewebe PS in der äußeren Zellmembran aufweisen. Darüber hinaus deutet die Studie an unterschiedlich malignen Hauttumoren darauf hin, dass der exponierte PS-Gehalt mit der Bösartigkeit des Gewebes zunimmt. Die Ergebnisse dieses ersten Teils der Studie beweisen, dass PS als universeller Marker für Krebs und als Angriffspunkt für Immunabwehrpeptide dienen kann. Im weiteren Verlauf des Projekts wurden etliche hLFcin Derivate auf ihre Wirkung gegen Krebszellen und das von ihnen exponierte PS untersucht. Diese Peptide unterschieden sich

hauptsächlich in Art und Länge ihrer Aminosäurezusammensetzung, Anzahl an positiven Ladungen, Hydrophobizität und daraus resultierend unter anderem in ihrer Sekundärstruktur und Wirkungsweise. *In vitro* Daten korrelierten sehr gut mit durchgeführten Modelstudien, in denen Krebszellen mittels PS-Liposomen und gesunde Zellen mittels PC-Liposomen imitiert wurden. Biophysikalische Untersuchungen dienten zum Beweis, dass die wirksamen und für Krebszellen selektiven Peptide tatsächlich PS als Angriffspunkt nutzen. Die durchgeführten *in vitro* Studien ließen erkennen, dass die Peptide unterschiedliche Mechanismen anwenden, um Krebszellen zu zerstören. Einige Peptide erreichten bereits nach wenigen Minuten ihre höchste Aktivität, was auf einen Zelltod durch Membranolyse schließen lässt. Andere untersuchte Peptide hingegen benötigten eine längere Inkubationszeit von bis zu einigen Stunden. Dies deutet auf einen Zelltod durch Apoptose hin. Interessanterweise scheinen Peptide mit einer langsameren Wirkungsweise spezifischer zu sein als jene, die nur durch Membranolyse wirken. Zusätzlich ist die Einnahme einer bestimmten Sekundärstruktur in Gegenwart der Zielmembran von offenbar großer Bedeutung.

Summary

In the 21st century cancer is still a major problem and accounts for 13% of all deaths worldwide. Regarding therapy, much progress has been achieved but cancer is still not curative in more than 50% of the cases. Major problems of therapy are reoccurrence, (multi-) drug resistance, metastases and severe side effects due to inadequate specificity.

A new strategy uses host defense peptides like human lactoferricin (hLFcin) and derivatives thereof to specifically target the cancer cell membrane. Host defense peptides which are part of the innate immune system of many diverse species mostly comprise short stretches of amino acids and are cationic and amphipathic. These peptides target the negatively charged phospholipid phosphatidylserine (PS) which is only exposed in the outer leaflet of the cell membrane during malignant transformation, whereas the outer leaflet of the healthy cell membrane comprises the neutral phospholipids phosphatidylcholine (PC) and sphingomyelin (SM).

In this study we could prove PS exposure of cancer cells of various cancer types of primary as well as of metastatic lesions, of cancer cell lines as well as of primary cancer cells. Furthermore we demonstrated that PS exposure correlates with stage of malignity. Our findings demonstrate that PS could serve as a uniform marker for cancer cells and as potent target for anticancer peptides. Therefore we tested several hLFcin derivatives, differing in content and length of primary sequence, number of positive charges, hydrophobicity and consequently secondary structure and efficacy, for their toxicity against cancer cells and their interaction with exposed PS. *In vitro* data nicely correlated with the model studies in which cancer cells are mimicked by PS- and non-cancer cells by PC-liposomes. Biophysical investigations proved that PS is the target for the studied active and cancer selective peptides. *In vitro* studies revealed different modes of action of the peptides. Some peptides reached their highest activity already after several minutes indicating cell death through membrane lysis, whereas others needed prolonged incubation times up to several hours supporting the idea of a

killing mechanism through apoptosis. Interestingly, peptides performing a slow killing seem to be more specific than those who kill through lysis of the cell membrane. Additionally, secondary structure seems to play an important role for the cancer specificity.

Abbreviations

hLF	human Lactoferrin
hLFcin	human Lactoferricin
bLFcin	bovine Lactoferricin
PS	phosphatidylserine
PC	phosphatidylcholine
PG	phosphatidylglycerol
SM	sphingomyelin
PE	phosphatidylethanolamine
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dihexadecanoyl-sn-glycero-3-phosphocholine
DPPS	1,2-dihexadecanoyl-sn-glycero-3-phospho-L-serine
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
DPX	p-xylene-bis-pyridinium bromide
LUV	large unilamellar vesicle
ATP	adenosine-5’-triphosphate
DSC	differential scanning calorimetry
PI	propidium iodide
FBS	fetal bovine serum
Gal	galactose
GlcNAc	*N*-acetylglucosamine
AcNeu	N-acetylneuramin
GlcNeu	*N*-glycosylneuraminic acid;

Content

Statutory declaration ii

Acknowledgments iii

Zusammenfassung iv

Summary vi

Abbreviations viii

Content ix

Aim of the study 1

Introduction 5

Membrane-active host defense peptides – challenges and perspectives for the development of novel anticancer drugs 5

Results 55

In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy 56

Killing of melanoma cells and metastasis by human Lactoferricin derivatives correlates with effect on Cancer Models composed of Phosphatidylserine 86

Slow killing of cancer cells, phosphatidylserine interaction and formation of a ß-sheet structure by hLFcin derived anticancer peptides is the key for high cancer specificity 118

Summary and Outlook 147

Aim of the study

Every year millions of people are diagnosed with cancer worldwide. Notwithstanding in the last decades much progress has been achieved in cancer therapy, cancer remains a leading cause of death (http://www.who.int/mediacentre/factsheets/fs297/en/). Nowadays, surgery, chemotherapy, radiation, hormone ablation therapy and targeted therapy are the standard treatments, but in the year 2008 they were not curative in more than 50% of the cases (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900). Furthermore, the use of these types of therapy can be limited due to resistance and accompanied by potential toxicity and diverse side effects due to inadequate specificity for tumor cells. Obviously, the discovery of new and more specific targets, together with the design of specific antitumor drugs, is one of the major interests in cancer research.

Cancer cells are often well characterized, but little is known about the plasma cell membrane, or to be more precise, the arising differences in the lipid composition in carcinogenesis. Eukaryotic plasma membranes usually comprise an overall neutral charge on the outer leaflet due to the zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM) (1). The negatively charged phospholipid phosphatidylserine (PS) together with the major part of phosphatidylethanolamine (PE) normally only assembles in the inner leaflet of eukaryotic plasma membranes (1). This asymmetric distribution of phospholipids is well documented (2;3) and is maintained by an ATP-dependent aminophospholipid translocase (4;5). This asymmetry can get lost due to exposure of the negatively charged phosphatidylserine on the surface of cancerous (6-10) and other pathological cells (11), apoptotic cells (12;13), as well as platelets and erythrocytes upon activation (14;15). One aim of this study was to prove PS exposure of cells of different types of cancer, of primary and metastatic lesions, cancer cell lines and primary cancer cells freshly derived from tumor tissue as well as the lack of exposed PS of non-cancerous cells.

Based on the knowledge of PS exposure, new strategies for the design of anticancer drugs can be considered, especially cationic host defense derived peptides interacting with negatively charged phospholipids. Various peptides are known to exhibit e.g. antimicrobial activity which is based on interactions with the anionic phospholipid phosphatidylglycerol (PG) (16). Thus, it is obvious to test and further improve antimicrobial peptides in respect of their anticancer activity.

Human Lactoferrin (hLF), an iron binding glycoprotein found in milk, exocrine secretions, granules and neutrophils during inflammatory response, is one prominent member of antimicrobial peptides which originally exhibits anticancer activity (for reviews see (17;18) and following chapter). A small peptide stretch of hLF (corresponding to residues 21-31), namely LF11 (FQWQRNIRKVR-NH_2), has already been optimized in its antimicrobial activity regarding electrostatic and hydrophobic interactions (19;20), resulting in a peptide library of 173 derivatives of LF11. The aim of this study was to further improve peptide sequences in terms of anticancer activity based on the results obtained in the former antimicrobial studies. For peptide optimization in respect of activity and selectivity, cancer membrane mimetic systems composed of phosphatidylserine were used to get further insight into peptide/lipid interactions. For this purpose, the following biophysical methods were applied: differential scanning calorimetry (DSC), fluorescence spectroscopy (tryptophan quenching, ANTS/DPX leakage) and circular dichroism spectroscopy (CD). Most promising peptide candidates were then selected for *in vitro* experiments, including fluorescence spectroscopy and flow cytometry (PI-uptake assay), fluorescence microscopy (PI-uptake, Caspase-3 cleavage) and photometry (MTS viability assay) with cancerous and non-cancerous cells of various tissues. Besides peptide optimization the aim of the study was to prove PS as a potential target for the designed LFcin derived peptides and elucidate the structural and mechanistic conditions for highly specific killing of cancer cells. As it had already been shown for bLFcin that necrosis (21) as well as apoptosis (22) might be induced by derivatives of the same peptide, elucidation of the mode of action of selected peptides was aspired.

Reference List

1. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 1996: Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications; Lupus, 5(5): 480-487

2. Zwaal, R. F. and Schroit, A. J., 1997: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells; Blood, 89(4): 1121-1132

3. Bevers, E. M., Comfurius, P., Dekkers, D. W., Harmsma, M., and Zwaal, R. F., 1998: Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling; Lupus, 7 Suppl 2(S126-S131

4. Seigneuret, M. and Devaux, P. F., 1984: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes; Proc.Natl.Acad.Sci.U.S.A, 81(12): 3751-3755

5. Frey, B. and Gaipl, U., 2010: The immune functions of phosphatidylserine in membranes of dying cells and microvesicles; Seminars in Immunopathology, 1-20

6. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J., 1989: Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation; Proc.Natl.Acad.Sci.U.S A, 86(9): 3184-3188

7. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

8. Woehlecke, H., Pohl, A., Alder-Baerens, N., Lage, H., and Herrmann, A., 2003: Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2); Biochem.J., 376(Pt 2): 489-495

9. Schroder-Borm, H., Bakalova, R., and Andra, J., 2005: The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine; FEBS Lett., 579(27): 6128-6134

10. Ran, S., Downes, A., and Thorpe, P. E., 2002: Increased exposure of anionic phospholipids on the surface of tumor blood vessels; Cancer Res., 62(21): 6132-6140

11. Zwaal, R. F., Comfurius, P., and Bevers, E. M., 2005: Surface exposure of phosphatidylserine in pathological cells; Cell Mol.Life Sci., 62(9): 971-988

12. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M., 2005: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages; J.Immunol., 148(7): 2207-2216

13. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R., 1995: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl; J.Exp.Med., 182(5): 1545-1556

14. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 2005: Changes in membrane phospholipid distribution during platelet activation; Biochim.Biophys.Acta, 736(1): 57-66

15. Williamson, P., Bevers, E. M., Smeets, E. F., Comfurius, P., Schlegel, R. A., and Zwaal, R. F., 1995: Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets; Biochemistry, 34(33): 10448-10455

16. Lohner, K., 2003: Development of Novel Antimicrobial Agents: Emerging Strategies; Journal of Antimicrobial Chemotherapy, 51(5): 1321-1321

17. Farnaud, S. and Evans, R. W., 2003: Lactoferrin--a multifunctional protein with antimicrobial properties; Molecular Immunology, 40(7): 395-405

18. Vorland, L. H., 1999: Lactoferrin: A multifunctional glycoprotein; APMIS, 107(7-12): 971-981

19. Zweytick, D., Pabst, G., Abuja, P. M., Jilek, A., Blondelle, S. E., Andra, J., Jerala, R., Monreal, D., Martinez, d. T., and Lohner, K., 2006: Influence of N-acylation of a peptide derived from human lactoferricin on membrane selectivity; Biochim.Biophys.Acta, 1758(9): 1426-1435

20. Zweytick, D., Tumer, S., Blondelle, S. E., and Lohner, K., 2008: Membrane curvature stress and antibacterial activity of lactoferricin derivatives; Biochem.Biophys.Res.Commun., 369(2): 395-400

21. Eliassen, L. T., Berge, G., Sveinbjornsson, B., Svendsen, J. S., Vorland, L. H., and Rekdal, O., 2002: Evidence for a Direct Antitumor Mechanism of Action of Bovine Lactoferricin; Anticancer Res, 22(2703-2710

22. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W., 2005: Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines; Mol Cancer Ther, 4(4): 612-624

Introduction

The following review focuses on anticancer peptides as alternative strategy in cancer therapy, describes the need of new therapies and highlights the differences between cancerous and non-cancerous cell membranes.

Membrane-active host defense peptides – challenges and perspectives for the development of novel anticancer drugs

Sabrina Riedl, Dagmar Zweytick and Karl Lohner

Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstraße 6, A-8042 Graz, Austria

Invited review submitted to Chemistry and Physics of Lipids

Abstract

Although in the last decades much progress has been achieved in cancer therapies, problems arise particularly in chemotherapy due to resistance to current drugs and their low specificity. Host defense peptides, effector molecules of innate immunity, represent a novel strategy for the development of alternative anticancer drug molecules. These cationic amphipathic peptides can discriminate between neoplastic and non-neoplastic cells interacting specifically with negatively charged membrane components such as phosphatidylserine (PS), sialic acid or heparan sulfate, which differ between cancer and non-cancer cells. Moreover, increased number of microvilli has been found on cancer cells leading to an increase of cell surface area, which in turn may enhance their susceptibility to anticancer peptides. Thus, part of the review will be devoted to the differences in membrane composition of non-cancer and cancer cells with a focus on the exposure of PS on the outer membrane. Normally, surface exposed PS triggers apoptosis, which however can be circumvented by cancer cells by various means.

Host defense peptides targeting selectively differences between cancer and non-cancer cell membranes have an excellent tumor tissue penetration and can reach the site of both primary tumor and distant metastasis. Since these molecules kill their target cells rapidly mainly by perturbing the integrity of the plasma membrane, resistance is less likely to occur. Hence, a chapter will also describe studies related to the molecular mechanisms of membrane damage as well as alternative non-membrane related mechanisms. *In vivo* studies have demonstrated that host defense peptides display anticancer activity against a number of cancers such as e.g. leukemia, prostate, ascite and ovarian tumors, but so far, none of these peptides has made it on the market. Nevertheless, optimization of host defense peptides using various strategies to enhance further selectivity and serum stability is expected to yield novel anticancer drugs with improved properties in respect of cancer cell toxicity as well as reduced development of drug resistance.

Introduction

Cancer is a leading cause of death worldwide representing about one eighth of all deaths (http://www.who.int/mediacentre/factsheets/fs297/en/) being strongly affected by demographic changes especially ageing of the population. In 2008 more than 12.7 million people were newly diagnosed with cancer accounting for 7.6 million deaths, whereby over one quarter of the global cancer incidences occur in Europe. In addition, cancer has also emerged as a major public health problem in developing countries. According to the World Health Organization new cases of cancer impairment will strongly increase (Figure 1) with estimated death rates up to 11 million in the year 2030. Although in the last decades much progress has been achieved in respect of therapies, like surgery, chemotherapy, radiation or hormone ablation therapy, they are not successful in more than 50% of the cases (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900).

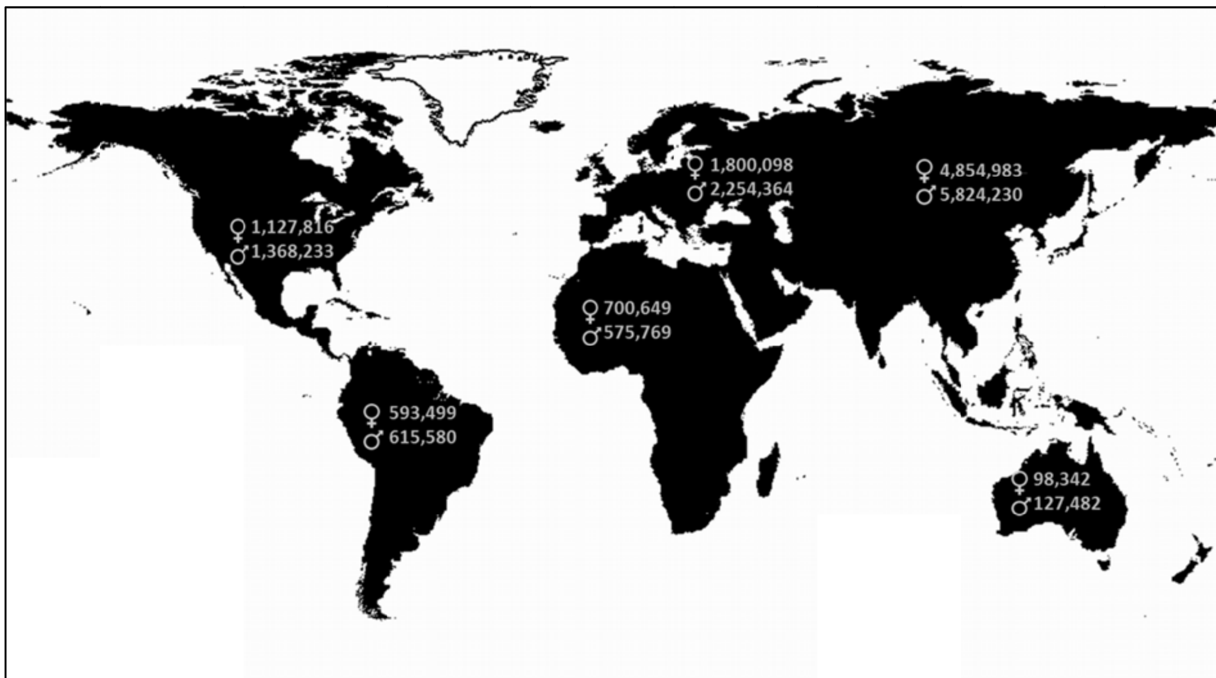

Figure 1: Prospects of cancer in 2030: Estimation of new cases of cancer impairment of men and women in the year 2030 adapted from GLOBOCAN (2008) IARC.

Moreover, for those who survive, the risk of reoccurrence of the disease is a major problem. If the tumor progresses or reoccurs, chemotherapy is the standard treatment. However, resistance as well as potential toxicity and many side effects of chemotherapeutics, which are mainly due to inadequate specificity for tumor cells, represent a major limitation of this type of therapy. Hence, increasingly the retrieval of more specific targets generally expressed within a certain tumor is a major issue in anticancer research. Receptors of growth factors and proteins involved in cell-cell-signaling are of broad interest, but relatively little effort has been devoted to target per se cancer cell membranes. Thus, this review will focus on a promising new approach in cancer therapy which is based on host defense peptides, effector molecules of innate immunity that target specifically cancer cells and destroy them within minutes mostly by damaging its cell membrane without prior binding to specific receptors.

Deficits and drawbacks of conventional cancer therapies – need of novel treatment strategies

Before development of chemotherapeutics in the 1940s (1) surgical removal of the tumor tissue was the only available treatment and still can often be successfully applied for localized cancers, for which radiation therapy is also widely used targeting cells by directly damaging DNA (2). Nevertheless many cancer types advance rapidly, reoccur or tend to metastasize making chemotherapy the treatment of choice (3). Currently used anticancer drugs are mostly based on alkylating agents, antimetabolites as well as natural products and derivatives thereof. From 1940 to 2006 175 new anticancer drugs have been approved (4). More than 50% thereof are biological (10%, usually large peptides and proteins), natural (14%) or naturally derived products (28%, semisynthetic modifications) (Figure 2).

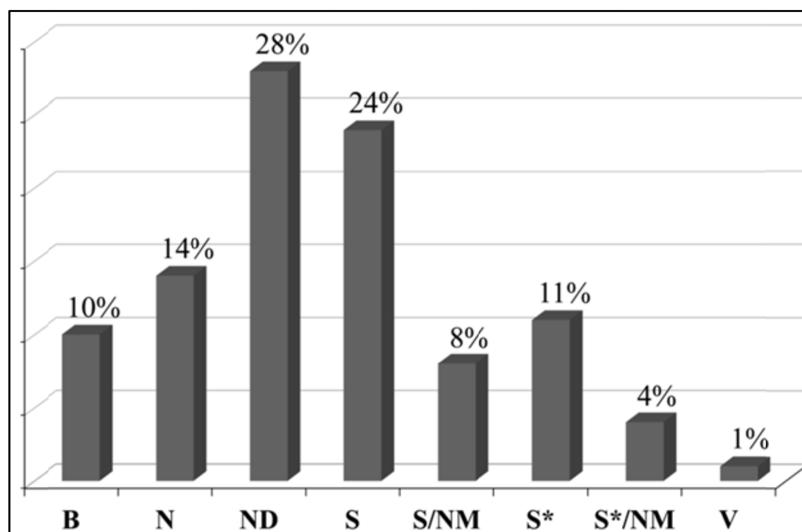

Figure 2: New anticancer drugs (1940s-06/2006) subdivided by source adapted from Newmann et al. (187): (B) biological; (N) natural product; (ND) derived from natural product – semisynthetic modifications; (S) synthetic drug; (S/NM) synthetic/natural drug mimic; (S*) made by total synthesis; (S*/NM) made by total synthesis/natural drug mimic; (V) vaccine.

Early compounds were based on the nitrogen mustards, a powerful class of agents that alkylate bases of DNA (5), leading to cell death. A second class of chemotherapeutics, the antimetabolites, like aminopterin and amethopterin, do not directly interact with DNA bases but interfere with synthesis of precursors as folate and cause mistakes during DNA replication of cancer cells (1). In the 1960s the development of natural-product anticancer drugs has started such as e.g. Vinca alkaloids that inhibit microtubule polymerization and thereby cell division or antracyclines that promote cell death upon intercalation in DNA and inhibition of replication (6,7). In the following decades a large number of further chemotherapeutics such as taxol or halychondrines and E7389 have been developed that interfere with a variety of biological targets (8).

Side effects owing to insufficient selectivity

Most of the conventional chemotherapeutics however exhibit insufficient selectivity against healthy mammalian cells causing deleterious side effects (9-11). Commonly, these agents act on cells that divide rapidly, one of the main properties of cancer cells. Therefore, damage of in particular rapidly-dividing normal cells, like bone marrow, gastrointestinal mucosa and hair follicles, can be expected resulting in the most common side effects of chemotherapy like myelosuppression (decreased production of blood cells), mucositis (inflammation of the lining of the digestive tract) and alopecia (hair loss). Newer chemotherapies such as the use of monoclonal antibodies (12), immunotoxins (13) or angiogenesis inhibitors (14), target specific differences between tumors and healthy cells and tissues, thereby exhibiting lower toxicity. However, side effects have been still observed.

Formation of resistance

Besides toxicity, resistance to treatment with anticancer drugs is a huge problem. Resistance can be intrinsic and result from individual variations in patients and somatic cell genetic differences in tumors (15). In addition acquired resistance has become common, by e.g. expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells before interacting with intracellular targets, or acquired insensitivity to drug induced apoptosis and induction of drug-detoxifying mechanism (15-17). Hence, many cancer types e.g. malignant melanoma (18,19) show only weak sensitivity to anticancer drugs. Treatment with methylating agents dacarbazine and its oral analogue temozolomide exhibit a response of only 15% (20,21). In metastatic melanoma resistance is reported to be linked to defective apoptosis due to inhibition of expression of a gene encoding Apaf-1, the apoptotic protease activating factor-1 (22). A number of multidrug-resistant cancer have been detected and thus various approaches including the development of drugs that engage, evade or exploit efflux by ABC transporters have been described (23).

Despite all the progress in the design of cancer therapy yet there is no cancer treatment that is 100% effective against disseminated cancer (15). In contrast, the overall contribution of curative and adjuvant cytotoxic chemotherapy to 5-year survival in adults was estimated to be only 2.3% in Australia and 2.1% in the USA (24). Therefore, the development of new anticancer drugs based on novel mode of actions with improved cancer cell selectivity is urgently needed. Furthermore, patients suffering of cancer with poor treatability like glioblastoma, a cancer of the brain, do not survive longer than 1-2 years even if detected early and treated by surgery, chemotherapy, and radiotherapy, (25). Cancer of stomach or ovary and others also demand for new effective treatment.

Host defense peptides targeting selectively differences between cancer and non-cancer cell membranes

In search of novel anticancer agents host defense peptides have emerged as potential alternative anticancer therapeutics offering many advantages over other therapies. Because of their mode of action and specificity – the cell membrane being the major target – resistance and non-neoplastic toxicity is less likely to occur (26-29) and thus, in addition they are expected to cause fewer side effects. Moreover, these peptides mostly damage cell membranes within minutes (30), which would hinder formation of resistance (27). Host defense peptides being part of the innate immune system of many diverse species (e.g. mammals, insects, amphibians) were initially discovered because of their antimicrobial activity (31). Currently, the antimicrobial peptide database lists more than 100 natural host defense peptides, which have antitumor activity (32,33). Selected host defense peptides, for which *in vitro* studies were reported, are listed in Table 1. The most intensively studied antimicrobial peptides that also exhibit cytotoxic activity against cancer cells were described in detail in terms of structure, mode of action and anticancer activity in a recent review by Hoskin and Ramamoorthy (26). These host defense peptides are characterized by low molecular weight (in the majority of cases less than 30 amino acids), low antigenicity (34) and exhibit a predominantly cationic

amphipathic structure making them prone to interact with anionic cell membrane surfaces (35,36). Indeed, various anionic molecules, discussed in the following, are more abundant on the surface of cancer cells as compared to non-cancer cells. This increased level of negative charge seemingly accounts for the selectivity of these cationic membrane-active peptides towards cancer cells. In addition, one can envisage that anticancer peptides, which translocate to the cytosol, interact preferentially with the mitochondrial membrane owing to its high content of negatively charged lipids such as cardiolipin. As a consequence the peptides may trigger apoptosis. In addition, the susceptibility of cancer and non-cancer cells towards anticancer peptides will be governed by differences in their membrane fluidity and/or by morphological changes such as the increased number of microvilli found on cancer cells. Figure 3 sketches these and additional parameters such as acidification of cancer cell environment that may be of benefit for the anticancer activity of histidin-rich peptides. Moreover, some important processes that suppress apoptosis in cancer cells are indicated.

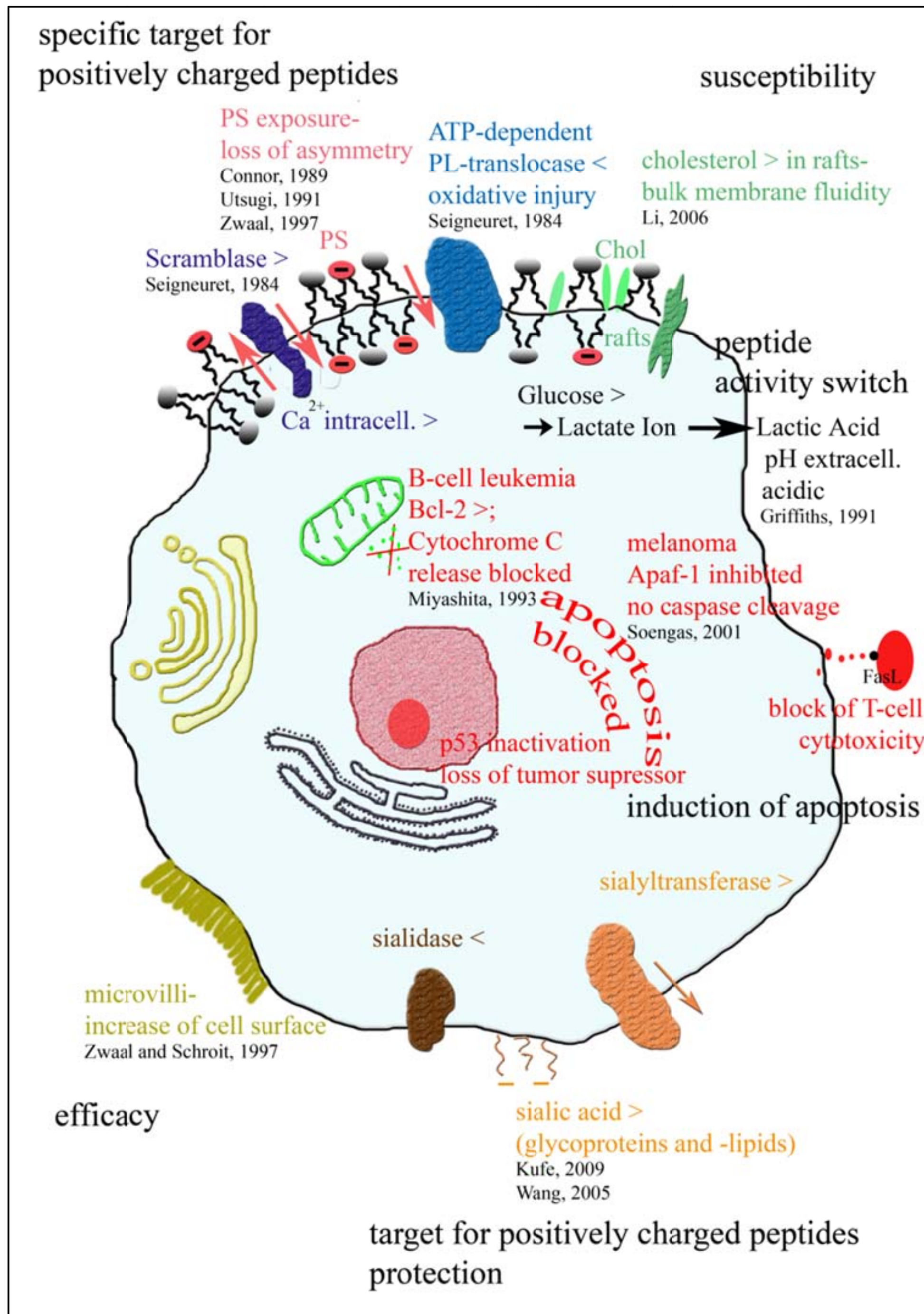

Figure 3: Sketch of specific characteristics of cancer cells concerning membrane and genetic changes: Selectivity of anticancer peptides can be driven by direct interaction with some of the targets indicated, i.e. these cationic peptides will bind to anionic molecules exposed on the surface such as PS. Exposure of PS is related to several cell processes, which involves activation of scramblase and inactivation of ATP-dependent phospholipid (PL) - translocase. Increased levels of sialic acid of glycolipids and – proteins on cancer surfaces, normally providing a protective shield, can also be targets for the positively charged peptides. Peptides, which translocate into the cytosolic compartment, may interact with cardiolipin, a major anionic lipid of

mitochondria, triggering apoptosis, a process normally blocked by several changes in tumor cells such as e.g. inactivation of the tumor suppressor p53. Changes in membrane fluidity and pH further influence susceptibility and activity of peptides. Furthermore, these peptides may also benefit by the increased surface area of cancer cells owing to the increased number of microvilli present.

Anionic phosphatidylserine exposed on the outer membrane leaflet of cancer cells

Indeed one of the major differences between cancer and non-cancer cell-surfaces is the exposure of the negatively charged lipid phosphatidylserine (PS) on the outer leaflet of the cancer cell membrane, which in non-cancer cells exhibits an overall neutral charge due to the zwitterionic phosphatidylcholine and sphingomyelin (37). Phosphatidylserine together with the major part of phosphatidylethanolamine is normally located in the inner leaflet of eukaryotic plasma membranes (37). This asymmetric distribution of the major phospholipids between the two membrane leaflets is well documented (38,39) and is maintained by an ATP-dependent aminophospholipid translocase which moves PS and PE to the inner leaflet (40) and/or abolished by a Ca^{2+} activated scramblase, which induces non-specific movement of phospholipids to both leaflets (38). The loss of membrane asymmetry was demonstrated for several cancer types, revealing a promising uniform marker for cancer. Within some studies PS exposed on the surface could even be visualized *in vitro* and *in vivo* by specific interaction with Annexin V, linked to a fluorophore (41-43). Since the pioneering work of Utsugi et al. (44) showing that tumorigenic Friend erythroleukemic cells expose 3-7-fold more PS than normal keratinocytes, PS exposure has been described for human ovarian carcinoma (45), human gastric carcinoma (46), different melanomas (47,48) and leukemias (49,50). In an extension of these studies, our laboratory focused on cancer with poor outcome and treatment efficacy including metastases as well as primary cell cultures and demonstrated that differently malignant melanoma cell lines expose 4-11 times more PS than melanocytes (Figure 4), whereby the amount of PS exposed correlated with tumor progression (51,52). Furthermore, we proved that not only cell lines derived

from primary lesions and metastasis show PS on the outer leaflet of the cell membrane but already cells from primary cancer cell cultures do. The number of different cancer types used in our study including (malignant) melanoma, a rhabdomyosarcoma, a malignant soft tissue tumor of mesenchymal origin, prostate cancer, renal cancer and a glioblastoma extended and strongly supported the earlier findings suggesting that PS exposure is a general phenomenon of cancer cells (51, 52).

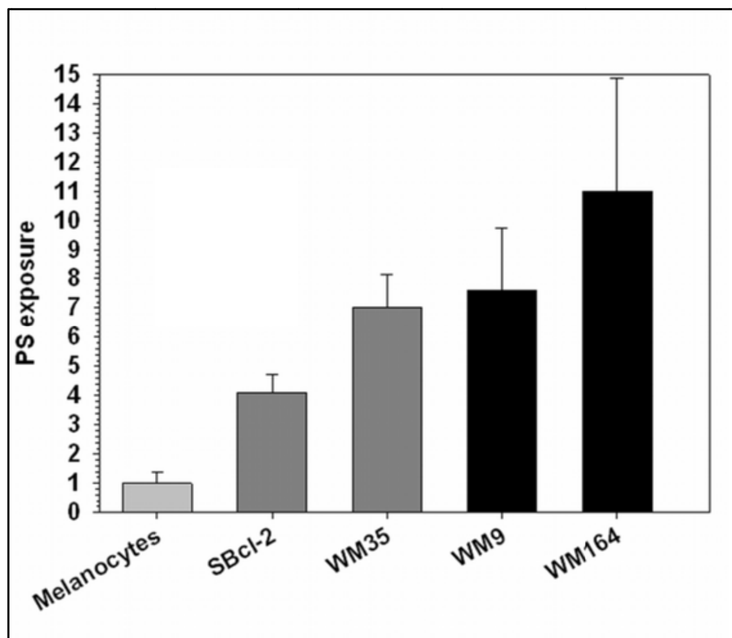

Figure 4: PS exposure of melanoma cell lines: Correlation of PS exposure with tumor progression of melanoma cell lines expressed as multiples of PS exposure of normal melanocytes as determined by Annexin V binding (SBcl-2, WM35 from primary and WM9, WM164 from metastatic lesions; error bars resulting from four independent experiments). Data adopted from (51;52).

In general, cells expressing PS in the outer leaflet of the plasma membrane are specifically recognized by macrophages (53,54) and dendritic cells (55) triggering apoptosis. However tumor cells, although exposing PS, circumvent apoptosis and prevent the recognition by macrophages (Figure 3). For

example, lung and colon cancer cells secrete elevated levels of a molecule that binds to FasL, a death activator on T-cells, and prevents being killed. While melanoma inhibit expression of a gene encoding Apaf-1, the apoptotic protease activating factor-1, certain B-cell leukemias and lymphomas express high levels of Bcl-2, a protein that blocks apoptotic signals (22,56). Therefore, cationic host defense peptides, which show an excellent tumor tissue penetration reaching the site of both primary tumor and distant metastasis (57) and specifically interact with anionic membrane lipids (58-60), represent a promising approach in the development of novel anticancer drugs (26-29). Finally it is also of interest to note that anionic phospholipids are also present on the surface of tumor blood vessels connected to tumor tissue (61). The observation of negatively charged tumor vasculature provides an additional potential target for anticancer peptides, which may be also used for imaging. For example, selective staining of vascular endothelium of different tumors and also tumor cells in and around regions of necrosis was shown using 9D2, a monoclonal antibody for anionic phospholipids or Annexin V (61). Further it is reported that radiolabeled Annexin V may allow for repetitive and selective *in vivo* identification of apoptotic cell death without the need for invasive biopsy (62), which should also be applicable for imaging of tumor tissue.

Enhancement of sialic acid residues on cancer cell surface

Another source of negative charges on the surface of human cells is represented by sialic acid residues, which are linked to glycoproteins (e.g. mucins) and glycolipids. An overexpression of transmembrane mucins was shown e.g. for carcinoma cells from epithelia (63,64) and transmembrane mucin 1 (MUC1) was overexpressed in more than 90% of breast carcinomas and frequently in others such as ovarian, lung, colon and pancreatic carcinoma (65). The extent of surface sialylation seems to be directly correlated with the metastatic potential of different cancer cells exhibiting a protective function against the host immune system and facilitating migration and invasion via modulation of adhesiveness (66-68). The effect of different degrees of sialylation in

terms of peptide interaction has been studied less intensively. For example, BMAP-27 and BMAP-28, host defense peptides from the cathelicidin family, exhibited lower activity on cancer cells, when sialic acid had been cleaved off (69) suggesting that the outside charge seems to act as an initial interaction site for the peptides. In contrast, in a preliminary study performed in our laboratory the activity of peptides derived from a fragment of human lactoferricin, was unaffected by cleavage of sialic acid moieties of a rhabdomyosarcoma (unpublished data), suggesting another than sialic acid as preferred target for this tumor type. Summarizing, it is obvious that glycosylation can play a role in the interaction with peptides. For more details about changes in the glycosylation pattern in cancer cells and its impact on cell processes like cancer progression, impairment of signaling pathways see Hoskin and Ramamoorthy (26).

Heparan sulfate

In addition to the above mentioned sources of negative charges on the surface of cancer cells, proteoglycans should be noted (70). These proteins contain highly negatively charged glycosaminoglycan side chains mainly in the form of heparan sulfate and chondroitin sulfate composed of linear repeats of sulfated disaccharides (71,72). These disaccharides contain a high number of sulfate groups yielding highly negatively charged molecules (73). It has been reported for several cancer cells that proteoglycan expression and sulfation is altered (74-80). Fadnes et al. (81) reported that chondoitrin sulfate had no effect on the activity of bovine lactoferricin (bLFcin), which however was improved after depletion of heparan sulfate on cancer cells and lowered upon addition of exogenous heparan sulfate. Although this observation seemingly implies that heparan sulfate may inhibit the activity of anticancer peptides, the same group showed recently that small lytic peptides derived from bLFcin are not affected by heparan sulfate or even possess increased activity in their presence (82).

Altered membrane fluidity in cancer cell membranes

Besides of changes in the pattern of the surface charge membrane fluidity also appears to be altered in tumorigenic cells, though it is not clear, if this alteration is uniform throughout all carcinomas. The majority of studies indicate an increased fluidity of the cancer cell membrane as described for lymphomas, lung carcinomas, and neural tumors (83-86). Contrariwise it has been reported that cells from solid tumor tissues (e.g. hepatoma) exhibit less membrane fluidity than their healthy counterparts (87). In this context it is important to note that already Sherbet et al. (88) suggested that membrane fluidity is not uniform all over the cell itself. This is in accordance with more recent observations showing that some breast and prostate cancer cell lines possess elevated levels of cholesterol-rich lipid rafts, raising the question if only some parts of the cancer membrane comprise increased fluidity by different lateral lipid arrangement (89). In the case of unaltered total cholesterol content this would imply a cholesterol-depleted bulk membrane harboring PS that may exhibit higher susceptibility to peptide attack, because of its increased fluidity and hence less tightly packed lipids. Interestingly, also the metastatic cascade seems to be influenced by alterations in membrane fluidity. Thus, increased fluidity was reported for metastatic cells (90) due to differences in the cholesterol to phospholipid ratio (91). According to some reports resistance to drugs seems to be also related to cell membrane fluidity. May et al. (1988) (92) for example reported that vinblastine resistant leukemic cells possess 50% more cholesterol, corresponding to decreased fluidity, than vinblastine sensitive cells. This finding is supported by Mazzoni et al. (1993) (93), who showed higher cholesterol levels in multidrug-resistant ovarian cancer cells (93). It is evident that knowledge of membrane fluidity - and cholesterol content is only one parameter besides of degree of hydrocarbon chain saturation etc. - is an important physical membrane parameter that interferes with insertion of amphipathic peptides being facilitated in more fluid membranes.

Microvilli increasing the cell surface area of cancer cells

Another important difference between cancer and non-cancer cells is the increase of surface area of tumorigenic cells, triggered by the increase of microvilli on these cells (27,38,94,95). This should be beneficial for the application of membrane-active peptides, since efficiency can be increased by enabling the binding of a larger amount of peptides per cell (27,38). Support for this notion comes from a comparative study on the cytolytic effect of cecropin B on tumor cells such as KG-1 leukemia and AGS stomach carcinoma and non-tumor cells like fibroblasts and red blood cells, which demonstrated that the peptide was more effective on the cancer than normal cells (96). Based on scanning electron microscopy data it was suggested that this may mainly be due to the high population of irregular microvilli on the cell surface of the cancer cells and that the attraction of peptides by microvilli may be one of the main driving forces before membranolysis can be efficiently initiated.

In summary, accumulation of cationic amphipathic peptides will be triggered by the presence of anionic components on the cell surface as well as by the increase of cell surface owing to increased number of microvilli, while insertion of peptides will be mainly governed by membrane fluidity, which in eukaryotic membranes is largely regulated by its cholesterol content. Therefore, the differences in lipid composition and morphology of cell membranes will govern the interaction with membrane-active peptide and furthermore, may be also related to the different susceptibility of different types of cancer cells against a certain antitumor peptide (97,98).

Table 1: Selected host defense peptides tested in *in vitro* studies for anticancer activity[a]

Peptide / *Source*	**Sequence**	***in vitro* study**
α-helical		
Aureins *Southern bell frog*	GLFDIIKKIAESF (aurein 1.2)	60 cancer cell lines tested (human tumor line testing program of the US National Cancer Institute) (~50 active) (99)
BMAP-27 / BMAP-28 *bovine*	GRFKRFRKKFKKLFKKLSPVIPLLHLG/ GGLRSLGRKILRAWKKYGPIIVPIIRIG	human tumor cells, leukemic cells (69)
cecropin A *Hyalophora cecropia*	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	bladder cancer (100)
cecropin B *Chinese oak silk moth*	KWKIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL	leukemia (101), bladder cancer (100)
citropins *Australian blue mountains tree frog*	GLFDVIKKVASVIGGL (citropin 1.1)	60 human cancer cell lines (human tumor line testing program of the US National Cancer Institute) (102); human histiocytic lymphoma cell line (103)
epinicidin-1 *fish*	GFIFHIIKGLFHAGKMIHGLV	human lung carcinoma, cervix adenocarcinoma , hepatocellular carcinoma, fibrosarcoma, histiocytic lymphoma cells (104)
gaegurin-6/-5 *Korean wrinkled frog*	FLPLLAGLAANFLPTIICKISYKC	human lung, prostate, liver, kidney and breast cancer cell lines (105)
LL-37 *Homo sapiens*	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Jurkat human T leukemia cells, HeLa cells (106)
magainins *African clawed frog*	GIGKFLHSAKKFGKAFVGEIMNS (magainin 2)	hematopoietic cell lines (30), Ehrlich ascites tumor cells, human adenocarcinoma (107), bladder cancer line (108), histiocytic lymphoma cell line (103)
Pexiganan (MSI-78)	GIGKFLKKAKKFGKAFVKILKK	
melittin *Honey bee venom*	GIGAVLKVLTTGLPALISWIKRKRQQ	human hepatocellular carcinoma (109); osteosarcoma (110); leukemic cells (111); prostate and ovarian (112), neuroblastoma cancer cells (113)
NK-2 *porcine leukocytes*	KILRGVCKKIMRTFLRRISKDILTGKK	human lymphocytes and seven human cancer cell lines (50); human neuroblastoma cancer cells (Drechsler and Andrä, 2011)
NKCS and derivatives	KILRGVSKKIMRTFLRRISKDILTGKK	human prostate carcinoma cells (114)
Pep27 *Strep. pneumonia*	MRKEFHNVLSSGQLLADKRPARDYNRK	leukemia cell lines (115)
polybia-MPI *Polybia paulista*	IDWKKLLDAAKQIL	human bladder cancer and prostate cancer cell line (116),
temporin A *frog Rana temporaria*	FLPLIGRVLSGIL	human histiocytic lymphoma cell line (103)

β-sheet		
buforin II *Bufo bufo gargarizans*	TRSSRAGLQFPVGRVHRLLRK	62 cell lines (117); human histiocytic lymphoma cell line (103)
human α-defensins *Homo sapiens*	ACYCRIPACIAGERRYGTCIYQGRLWAFCC (HNP1)	human lung adenocarcinoma (118)
defensins	diverse sequences	HeLa, glioma, kidney cancer, lung cancer, myeloma (34)
gomesin *Acanthoscurria gomesiana*	ECRRLCYKQRCVTYCRGR	breast and colon adenocarcinoma, HeLa (86)
lactoferricin B *bovine*	FKCRRWQWRMKKLGAPSITCVRRAF	neuroblastoma (119), melanoma, mammary carcinoma, colorectal adenocarcinoma (120)
protegrin-1 *porcine*	RGGRLCYCRRRFCVCVGR	human histiocytic lymphoma cell line (103)
tachyplesin I *Southeast Asian horseshoe crab*	KWCFRVCYRGICYRRCR	human gastric adenocarcinoma (121); human hepatocarcinoma cells (122);
Others		
PR-39 *porcine small intestine*	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	human hepatocellular carcinoma cells (123)
alloferon-1/-2 *insects*	HGVSGHGOHGVHG / GVSGHGVHG	mice (IFN-production) (124)
dolastatin 10 *Dolabela auricularia*	Dov-Val-Dil-Dap-Doe[b]	murine PS leukemia cells (125)

[a] for a complete list of anticancer peptides check http://aps.unmc.edu/AP/database/antiC.php
[b] dolavaline (Dov*)*, dolaisoleuine (Dil*)*, dola- proine (Dap*)*, dolaphenine (Doe)

Mode of action of anticancer peptides

Two general mechanisms of anticancer peptides, triggering necrosis or apoptosis as a consequence of their membrane related mode of killing of cancer cells, have been discussed (27,29,99). Both, killing based on necrosis via cell membrane lysis and killing based on apoptosis via the mitochondrial lytic effect seem to be dependent on the presence of anionic lipids. As described above negatively charged PS located in the outer leaflet of cancer cells can represent such a specific target, as can be cardiolipin, a major anionic phospholipid of the mitochondrial membrane. Other sources of negatively charged molecules, which are also exposed on non-cancer plasma membranes such as sialic acid, may play a minor role. This is reflected by the high selectivity of cationic peptides, like NK-2 (50) or human lactoferricin derivatives (see 2 last manuscripts of the thesis for different cancer cell lines over normal cells. Similarly, selective binding of the synthetic host defense-like membranolytic peptide, [D]-K6L9, to PS was demonstrated by co-localization of PS and peptides on the outside of cancer cells (100). Therefore, it was suggested that the exclusive selectivity of the peptide towards cancer cells derives from its binding to surface PS and that killing of the cancer cells occurs via cytoplasmic membrane depolarization. Consequently, it appears that for effective disruption of the cell membrane peptide-lipid interaction is the crucial step for which the peptides need to meet the proper structural requirements. Thus, in order to engineer more potent antitumor peptides in respect of efficacy as well as selectivity and thus reduced toxicity, it is necessary to further unravel their molecular mode of action.

In addition, non-membrane related mechanisms have been considered. For example, stimulation of NK cells as well as interferon synthesis by the insect antimicrobial peptide alloferon (101) or the transcriptional inhibitory effect of melittin and cecropin (102) were attributed to be probably due to an indirect mechanism, which is mediated by the ability of the cytolytic peptide to interfere with signal transduction pathways. Further, tachyplesin conjugated to an integrin homing domain

(103,104) was shown to interact with the mitochondrial membranes of cancer cells and to up-regulate members of the death receptor pathway, which suggests that this peptide-conjugate has also more than one effect on cancer cells. Therefore, Papo and Shai (27) proposed that these observations may imply that, in addition to their being active on their own, cytolytic peptides may activate or act synergistically with other host defense components in order to clear tumors. Nevertheless, since killing takes place very rapidly a non-receptor mediated membranolytic killing mechanism seems to be quite reasonable for the majority of anticancer peptides derived from host defense peptides.

Membrane related molecular mechanisms of cell killing

The discovery that naturally occurring antimicrobial peptides mostly kill bacteria by damaging their cell membrane has initiated much effort to study peptide-membrane interaction in detail. The concept of a characteristic lipid composition for a given cell membrane is well accepted and hence the different physicochemical properties of the lipids found in Biological membranes allow host defense peptides to discriminate between e.g. bacterial, cancer and non-cancer cell membranes (43,60,105). In a simplified view these cationic amphipathic peptides will exhibit a higher affinity to cell membranes that expose negatively charged lipids on their outer membrane leaflet. The molecular mechanism(s) of membrane damage mutually depends on the nature of both peptides and membrane lipids (106).

Several models (Figure 5) have been suggested to explain their biological activity (for reviews see e.g. (35,36,107,108). The most frequently discussed mode of action includes the formation of toroidal pores (109,110) and the carpet model (111). In the toroidal pore model peptides, together with the lipid, assemble into a supra-molecular arrangement of high curvature forming a water-filled pore.

Although a number of molecules including α-helical and β-sheet type peptides have been shown to adopt such a pore under the given experimental conditions (112), there is little evidence that the majority of these peptides act *in vivo* via transmembrane pores (113). In order to explain membrane disintegration by a non-pore model the carpet model is the most commonly cited one. In this model the amphipathic peptides accumulate at the membrane being aligned parallel to the bilayer surface. Once this "peptide carpet" becomes too dense, membrane permeabilization occurs via destabilization of the membrane bilayer (111). Thereby, at the molecular level different scenarios may apply, which were recently summarized in an excellent review by Wimley and Hristova (108). Briefly, membrane permeabilization has been proposed to be due to (a) peptide induced clustering or phase separation of lipids creating defects at the peptide-rich and -poor domains (36,59,114-117), (b) the sinking raft model, which allows a formal thermodynamic analysis to predict membrane perturbation (118-121) (c) disruption of the normally strict segregation of polar and non-polar groups of the lipids driven by the partitioning of an imperfectly amphipathic peptide into the bilayer interface (122,123) or (d) a detergent-like action in particular at high peptide concentration (124-126). Furthermore, recently a complex generic phase diagram has been developed rationalizing the membrane interactions of cationic amphipathic peptides by their molecular shape to explain in a more general sense the mode of action of these cationic amphipathic peptides (107,125). Besides electrostatic interactions, molecular properties of lipids such as their molecular shape, being related to the spontaneous curvature, are known to play a significant role for the aggregation state of lipids (107,127,128) and, thus, also affect interactions of these assemblies with membrane-active peptides. In fact, in terms of structural membrane changes insertion of antimicrobial peptide molecules, some of them exhibiting also antitumor activity such as NK-2 (50) or lactoferricin derivatives (43), promoted the formation of inverted hexagonal or bi-continuous cubic phases in membrane mimetic systems (59,129-132).

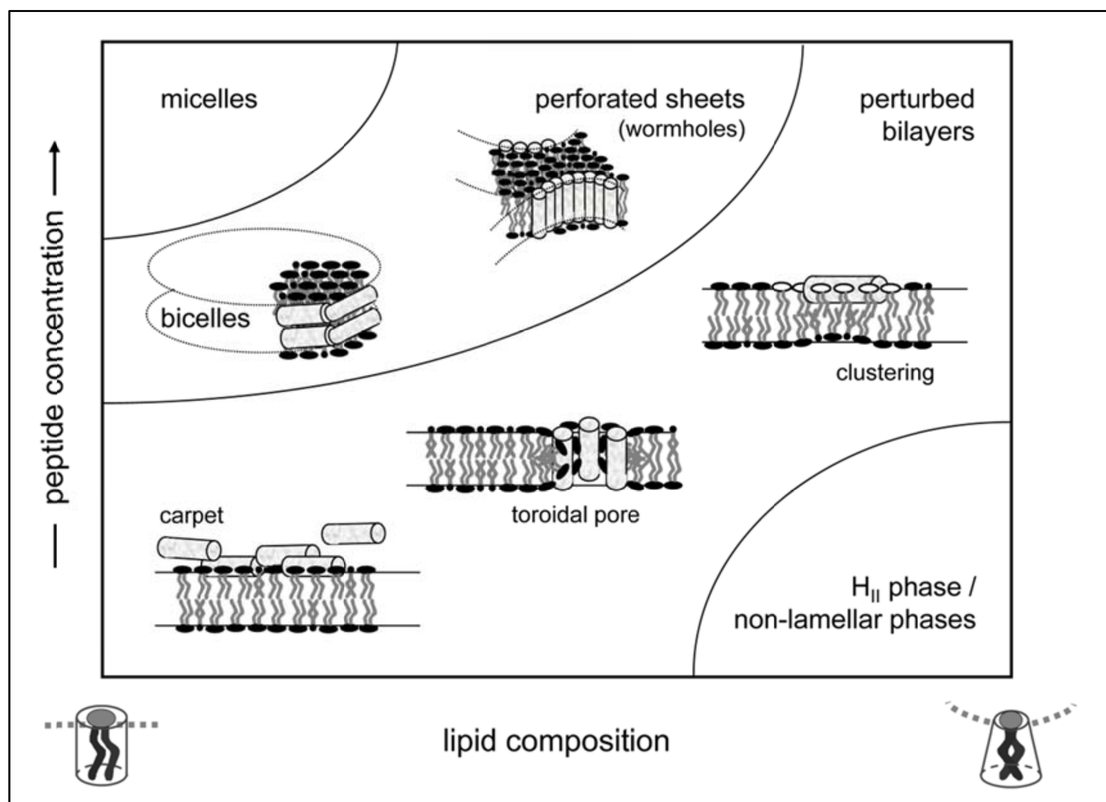

Figure 5: Mode of action of membrane-active host defense peptides: Boundaries of the schematic phase diagram of amphipathic peptide/phospholipid aggregates are given as a function of the concentration of membrane-associated peptide and the composition of the membrane from mixtures of cylindrical (phosphatidylserine, phosphatidylcholine) and truncated inverted cone lipids (cardiolipin, phosphatidylethanolamine); modified from (133). Membrane association of the cationic peptides is strongly increased in the presence of anionic phospholipids such as PS or cardiolipin. In the presence of cholesterol, an abundant membrane component in mammalian cells, the bilayer will be stabilized, i.e. the phase region of stable bilayers will be enhanced. Some molecular mechanisms of bilayer perturbation are schematically shown (for details see text above).

Supplementary, solid tumors are discussed to exhibit an acidic extracellular environment (for a review see (133)). This acidification seems to be a consequence of lactic acid accumulation, which is produced during aerobic and anaerobic glycolysis and, due to inadequate vasculature in growing tumors, its insufficient removal. However, lactic acid accumulation does not seem to be the only

reason for the acidification of the extracellular environment of tumors. Newell et al. (134) reported that glycolysis-deficient cells are also more acidic than cells from normal tissues without accumulating lactic acid above serum levels. In any case, an acidic tumor environment might be a useful “activation” switch for anticancer peptides comprised of amino acids like histidine, which are only positively charged and thus active at low pH (135). Such an approach was reported for the synthetic host defense-like lytic peptide [D]-K6L9, where the lysine residues were exchanged by histidine resulting in reduced systemic toxicity compared to the parent peptide. It was suggested that the pH-dependent activity and lytic mode of action of the His-rich peptide, hindering development of resistance, will make them potential candidates for treatment of solid tumors. In fact, intra-tumor and systemic inoculation of [D]-H6L9 significantly reduced the 22RV1 prostate cancer tumor size in a xenograft mice model (135).

In the context of taking advantage of the acidic environment of solid tumors it is worthwhile to report on pHLIP (pH (Low) Insertion Peptide), a peptide derived from the C-helix of bacteriorhodopsin, although this peptide neither displays a membranolytic mechanism nor is derived from a host defense peptide. pHLIP shows interesting pH-sensitive properties in a way that it only forms a transmembrane helix in the acidic environment of solid tumors, whereas at neutral or high pH it is in equilibrium between an unstructured form and bound to the surface of a lipid bilayer (136,137). pHLIP itself does not exhibit direct toxicity to cells (138) or mice (139) at concentrations below 50 µM. However, the peptide in its helical form possesses useful drug delivery efficiency facilitating translocation of cell-impermeable polar molecules into the cytoplasm (137-141). One example for efficient drug delivery into cancer cells through translocation with pHLIP is phalloidin toxin, which inhibits cancer cell proliferation after cleavage from pHLIP inside the cells (142).

Non-membrane related mechanisms

Here, we briefly summarize some mechanisms, which do not involve permeabilization/disruption of plasma or mitochondrial membranes in cancer cells. There is growing evidence that the antimicrobial activity of host defense peptides is not solely related to cell membrane damage but some act via immunomodulatory effects (143-145). Less knowledge has been gained so far, if this may be also the case for the antitumor activity of these cationic amphipathic peptides. In particular for cancers, where the immune response is often diminished or strongly suppressed, it was suggested that cancer therapies might be supported by the simultaneous administration of immunoadjuvants, which activate innate and acquired immune system (146). Although many small peptides of microbial origin possess a nonspecific immunostimulatory response (for a review see (29)), reports for an immunomodulatory effect of host defense peptides in cancer cells are scarce. One example are alloferons, histidine-rich peptides from insects, shown to stimulate natural killer cells of human peripheral blood lymphocytes and to induce synthesis of interferon in mice model (101). This cytokine-like modulating property resulted in antitumor resistance.

Specific targets have been reported so far only for a few anticancer peptides. PR-39, a peptide of the cathelicidin family being rich in proline and arginine residues, can translocate into the cytosolic compartment of cells without permeabilizing the plasma membrane, where it interacts with proteins containing SH3-binding motifs (147,148). Experiments using derivatives of PR-39 indicated that the N-terminal arginine is crucial for binding to the SH3-domain and subsequent induction of syndecan-1 production (149). Indeed, elevated levels of syndecan-1 were observed upon treatment of human hepatocellular carcinoma cells with either PR-39 or upon transfection of this cell line with the PR-39 gene (150). Expression of syndecan-1 has been related to inhibition of cell invasion (151) and thus it was suggested that this linear cationic peptide may suppress the invasive activity of these cancer cells preventing metastasis (150). Another target of many anticancer drugs is tubulin, which was also

shown to be the site of action for linear peptides derived from the host defense-like peptide dolastatin 10 (US Pat. No. 4,816,444) or dolastatin 15 (EP-A-398558) (152). Thereby, the mechanism of action, as for all anti-tumor tubulin ligands, involves the perturbation of microtubule dynamics during the G2/M phase of cell division and subsequent entry into apoptosis (153).

Finally, a specific case was reported for bee venom melittin, which preferentially counter-selects mammalian cells with the overexpressed ras oncogene, where it acts through hyperactivation of phospholipase A2 (154). It should be noted however that melittin is also known for its toxicity to normal mammalian cells (e.g. (155)). In fact, for *in vivo* studies a melittin/avidin conjugate was used, which did not exert its anticancer activity by membranolysis but by targeting cancer cells with high matrix metalloproteinase 2 (156) as described below.

***In vivo* studies of anticancer peptides**

Based on successful *in vitro* experiments a number of peptides have been tested also *in vivo* to explore their therapeutic potential (Table 2). In addition to the administration of anticancer peptides per se strategies have been developed that may improve pharmacokinetics and/or selectivity and hence reduce toxicity. This includes the application of vector mediated delivery of genes encoding membranolytic peptides and the use of homing domains transporting the anticancer peptides into the cytosolic compartment of cancer cells. In the following some of the respective *in vivo* studies will be briefly described.

Table 2: *In vivo* studies of anticancer peptides and proposed mode of action

Peptide[a]	Mode of action	Type of tumor
α-helical		
magainin and analogues	lytic	murine ascites tumors (107); melanoma xenograft in nude mice (181)
melittin/avidin-conjugate	lytic	melanoma in mice (112)
polybia-MPI / MPI-1	necrosis	sarcoma xenograft tumors in mice (182)
β-sheet		
human α-defensins	apoptosis	human lung adenocarcinoma xenograft in nude mice (118)
gomesin	necrosis	murine melanoma (183)
lactoferricin B	necrosis / apoptosis	fibrosarcoma, melanoma, colon carcinoma (184); xenografting of SH-SY-5Y cells in nude rats (119); inhibition of metastasis of melanoma and lymphoma in mice (185)
RGD-tachyplesin	apoptosis	melanoma mice (130)
others		
dolastatin 10	antiproliferative	indolent lymphoma, Waldenstrom's macroglobulinemia and chronic lymphocytic leukemia (177)
synthetic		
[D]-K_6L_9	necrosis	breast cancer in SCID/NCr mice (127)
[D]-H_6L_9	lytic	22RV1 prostate cancer in mice (161)

[a] source and amino acid composition of peptides are listed in Table 1.

Anticancer activity of peptides related to membrane damage

Magainins, originally isolated from the skin of the African clawed frog, Xenopus laevis (157), were among the first peptides that have been tested for their *in vivo* anticancer activity. Magainin 2 was shown to exhibit an amphiphilic α-helical structure that facilitates the formation of ion-permeable pores in membranes, consequently inducing depolarization and cytolysis (30). Baker et al. (158) demonstrated that magainin analogues were able to elevate the survival level of animals with ascites-producing tumors. Furthermore, magainin 2 has been tested for local treatment in a subcutaneous xenograft model of melanoma in nude mice, where it completely eliminated the tumor with only minimal damage to surrounding tissue (159).

Another prominent member of anticancer peptides is bovine lactoferricin (bLFcin), which is generated from lactoferrin through pepsin cleavage. Unlike magainins, bLFcin possesses an acyclic

twisted antiparallel β-sheet structure due to a disulfide bridge between two cysteine residues (160). Yoo et al. (161) demonstrated that the peptide inhibits liver and lung metastasis in mice. Additionally, bLFcin showed antiangiogenic activity leading to suppression of tumor growth. *In vivo* studies with bLFcin on fibrosarcoma, melanoma and colon carcinoma tumors revealed massive necrosis of the tumor tissue after exposure to the peptide (162). Eliassen et al. (163) also showed that bLFcin inhibits significantly tumor growth of neuroblastoma xenografts in nude rats. Clarification of the mechanism revealed that bLFcin induces apoptosis in human tumor cells through a pathway mediated by production of the intracellular ROS and activation of Ca^{2+}/Mg^{2+}-dependent endonucleases (161), which was partially confirmed by studies of Mader et al. (98), who also documented the mitochondrial pathway of apoptosis for bLFcin. Other studies reported that induction of apoptotic or necrotic cell death was dependent on the concentration of the peptide (163,164). Recently, it was shown that *in vitro* treatment with bLFcin killed Raji and Ramos human B-lymphoma cells via a caspase-independent apoptotic pathway being more efficient at low serum concentration of the peptide (165), Notably, in the presence of exogenous bovine serum albumin the activity was inhibited, which suggests partial neutralization of the cationic peptide by anionic serum components, as described earlier for the inhibition of the tumoricidal activity of amphiphilic alpha helical peptides (166). Nevertheless, immune-deficient SCID/beige mice exhibited extended survival using systemic administration of bLFcin emphasizing the potential of this peptide for further development as novel anticancer agent.

Gomesin (Gm), an antimicrobial peptide isolated from the hemocytes of the unchallenged Brazilian spider Acanthoscurria gomesiana, also exhibits anticancer activity (167). This cationic peptide adopts a hairpin-like two-stranded antiparallel β-sheet structure, which is maintained by two internal disulfide bridges (168). Topical treatment of subcutaneous murine melanoma with gomesin significantly retarded tumor growth and increased the number of animals with tumors below a

critical size (169). Importantly, Gm did not exhibit any toxic effect on the peripheral healthy skin of mice after repeated applications of the peptide. Gm does not kill tumor cells through apoptosis rather through induction of morphologic cell alterations with increased granularity, loss of cytoplasmic content by membrane permeabilization, and partial collapse of the proton gradient (169).

Polybia-MPI (MPI) is an anticancer peptide derived from the venom of social wasp Polybia paulista. In order to improve the activity and stability of MPI, the C terminal amide of the peptide was replaced by a thioamide group (MPI-1) (170). Indeed, MPI-1 suppresses more efficiently tumor growth of sarcoma xenograft in mice than MPI, which was attributed to its higher serum stability. However, the modification did not affect the cytotoxic behavior of the peptide. *In vitro* studies give evidence for a killing through necrosis as a result of acute cell injury, swelling and bursting via disrupting the membrane or forming transmembrane pores (171).

An alternative approach for *in vivo* use of membranolytic peptides to maintain therapeutic levels and reduce cytotoxicity was suggested by Winder et al. (102) introducing vector-mediated delivery of genes encoding cecropin or melittin into a human bladder carcinoma derived cell line(102). The resultant cell clones were analyzed for tumorigenicity in nude mice showing that expression of cecropin resulted in either a complete loss of tumorigenicity in some clones or reduced tumorigenicity, as measured by latency of tumor formation. Similarly, a plasmid that expressed secretable human α-defensin-1, also known as the human neutrophil peptide-1 (HNP1) was used for gene therapy of a human lung adenocarcinoma xenograft in nude mice (172). In this study, Xu et al. (172) showed that HNP1 can be intracellularily expressed in tumor cells resulting in significant inhibition of tumor growth by induction of apoptosis.

Anticancer agents based on anticancer peptide-conjugates

Melittin, extracted from the European honey bee Apis mellifera, is a mostly α-helical peptide that induces cell lysis supposedly by pore formation (173,174). Since melittin is highly toxic to red blood cells and untransformed cells, a melittin/avidin conjugate was designed to gain an inactive peptide towards healthy cells, as it specifically targets cancer cells with high matrix metalloproteinase 2 (MMP2) activity (156). MMP2 is known to be over-expressed in several cancers and tumor microvascular endothelial cells and has the ability to reactivate the peptide activity by cleavage of the conjugate at the tumor site. *In vivo* studies demonstrated that the size of B16 tumors in mice decreased significantly after treatment with the melittin/avidin conjugate. Most likely, activity of the conjugate arises from its ability to target tumor vasculature since levels of cell lysis in cultured cells was relatively low (156).

Tachyplesin, a peptide isolated from hemocytes of the horseshoe crab (Tachypleus tridentatus), exhibits a structure with exposed basic amino acids on the peptide surface (175), which enables binding to anionic phospholipids (103,176). The peptide was shown to have anticancer activity *in vivo* in a B16 mouse model (104). In this study, a synthetic tachyplesin was conjugated to the integrin homing domain RGB, which enables internalization of the peptide.

Anti-proliferative peptides

Dolastatin 10 (US Pat. No. 4,816,444) derived from the family of dolastatins, a peptide isolated from the marine shell-less mollusk Dolabela auricularia , finished Phase II trial against indolent lymphoma, Waldenstrom's macroglobulinemia and chronic lymphocytic leukemia in 2000 (152). At the time of discovery it was shown to be the most potent anti-proliferative agent against murine PS leukemia cells (177). Eventually, the peptide had to be dropped of clinical trials because of moderate

peripheral neuropathy in 40% of patients. Therefore, derivatives of dolastatin 10, like TZT-1027 (Soblidotin) (178) or of dolastatin 15 (antimitotic), like ILX-651 (Synthadotin) (179) showing reduced toxicity but still potent antitumor activity have been further explored in clinical studies.

Challenges for the development of host defense peptides as novel anticancer agents

Interestingly, despite the numerous successful *in vivo* studies none of the membrane-active host defense peptides has made it to the market so far. This may have several reasons, whereby their development as novel anticancer agent may be mainly hampered by the poor bioavailability and potential toxicity of such peptides (143). Peptide degradation in serum owing to the presence of proteases is one major obstacle, which besides of unspeficic binding to serum components limits the half time of these molecules in serum. In order to improve the pharmacodynamic properties different strategies may be applied to result in enhanced cell specificity and serum stability.

Enhancing selectivity to further reduce cytotoxicity

Not all host defense peptides showing antitumor activity are sufficiently selective against cancer cells. Accordingly, a more detailed knowledge of their structure-activity relationship is required to understand the molecular peptide parameters for the design of promising novel antitumor agents. As outlined before host defense peptides exhibit great structural diversity, but share some common features. They are typically 10 to 40 amino acids long and positively charged (net charge of +2 to +9). While differing in the percentage of hydrophobic residues, these peptides share an amphiphilic character, i.e. they have the ability to adopt a structure in which hydrophobic and polar/charged residues are spatially organized in discrete sectors of the molecule. Although host defense peptides belong to different structural classes, they often exhibit an intrinsic flexibility and structural adaptability, which is thought to be responsible for different modes of interaction with membrane

bilayers (180). Thus net charge, amphipathicity, hydrophobicity, and structural propensity are among the most important physicochemical and structural parameters that govern the ability of these peptides to interact with cell membranes. A statistical analysis of the antitumor peptides listed in the Antimicrobial Peptide Data Base (32,33) in terms of secondary structure, length of the peptide, distribution of charged and hydrophobic amino acid residues and determination of hydrophobicity expressed as transfer free energy of peptides from water to n-octanol (ΔG_{woct}) (181) shows that these parameters can vary within a wide range (Figure 6). It is interesting to note that the fraction of peptides with unknown structure (~39%) is comparable to the fraction of peptides with α-helical structure (~34%), which seems to be the predominant one. However, as can be deduced from Table 1 and 2 there have not been much less peptides tested successfully in *in vitro* and *in vivo* studies, which exhibit a β-sheet structure, although these peptides account only for ~2% of the total antitumor peptides listed. Nevertheless, a cluster analysis of a database of 158 peptides, which form an amphipathic α-helix, was performed to reveal detailed structural characteristics of anticancer peptides (182). This database was constructed using data presented by Owen (US Patent 6875744), and contains 14 peptides, which show selectivity for cancer cell lines only, 123 peptides showing cytotoxicity also to non-cancer cells and 21 inactive peptides. Three-dimensional clustering techniques were used to group these peptides with respect to similarity in net positive charge, hydrophobicity and hydrophobic moment producing 21 clusters. The observation that the inactive peptides were found across seven clusters emphasizes that these three parameters alone cannot be used as predictors of activity. However, as hydrophobicity of peptides is important for membrane penetration, in addition an extended hydrophobic moment plot analysis was performed. Although peptides with arc sizes >270° appeared to be less toxic, statistical analysis could not find a direct correlation between arc size and toxicity. Furthermore, using this approach it was predicted that over 50% of the active peptides would be surface active. This suggests that amphiphilicity is a key driver of the membrane interactions for these peptides. Thereby, peptides showing cancer cell specificity fell within a narrow range of amphiphilicity of 0.53-0.78 and had no tilted helical peptide structure,

which within the analysis was more commonly found for peptides being toxic to both cancer and non-cancer cells. Such a tilted structure has been associated already earlier with relatively non-specific means of cell membrane lysis (26). This cluster analysis in conjunction with an earlier work of the same group (183) suggests that the interplay of a range of physicochemical characteristics rather than any single overriding factor determines the Biological activity of these peptides. Moreover, to add to the complexity of the system one has to keep in mind that the lipid composition of the target membrane has not to be neglected, as in turn it strongly affects the penetration behavior of peptides. Therefore, application of newly developed technologies for high-throughput screening as described recently for antimicrobial peptides (184) may be beneficial to unravel the molecular parameters for anticancer peptides being more suitable for clinical use, i.e. to exhibit high specificity and activity, low susceptibility to proteolysis and reduced toxicity.

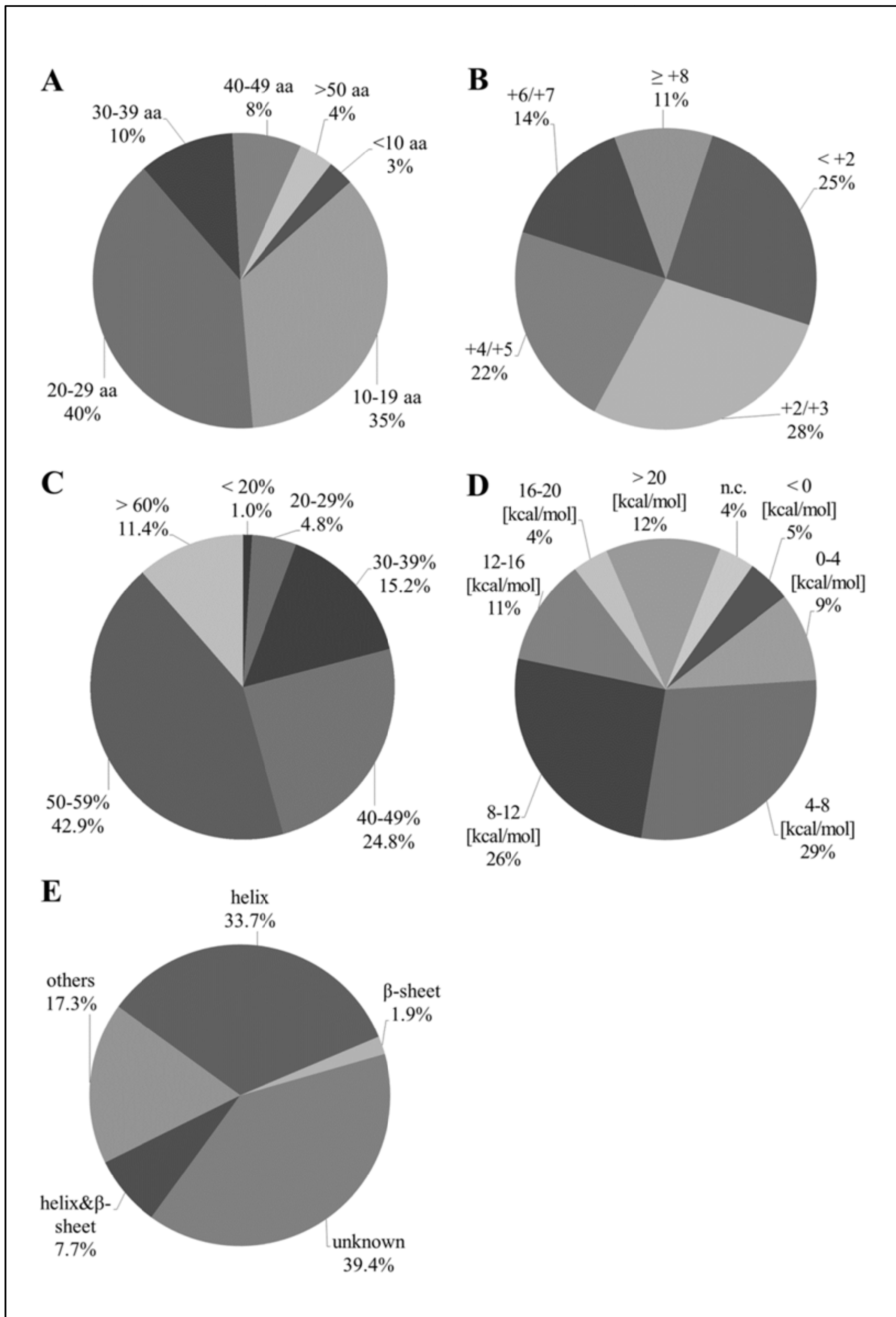

Figure 6: Characteristics of anticancer peptides: (A) peptide length; (B) net charge; (C) hydrophobic content (% hydrophobic amino acids; http://aps.unmc.edu/AP/design/design_improve.php); (D) hydrophobicity of peptides expressed as transfer free energy of peptides from water to n-octanol (ΔGwoct) (http://blanco.biomol.uci.edu/mpex/) (201); (E) structural characteristics.

Improving serum stability

The *in vivo* potency of antitumor peptides is often hindered by proteolysis of the peptides, which reduces their half time in serum and hence bioavailability. One strategy to overcome this problem is the application of vector-mediated delivery of genes that encode the active anticancer peptide (102) as outlined in section *Anticancer activity of peptides related to membrane damage*. Another simple but efficient modification is the incorporation of D-amino acids or replacement of all naturally L-amino acids by its diastereomer (27). Early studies on magainin 2 and its all D-amino acid analogue MSI-238 demonstrated the feasibility of this approach (158). Both peptides were found to be active against P388 leukemia, S180 ascites and spontaneous ovarian tumor of mice upon intra-peritoneal injection of peptides, but with the all-D peptide showing significantly higher *in vitro* activity than the all L-peptide (158). A series of studies were initiated using systematically de novodesigned antimicrobial peptides composed of both D- and L-amino acids mimicking the lytic activity of host defense peptides (185). The resulting diastereomeric peptides lost their cytotoxic effect against normal cells but preserved anticancer activity with reduced serum inactivation and enzymatic degradation *in vivo*. For instance, one specific diastereomer composed of 15 amino acids ([D]-K6L9) showed selectivity to primary and metastatic tumors in human prostate and breast xenografts, when administered systemically (100,186). A histopathological evaluation revealed that the diastereomer did not cause any damage to organs of mice emphasizing the potential of such peptides to be developed for therapeutic application.

Perspectives

Cancer treatment with chemotherapeutics still has many severe side effects and can induce drug resistance adversely affecting their successful use. Therefore, novel therapeutic approaches with improved cancer cell selectivity and thus reduced toxicity as well as hindered development of

resistance are urgently needed as has been observed for membrane-active host defense peptides in a number of *in vivo* studies (see section 5). It is also advantageous that they have an excellent tumor tissue penetration, which enables them not only to reach primary but also metastatic tumor sites. However, despite the wealth of information the molecular basis for a selective targeting of cancer cells and the mechanism of cell killing by host defense peptides remain questions to be solved. Since there is a wide variety of host defense peptides, but many of them do not possess sufficient anti-tumor activity, optimization of peptides including innovative types of peptide modification to fine-tune their selective interaction with their target cell membrane together with a profound knowledge of its characteristic lipid composition will be necessary to get a highly active and specific peptide. Biophysical studies considering the peptides as well as membrane characteristics will provide new insight for the rationale design of anticancer peptides. The progress may be facilitated by recent advances in high-throughput screening techniques that can be also implemented in academic laboratories. Based on such knowledge, the field of peptidomimetics may offer alternative means for the development of anticancer drugs with optimized pharmacodynamic profiles. In summary, these strategies are expected to yield novel anticancer drugs with improved properties in respect of selectivity and thus reduced toxicity, as well as of hindered development of drug resistance. These peptides may be also used in combination with chemotherapeutics, as some peptides have shown synergy with classical chemotherapy (27). Achievement of this goal would be a major advancement in cancer treatment.

Acknowledgement: Financial support from the Austrian Science Foundation FWF (grant no. P20760-B11 to D.Z) and the Sonnleitner Stiftung of the Austrian Academy of Sciences is acknowledged.

Reference List

1. Shewach, D. S. and Kuchta, R. D., 2009: Introduction to Cancer Chemotherapeutics; Chem Rev., 109(7): 2859-2861

2. Tofilon, P. J. and Camphausen, K., 2009: Molecular targets for tumor radiosensitization; Chem Rev., 109(7): 2974-2988

3. Espinosa, E., Zamora, P., Feliu, J., and González Barón, M., 2003: Classification of anticancer drugs-a new system based on therapeutic targets; Cancer Treat Rev, 29(6): 515-523

4. Newman, D. J. and Cragg, G. M., 2007: Natural Products as Sources of New Drugs over the Last 25 Years; J Nat Prod., 70(3): 461-477

5. Shore, S., 1947: Review of the nitrogen mustards; Hahnemann Mon., 82(11): 461-470

6. Chaires, J. B., 1985: Thermodynamics of the daunomycin-DNA interaction: ionic strength dependence of the enthalpy and entropy; Biopolymers, 24(2): 403-419

7. Capranico, G., Kohn, K. W., and Pommier, Y., 1990: Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin; Nucleic Acids Res., 18(22): 6611-6619

8. Jackson, K. L., Henderson, J. A., and Phillips, A. J., 2009: The halichondrins and E7389; Chem.Rev., 109(7): 3044-3079

9. Smith, L. L., Brown, K., Carthew, P., Lim, C. K., Martin, E. A., Styles, J., and White, I. N., 2000: Chemoprevention of breast cancer by tamoxifen: risks and opportunities; Crit Rev.Toxicol., 30(5): 571-594

10. Cassidy, J. and Misset, J. L., 2002: Oxaliplatin-related side effects: Characteristics and management; Seminars in Oncology, 29(5, Supplement 15): 11-20

11. Kalyanaraman, B., Joseph, J., Kalivendi, S., Wang, S., Konorev, E., and Kotamraju, S., 2002: Doxorubicin-induced apoptosis: Implications in cardiotoxicity; Mol Cell Biochem, 234-235(1): 119-124

12. Nabholtz, J. M. and Slamon, D., 2001: New adjuvant strategies for breast cancer: meeting the challenge of integrating chemotherapy and trastuzumab (Herceptin); Semin.Oncol., 28(1, Suppl 3): 1-12

13. Pastan, I. and Kreitman, R. J., 1998: Immunotoxins for targeted cancer therapy; Adv.Drug Deliv.Rev., 31(1-2): 53-88

14. Miller, T. W., Isenberg, J. S., and Roberts, D. D., 2009: Molecular regulation of tumor angiogenesis and perfusion via redox signaling; Chem.Rev., 109(7): 3099-3124

15. Gottesman, M. M., 2002: Mechanisms of cancer drug resistance; Annu.Rev.Med., 53: 615-627

16. Gillet, J. P. and Gottesman, M. M., 2010: Mechanisms of multidrug resistance in cancer; Methods Mol.Biol., 596: 47-76

17. Perez-Tomas, R., 2006: Multidrug Resistance: Retrospect and Prospects in Anti-Cancer Drug Treatment; Curr Med Chem., 13(16): 1859-1876

18. Schadendorf, D., Worm, M., Algermissen, B., Kohlmus, C. M., and Czarnetzki, B. M., 1994: Chemosensitivity testing of human malignant melanoma. A retrospective analysis of clinical response and *in vitro* drug sensitivity; Cancer, 73(1): 103-108

19. Helmbach, H., Rossmann, E., Kern, M. A., and Schadendorf, D., 2001: Drug-resistance in human melanoma; Int J Cancer, 93(5): 617-622

20. Chapman, P. B., Einhorn, L. H., Meyers, M. L., Saxman, S., Destro, A. N., Panageas, K. S., Begg, C. B., Agarwala, S. S., Schuchter, L. M., Ernstoff, M. S., Houghton, A. N., and Kirkwood, J. M., 1999: Phase III Multicenter Randomized Trial of the Dartmouth Regimen Versus Dacarbazine in Patients With Metastatic Melanoma; Journal of Clinical Oncology, 17(9): 2745-2751

21. Middleton, M. R., Lee, S. M., Arance, A., Wood, M., Thatcher, N., and Margison, G. P., 2000: O6-methylguanine formation, repair protein depletion and clinical outcome with a 4 hr schedule of temozolomide in the treatment of advanced melanoma: Results of a phase II study; Int J Cancer, 88(3): 469-473

22. Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y. A., Cordon-Cardo, C., and Lowe, S. W., 2001: Inactivation of the apoptosis effector Apaf-1 in malignant melanoma; Nature, 409(6817): 207-211

23. Szakacs, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C., and Gottesman, M. M., 2006: Targeting multidrug resistance in cancer; Nat Rev Drug Discov, 5(3): 219-234

24. Morgan, G., Ward, R., and Barton, M., 2004: The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies; Clinical Oncology, 16(8): 549-560

25. CBTRUS2008: Statistical Report: Primary Brain Tumors in the United States, 2000-2004; Central Brain Tumor Registry of the United States; 2008

26. Hoskin, D. W. and Ramamoorthy, A., 2008: Studies on anticancer activities of antimicrobial peptides; Biochim.Biophys.Acta, 1778(2): 357-375

27. Papo, N. and Shai, Y., 2005: Host defense peptides as new weapons in cancer treatment; Cell Mol.Life Sci., 62(7-8): 784-790

28. Mader, J. S. and Hoskin, D. W., 2006: Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment; Expert Opin.Investig.Drugs, 15(8): 933-946

29. Schweizer, F., 2009: Cationic amphiphilic peptides with cancer-selective toxicity; Eur J Pharmacol, 625(1-3): 190-194

30. Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H. C., and Colamonici, O., 1991: Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation; Proc Natl Acad Sci U S A, 88(9): 3792-3796

31. Zasloff, M., 2002: Antimicrobial peptides of multicellular organisms; Nature, 415(6870): 389-395

32. Wang, Z. and Wang, G., 2004: APD: the Antimicrobial Peptide Database; Nucleic Acids Res., 32(suppl 1): D590-D592

33. Wang, G., Li, X., and Wang, Z., 2009: APD2: the updated antimicrobial peptide database and its application in peptide design; Nucleic Acids Res., 37(suppl 1): D933-D937

34. Iwasaki, T., Ishibashi, J., Tanaka, H., Sato, M., Asaoka, A., Taylor, D., and Yamakawa, M., 2009: Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface; Peptides, 30(4): 660-668

35. Lohner, K. and Blondelle, S. E., 2005: Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics; Comb.Chem.High Throughput.Screen., 8(3): 241-256

36. Lohner, K., 2009: New strategies for novel antibiotics: peptides targeting bacterial cell membranes; Gen.Physiol.Biophys., 28(2): 105-116

37. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 1996: Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications; Lupus, 5(5): 480-487

38. Zwaal, R. F. and Schroit, A. J., 1997: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells; Blood, 89(4): 1121-1132

39. Bevers, E. M., Comfurius, P., Dekkers, D. W., Harmsma, M., and Zwaal, R. F., 1998: Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling; Lupus, 7 Suppl 2(S126-S131

40. Seigneuret, M. and Devaux, P. F., 1984: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes; Proc.Natl.Acad.Sci.U.S.A, 81(12): 3751-3755

41. Dobrzynska, I., Kotynska, J., and Figaszewski, Z., 2007: Changes in electrical charge of phosphatidylcholine and phosphatidylserine liposomal membranes caused by adsorption of monovalent ions; Chemia Analityczna, 52(6): 931-944

42. Szachowicz-Petelska, B., Dobrzynska, I., Figaszewski, Z., and Sulkowski, S., 2002: Changes in physico-chemical properties of human large intestine tumour cells membrane; Mol Cell Biochem, 238(1-2): 41-47

43. Riedl, S., Rinner, B., Tumer, S., Schaider, H., Lohner, K., and Zweytick, D., 2011: Targeting the cancer cell membrane specifically with human lactoferricin derivatives; Annals of Oncology, 22 (suppl 3): 33

44. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

45. Rao, L. V., Tait, J. F., and Hoang, A. D., 1992: Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity; Thromb.Res., 67(5): 517-531

46. Woehlecke, H., Pohl, A., Alder-Baerens, N., Lage, H., and Herrmann, A., 2003: Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2); Biochem.J., 376(Pt 2): 489-495

47. Kirszberg, C., Lima, L. G., Da Silva, d. O., Pickering, W., Gray, E., Barrowcliffe, T. W., Rumjanek, V. M., and Monteiro, R. Q., 2009: Simultaneous tissue factor expression and phosphatidylserine exposure account for the highly procoagulant pattern of melanoma cell lines; Melanoma Res., 19(5): 301-308

48. Cichorek, M., Kozlowska, K., Witkowski, J. M., and Zarzeczna, M., 2000: Flow cytometric estimation of the plasma membrane diversity of transplantable melanomas, using annexin V; Folia Histochem.Cytobiol., 38(1): 41-43

49. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J., 1989: Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation; Proc.Natl.Acad.Sci.U.S.A, 86(9): 3184-3188

50. Schröder-Borm, H., Bakalova, R., and Andrä, J., 2005: The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine; FEBS Lett, 579(27): 6128-6134

51. Zweytick, D., Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., and Lohner, K., 2011: In search of new targets - the membrane lipid phosphatidylserine - the underestimated Achilles' heel of cancer cells; Annals of Oncology, 22 (suppl 3): 43

52. Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., Lohner, K., and Zweytick, D., 2011: In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy; Biochim Biophys Acta, accepted

53. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M., 1992: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages; J.Immunol., 148(7): 2207-2216

54. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R., 1995: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl; J.Exp.Med., 182(5): 1545-1556

55. Albert, M. L., Sauter, B., and Bhardwaj, N., 1998: Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs; Nature, 392(6671): 86-89

56. Miyashita, T. and Reed, J. C., 1993: Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line; Blood, 81(1): 151-157

57. Shadidi, M. and Sioud, M., 2003: Selective targeting of cancer cells using synthetic peptides; Drug Resist.Updat., 6(6): 363-371

58. Zweytick, D., Pabst, G., Abuja, P. M., Jilek, A., Blondelle, S. E., Andrä, J., Jerala, R., Monreal, D., Martinez, d. T., and Lohner, K., 2006: Influence of N-acylation of a peptide derived from human lactoferricin on membrane selectivity; Biochim.Biophys.Acta, 1758(9): 1426-1435

59. Zweytick, D., Deutsch, G., Andrä, J., Blondelle, S. E., Vollmer, E., Jerala, R., and Lohner, K., 2011: Studies on lactoferricin derived E. coli membrane active peptides reveal differences in the mechanism of N-acylated versus non-acylated peptides; Journal of Biological Chemistry, 24: 21266-21276

60. Lohner, K., Latal, A., Lehrer, R. I., and Ganz, T., 1997: Differential scanning microcalorimetry indicates that human defensin, HNP-2, interacts specifically with biomembrane mimetic systems; Biochemistry, 36(6): 1525-1531

61. Ran, S., Downes, A., and Thorpe, P. E., 2002: Increased exposure of anionic phospholipids on the surface of tumor blood vessels; Cancer Res., 62(21): 6132-6140

62. Van de, W. C., Lahorte, C., Vermeersch, H., Loose, D., Mervillie, K., Steinmetz, N. D., Vanderheyden, J. L., Cuvelier, C. A., Slegers, G., and Dierck, R. A., 2003: Quantitative tumor apoptosis imaging using technetium-99m-HYNIC annexin V single photon emission computed tomography; J.Clin.Oncol., 21(18): 3483-3487

63. Kufe, D. W., 2009: Mucins in cancer: function, prognosis and therapy; Nature Reviews Cancer, 9(12): 874-885

64. Carraway, K. L., Price-Schiavi, S. A., Zhu, X., and Komatsu, M., 1999: Membrane Mucins and Breast Cancer; Cancer Control, 6(6): 613-614

65. Bafna, S., Kaur, S., and Batra, S. K., 2010: Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells; Oncogene, 29(20): 2893-2904

66. Wang, F. L., Cui, S. X., Sun, L. P., Qu, X. J., Xie, Y. Y., Zhou, L., Mu, Y. L., Tang, W., and Wang, Y. S., 2009: High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer; Cancer Detect.Prev., 32(5-6): 437-443

67. Litynska, A., Przybylo, M., Pochec, E., Hoja-Lukowicz, D., Ciolczyk, D., Laidler, P., and Gil, D., 2001: Comparison of the lectin-binding pattern in different human melanoma cell lines; Melanoma Res., 11(3): 205-212

68. Cazet, A., Julien, S., Bobowski, M., Krzewinski-Recchi, M. A., Harduin-Lepers, A., Groux-Degroote, S., and Delannoy, P., 2010: Consequences of the expression of sialylated antigens in breast cancer; Carbohydr.Res., 345(10): 1377-1383

69. Risso, A., Zanetti, M., and Gennaro, R., 1998: Cytotoxicity and apoptosis mediated by two peptides of innate immunity; Cell Immunol., 189(2): 107-115

70. Kjellen, L. and Lindahl, U., 1991: Proteoglycans: Structures and Interactions; Annu Rev Biochem, 60(1): 443-475

71. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., and Kitagawa, H., 2003: Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate; Curr Opin Struct Biol, 13(5): 612-620

72. Tkachenko, E., Rhodes, J. M., and Simons, M., 2005: Syndecans: New Kids on the Signaling Block; Circ Res, 96(5): 488-500

73. Rabenstein, D. L., 2002: Heparin and heparan sulfate: structure and function; Nat.Prod.Rep., 19(3): 312-331

74. Jayson, G. C., Lyon, M., Paraskeva, C., Turnbull, J. E., Deakin, J. A., and Gallagher, J. T., 1998: Heparan Sulfate Undergoes Specific Structural Changes during the Progression from Human Colon Adenoma to Carcinoma *in Vitro*; Journal of biological Chemistry, 273(1): 51-57

75. Kleeff, J., Ishiwata, T., Kumbasar, A., Friess, H., Büchler, M. W., Lander, A. D., and Korc, M., 1998: The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer; J Clin Invest, 102(9): 1662-1673

76. Safaiyan, F., Lindahl, U., and Salmivirta, M., 1998: Selective reduction of 6-O-sulfation in heparan sulfate from transformed mammary epithelial cells; Eur J Biochem, 252(3): 576-582

77. Matsuda, K., Maruyama, H., Guo, F., Kleeff, J., Itakura, J., Matsumoto, Y., Lander, A. D., and Korc, M., 2001: Glypican-1 Is Overexpressed in Human Breast Cancer and Modulates the Mitogenic Effects of Multiple Heparin-binding Growth Factors in Breast Cancer Cells; Cancer Res, 61(14): 5562-5569

78. Sanderson, R. D., 2001: Heparan sulfate proteoglycans in invasion and metastasis; Semin Cell Dev Biol, 12(2): 89-98

79. Aviel-Ronen, S., Lau, S. K., Pintilie, M., Lau, D., Liu, N., Tsao, M. S., and Jothy, S., 2008: Glypican-3 is overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma; Mod Pathol, 21(7): 817-825

80. Nakatsura, T., Kageshita, T., Ito, S., Wakamatsu, K., Monji, M., Ikuta, Y., Senju, S., Ono, T., and Nishimura, Y., 2004: Identification of glypican-3 as a novel tumor marker for melanoma; Clin Cancer Res, 10(19): 6612-6621

81. Fadnes, B., Rekdal, O., and Uhlin-Hansen, L., 2009: The anticancer activity of lytic peptides is inhibited by heparan sulfate on the surface of the tumor cells; BMC Cancer, 9(1): 183-

82. Fadnes, B., Uhlin-Hansen, L., Lindin, I., and Rekdal, O., 2011: Small lytic peptides escape the inhibitory effect of heparan sulfate on the surface of cancer cells; BMC Cancer, 11(1): 116-

83. Sherbet, G. V., 1989: Membrane fluidity and cancer metastasis; Exp.Cell Biol., 57(4): 198-205

84. Sok, M., Sentjurc, M., Schara, M., Stare, J., and Rott, T., 2002: Cell membrane fluidity and prognosis of lung cancer; Ann.Thorac.Surg., 73(5): 1567-1571

85. Campanella, R., 1992: Membrane lipids modifications in human gliomas of different degree of malignancy; J.Neurosurg.Sci., 36(1): 11-25

86. Rodrigues, C. M., Sola, S., Castro, R. E., Laires, P. A., Brites, D., and Moura, J. J., 2002: Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis; J.Lipid Res., 43(6): 885-894

87. Van Blitterswijk, W. J., 1984: Alteration in lipid fluidity in the plasma membrane of tumor cells; 53-84

88. Sherbet, G. V. and Jackson, S., 1986: Temperature-dependent surface charge modulation, membrane fluidity, and metastatic ability of L51178Y lymphoma; Anticancer Res., 6(1): 129-134

89. Li, Y. C., Park, M. J., Ye, S. K., Kim, C. W., and Kim, Y. N., 2006: Elevated Levels of Cholesterol-Rich Lipid Rafts in Cancer Cells Are Correlated with Apoptosis Sensitivity Induced by Cholesterol-Depleting Agents; Am J Pathol, 168(4): 1107-1118

90. Nakazawa, I. and Iwaizumi, M., 1989: A role of the cancer cell membrane fluidity in the cancer metastases: an ESR study; Tohoku J.Exp.Med., 157(3): 193-198

91. Schroeder, F. and Gardiner, J. M., 1984: Membrane lipids and enzymes of cultured high- and low-metastatic B16 melanoma variants; Cancer Res., 44(8): 3262-3269

92. May, G. L., Wright, L. C., Dyne, M., Mackinnon, W. B., Fox, R. M., and Mountford, C. E., 1988: Plasma membrane lipid composition of vinblastine sensitive and resistant human leukaemic lymphoblasts; Int J Cancer, 42(5): 728-733

93. Mazzoni, A. and Trave, F., 1993: Cytoplasmic membrane cholesterol and doxorubicin cytotoxicity in drug-sensitive and multidrug-resistant human ovarian cancer cells; Oncol.Res., 5(2): 75-82

94. Domagala, W. and Koss, L. G., 1980: Surface configuration of human tumor cells obtained by fine needle aspiration biopsy; Scan Electron Microsc, 3(101-108

95. Chaudhary, J. and Munshi, M., 1995: Scanning electron microscopic analysis of breast aspirates; Cytopathology, 6(3): 162-167

96. Chan, S. C., Hui, L., and Chen, H. M., 1998: Enhancement of the cytolytic effect of anti-bacterial cecropin by the microvilli of cancer cells; Anticancer Res, 18(6A): 4467-4474

97. Leuschner, C. and Hansel, W., 2004: Membrane disrupting lytic peptides for cancer treatments; Curr.Pharm.Des, 10(19): 2299-2310

98. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W., 2005: Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines; Mol Cancer Ther, 4(4): 612-624

99. Bhutia, S. K. and Maiti, T. K., 2008: Targeting tumors with peptides from natural sources; Trends Biotechnol., 26(4): 210-217

100. Papo, N., Seger, D., Makovitzki, A., Kalchenko, V., Eshhar, Z., Degani, H., and Shai, Y., 2006: Inhibition of Tumor Growth and Elimination of Multiple Metastases in Human Prostate and Breast Xenografts by Systemic Inoculation of a Host Defense-Like Lytic Peptide; Cancer Res, 66(10): 5371-5378

101. Chernysh, S., Kim, S. I., Bekker, G., Pleskach, V. A., Filatova, N. A., Anikin, V. B., Platonov, V. G., and Bulet, P., 2002: Antiviral and antitumor peptides from insects; Proc Natl Acad Sci U S A, 99(20): 12628-12632

102. Winder, D., Günzburg, W. H., Erfle, V., and Salmons, B., 1998: Expression of Antimicrobial Peptides Has an Antitumour Effect in Human Cells; Biochem Biophys Res Commun, 242(3): 608-612

103. Park, N. G., Lee, S., Oishi, O., Aoyagi, H., Iwanaga, S., Yamashita, S., and Ohno, M., 1992: Conformation of tachyplesin I from Tachypleus tridentatus when interacting with lipid matrixes; Biochemistry, 31(48): 12241-12247

104. Chen, Y., Xu, X., Hong, S., Chen, J., Liu, N., Underhill, C. B., Creswell, K., and Zhang, L., 2001: RGD-Tachyplesin Inhibits Tumor Growth; Cancer Res, 61(6): 2434-2438

105. Lohner, K., 2001: The role of membrane lipid composition in cell targeting of antimicrobial peptides; 149-165

106. Sevcsik, E., Pabst, G., Richter, W., Danner, S., Amenitsch, H., and Lohner, K., 2008: Interaction of LL-37 with model membrane systems of different complexity: influence of the lipid matrix; Biophys.J., 94(12): 4688-4699

107. Bechinger, B., 2009: Rationalizing the membrane interactions of cationic amphipathic antimicrobial peptides by their molecular shape; Current Opinion in Colloid & Interface Science, 14(5): 349-355

108. Wimley, W. and Hristova, K., 2011: Antimicrobial Peptides: Successes, Challenges and Unanswered Questions; J Membr Biol, 239(1): 27-34

109. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K., 1996: An Antimicrobal Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation; Biochemistry, 35(35): 11361-11368

110. Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L., and Huang, H. W., 1996: Membrane Pores Induced by Magainin; Biochemistry, 35(43): 13723-13728

111. Shai, Y., 2002: Mode of action of membrane active antimicrobial peptides; Peptide Science, 66(4): 236-248

112. Huang, H. W., 2006: Molecular mechanism of antimicrobial peptides: The origin of cooperativity; Biochim Biophys Acta, 1758(9): 1292-1302

113. Wimley, W. C., 2010: Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model; ACS Chemical Biology, 5(10): 905-917

114. Epand, R. M., Epand, R. F., Arnusch, C. J., Papahadjopoulos-Sternberg, B., Wang, G., and Shai, Y., 2010: Lipid clustering by three homologous arginine-rich antimicrobial peptides is insensitive to amino acid arrangement and induced secondary structure; Biochim.Biophys Acta, 1798(6): 1272-1280

115. Epand, R. M. and Epand, R. F., 2011: Bacterial membrane lipids in the action of antimicrobial agents; J Pept Sci, 17(5): 298-305

116. Lohner, K. and Prenner, E. J., 1999: Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems; Biochim.Biophys.Acta, 1462(1-2): 141-156

117. Epand, R. M. and Epand, R. F., 2009: Lipid domains in bacterial membranes and the action of antimicrobial agents; Biochim Biophys Acta, 1788(1): 289-294

118. Pokorny, A. and Almeida, P. F. F., 2004: Kinetics of Dye Efflux and Lipid Flip-Flop Induced by δ-Lysin in Phosphatidylcholine Vesicles and the Mechanism of Graded Release by Amphipathic, α-Helical Peptides; Biochemistry, 43(27): 8846-8857

119. Pokorny, A., Kilelee, E. M., Wu, D., and Almeida, P. F. F., 2008: The Activity of the Amphipathic Peptide δ-Lysin Correlates with Phospholipid Acyl Chain Structure and Bilayer Elastic Properties; Biophys J, 95(10): 4748-4755

120. Gregory, S. M., Cavenaugh, A., Journigan, V., Pokorny, A., and Almeida, P. F. F., 2008: A Quantitative Model for the All-or-None Permeabilization of Phospholipid Vesicles by the Antimicrobial Peptide Cecropin A; Biophys J, 94(5): 1667-1680

121. Almeida, P. F. and Pokorny, A., 2009: Mechanisms of Antimicrobial, Cytolytic, and Cell-Penetrating Peptides: From Kinetics to Thermodynamics; Biochemistry, 48(34): 8083-8093

122. Rathinakumar, R. and Wimley, W. C., 2008: Biomolecular Engineering by Combinatorial Design and High-Throughput Screening: Small, Soluble Peptides That Permeabilize Membranes; J Am Chem Soc, 130(30): 9849-9858

123. Rathinakumar, R. and Wimley, W. C., 2010: High-throughput discovery of broad-spectrum peptide antibiotics; The FASEB Journal, 24(9): 3232-3238

124. Hristova, K., Selsted, M. E., and White, S. H., 1997: Critical Role of Lipid Composition in Membrane Permeabilization by Rabbit Neutrophil Defensins; Journal of biological Chemistry, 272(39): 24224-24233

125. Bechinger, B. and Lohner, K., 2006: Detergent-like actions of linear amphipathic cationic antimicrobial peptides; Biochim.Biophys.Acta, 1758(9): 1529-1539

126. Ostolaza, H., Bartolomé, B., de Zárate, I. O., de la Cruz, F., and Goni, F. M., 1993: Release of lipid vesicle contents by the bacterial protein toxin [alpha]-haemolysin; Biochimi Biophys Acta, 1147(1): 81-88

127. Israelachvili, J. N., Marcelja, S., and Horn, R. G., 1980: Physical principles of membrane organization; Q.Rev.Biophys., 13(02): 121-200

128. Marsh, D., 1996: Lateral pressure in membranes; Biochim Biophys Acta, 1286(3): 183-223

129. Staudegger, E., Prenner, E. J., Kriechbaum, M., Degovics, G., Lewis, R. N. A. H., McElhaney, R. N., and Lohner, K., 2000: X-ray studies on the interaction of the antimicrobial peptide gramicidin S with microbial lipid extracts: evidence for cubic phase formation; Biochim Biophys Acta, 1468(1-2): 213-230

130. Willumeit, R., Kumpugdee, M., Funari, S. S., Lohner, K., Pozo-Navas, B., Brandenburg, K., Linser, S., and Andrä, J., 2005: Structural rearrangement of model membranes by the peptide antibiotic NK-2; Biochim.Biophys.Acta, 1669(2): 125-134

131. Hickel, A., Danner-Pongratz, S., Amenitsch, H., Degovics, G., Rappolt, M., Lohner, K., and Pabst, G., 2008: Influence of antimicrobial peptides on the formation of nonlamellar lipid mesophases; Biochim.Biophys.Acta, 1778(10): 2325-2333

132. Zweytick, D., Tumer, S., Blondelle, S. E., and Lohner, K., 2008: Membrane curvature stress and antibacterial activity of lactoferricin derivatives; Biochem.Biophys.Res.Commun., 369(2): 395-400

133. Griffiths, J. R.1991: Are cancer cells acidic?; Br.J Cancer, 64(3): 425-427

134. Newell, K., Franchi, A., Pouysségur, J., and Tannock, I., 1993: Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity; Proc Natl Acad Sci U S A, 90(3): 1127-1131

135. Makovitzki, A., Fink, A., and Shai, Y., 2009: Suppression of Human Solid Tumor Growth in Mice by Intratumor and Systemic Inoculation of Histidine-Rich and pH-Dependent Host Defense-like Lytic Peptides; Cancer Res, 69(8): 3458-3463

136. Hunt, J. F., Rath, P., Rothschild, K. J., and Engelman, D. M., 1997: Spontaneous, pH-dependent membrane insertion of a transbilayer alpha-helix; Biochemistry, 36: 15177-15192

137. Reshetnyak, Y. K., Segala, M., Andreev, O. A., and Engelman, D. M., 2007: A Monomeric Membrane Peptide that Lives in Three Worlds: In Solution, Attached to, and Inserted across Lipid Bilayers; Biophys J, 93(7): 2363-2372

138. Reshetnyak, Y. K., Andreev, O. A., Lehnert, U., and Engelman, D. M., 2006: Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix; Proc Natl Acad Sci U S A, 103(17): 6460-6465

139. Andreev, O. A., Dupuy, A. D., Segala, M., Sandugu, S., Serra, D. A., Chichester, C. O., Engelman, D. M., and Reshetnyak, Y. K., 2007: Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues *in vivo*; Proc Natl Acad Sci U S A, 104(19): 7893-7898

140. Zoonens, M., Reshetnyak, Y. K., and Engelman, D. M., 2008: Bilayer Interactions of pHLIP, a Peptide that Can Deliver Drugs and Target Tumors; Biophys J, 95(1): 225-235

141. Thévenin, D., An, M., and Engelman, D. M., 2009: pHLIP-Mediated Translocation of Membrane-Impermeable Molecules into Cells; Chem Biol, 16(7): 754-762

142. An, M., Wijesinghe, D., Andreev, O. A., Reshetnyak, Y. K., and Engelman, D. M., 2010: pH-(low)-insertion-peptide (pHLIP) translocation of membrane impermeable phalloidin toxin inhibits cancer cell proliferation; Proc Natl Acad Sci U S A, 107(47): 20246-20250

143. Hancock, R. E. W. and Sahl, H. G., 2006: Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies; Nat Biotech, 24(12): 1551-1557

144. Easton, D. M., Nijnik, A., Mayer, M. L., and Hancock, R. E. W., 2009: Potential of immunomodulatory host defense peptides as novel anti-infectives; Trends Biotechnol., 27(10): 582-590

145. Wieczorek, M., Jenssen, H., Kindrachuk, J., Scott, W. R. P., Elliott, M., Hilpert, K., Cheng, J. T. J., Hancock, R. E. W., and Straus, S. K., 2010: Structural Studies of a Peptide with Immune Modulating and Direct Antimicrobial Activity; Chem Biol, 17(9): 970-980

146. Dredge, K., Marriott, B., Todryk, S., and Dalgleish, A., 2002: Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy; Cancer Immunol Immunother, 51(10): 521-531

147. Chan, Y. R. and Gallo, R. L., 1998: PR-39, a Syndecan-inducing Antimicrobial Peptide, Binds and Affects p130Cas; Journal of biological Chemistry, 273(44): 28978-28985

148. Tanaka, K., Fujimoto, Y., Suzuki, M., Suzuki, Y., Ohtake, T., Saito, H., and Kohgo, Y., 2001: PI3-kinase p85-Is a Target Molecule of Proline-rich Antimicrobial Peptide to Suppress Proliferation of ras-Transformed Cells; Cancer Science, 92(9): 959-967

149. Chan, Y. R., Zanetti, M., Gennaro, R., and Gallo, R. L., 2001: Anti-Microbial Activity and Cell Binding are Controled by Sequence Determinants in the Anti-Microbial Peptide PR-39; J Invest Dermatol, 116(2): 230-235

150. Ohtake, T., Fujimoto, Y., Ikuta, K., Saito, H., Ohhira, M., Ono, M., and Kohgo, Y., 1999: Proline-rich antimicrobial peptide, PR-39 gene transduction altered invasive activity and actin structure in human hepatocellular carcinoma cells; Br J Cancer, 81(3): 393-403

151. Liebersbach, B. F. and Sanderson, R. D., 1994: Expression of syndecan-1 inhibits cell invasion into type I collagen; Journal of biological Chemistry, 269(31): 20013-20019

152. Simmons, T. L., Andrianasolo, E., McPhail, K., Flatt, P., and Gerwick, W. H., 2005: Marine natural products as anticancer drugs; Mol Cancer Ther, 4(2): 333-342

153. Davis A, Jang, J., Middleton, KM., Wang, Y., Weisz, I., Ling, Y., and Bekesi, G., 1999: Novel suicide Ligands of Tubulin Arrest Cancer Cells in S-Phase; Neoplasia, 1(6): 498-507

154. Sharma, S. V., 1992: Melittin resistance: a counterselection for ras transformation.; Oncogene, 7(2): 193-201

155. Habermann, E. and Jentsch, T. J., 1967: Sequenzanalyse des Melttins aus den tryptischen und peptischen Spaltsstücken des Bienengiftes; Hoppe Seylers.Z.Physiol Chem., 348(37-50

156. Holle, L., Song, W., Holle, E., Wei, Y., Wagner, T., and Yu, X., 2003: A matrix metalloproteinase 2 cleavable melittin/avidin conjugate specifically targets tumor cells *in vitro* and *in vivo*; Int J Oncol, 22(1): 93-98

157. Zasloff, M., 1987: Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor; Proc Natl Acad Sci U S A, 84(15): 5449-5453

158. Baker, M. A., Maloy, W. L., Zasloff, M., and Jacob, L. S., 1993: Anticancer efficacy of Magainin2 and analogue peptides; Cancer Res, 53(13): 3052-3057

159. Soballe, P. W., Maloy, W. L., Myrga, M. L., Jacob, L. S., and Herlyn, M., 1995: Experimental local therapy of human melanoma with lytic magainin peptides; Int J Cancer, 60(2): 280-284

160. Vogel, H. J., Schibli, D. J., Jing, W., Lohmeier-Vogel, E. M., Epand, R. F., and Epand, R. M., 2002: Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides; Biochem.Cell Biol., 80(1): 49-63

161. Yoo, Y.-C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., and Azuma, I., 1997: Bovine Lactoferrin and Lactoferricin, a Peptide Derived from Bovine Lactoferrin, Inhibit Tumor Metastasis in Mice; Cancer Science, 88(2): 184-190

162. Eliassen, L. T., Berge, G., Sveinbjornsson, B., Svendsen, J. S., Vorland, L. H., and Rekdal, O., 2002: Evidence for a Direct Antitumor Mechanism of Action of Bovine Lactoferricin; Anticancer Res, 22(2703-2710

163. Eliassen, L. T., Berge, G., Leknessund, A., Wikman, M., Lindin, I., Lokke, C., Ponthan, F., Johnsen, J. I., Sveinbjornsson, B., Kogner, P., Flaegstad, T., and Rekdal, O., 2006: The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells *in vitro* and inhibits xenograft growth *in vivo*; Int.J Cancer, 119(3): 493-500

164. Onishi, J., Roy, M. K., Juneja, L. R., Watanabe, Y., and Tamai, Y., 2008: A lactoferrin-derived peptide with cationic residues concentrated in a region of its helical structure induces necrotic cell death in a leukemic cell line (HL-60); J.Pept.Sci., 14(9): 1032-1038

165. Furlong, S. J., Mader, J. S., and Hoskin, D. W., 2010: Bovine lactoferricin induces caspase-independent apoptosis in human B-lymphoma cells and extends the survival of immune-deficient mice bearing B-lymphoma xenografts; Experimental and Molecular Pathology, 88(3): 371-375

166. Peck-Miller, K. A., Darveau, R. P., and Fell, H. P., 1993: Identification of serum components that inhibit the tumoricidal activity of amphiphilic alpha helical peptides; Cancer Chemotherapy and Pharmacology, 32(2): 109-115

167. Silva, P. I., Daffre, S., and Bulet, P., 2000: Isolation and Characterization of Gomesin, an 18-Residue Cysteine-rich Defense Peptide from the Spider Acanthoscurria gomesiana Hemocytes with Sequence Similarities to Horseshoe Crab Antimicrobial Peptides of the Tachyplesin Family; Journal of biological Chemistry, 275(43): 33464-33470

168. Mandard, N., Bulet, P., Caille, A., Daffre, S., and Vovelle, F., 2002: The solution structure of gomesin, an antimicrobial cysteine-rich peptide from the spider; Eur.J Biochem., 269(4): 1190-1198

169. Rodrigues, E. G., Dobroff, A. S., Cavarsan, C. F., Paschoalin, T., Nimrichter, L., Mortara, R. A., Santos, E. L., Fazio, M. A., Miranda, A., Daffre, S., and Travassos, L. R., 2008: Effective topical treatment of subcutaneous murine B16F10-Nex2 melanoma by the antimicrobial peptide gomesin; Neoplasia, 10(1): 61-68

170. Zhang, W., Li, J., Liu, L. W., Wang, K. R., Song, J. J., Yan, J. X., Li, Z. Y., Zhang, B. Z., and Wang, R., 2010: A novel analog of antimicrobial peptide Polybia-MPI, with thioamide bond substitution, exhibits increased therapeutic efficacy against cancer and diminished toxicity in mice; Peptides, 31(10): 1832-1838

171. Wang, K. R., Yan, J. X., Zhang, B. Z., Song, J. J., Jia, P. f., and Wang, R., 2009: Novel mode of action of polybia-MPI, a novel antimicrobial peptide, in multi-drug resistant leukemic cells; Cancer Lett, 278(1): 65-72

172. Xu, N., Wang, Y. S., Pan, W. B., Xiao, B., Wen, Y. J., Chen, X. C., Chen, L. J., Deng, H. X., You, J., Kan, B., Fu, A. F., Li, D., Zhao, X., and Wei, Y. Q., 2008: Human alpha-defensin-1 inhibits growth of human lung adenocarcinoma xenograft in nude mice; Mol.Cancer Ther., 7(6): 1588-1597

173. Tosteson, M. T. and Tosteson, D. C., 1981: The sting. Melittin forms channels in lipid bilayers; Biophys J, 36(1): 109-116

174. Matsuzaki, K., Yoneyama, S., Miyajima, and K., 1997: Pore Formation and Transition of Melittin; Biophys.J., 73(831-838

175. Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y., 1988: Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (Tachypleus tridentatus). Isolation and chemical structure; Journal of biological Chemistry, 263(32): 16709-16713

176. Katsu, T., Nakao, S., and Iwanaga, S., 1993: Mode of action of an antimicrobial peptide, tachyplesin I, on biomembranes.; Biol Pharm Bull., 16(2): 178-181

177. Pettit, G. R., Kamano, Y., Herald, C. L., Tuinman, A. A., Boettner, F. E., Kizu, H., Schmidt, J. M., Baczynskyj, L., Tomer, K. B., and Bontems, R. J., 1987: The isolation and structure of a remarkable marine animal antineoplastic constituent: dolastatin 10; J Am Chem Soc, 109(22): 6883-6885

178. Miyazaki, K., Kobayashi M, Natsume T, Gondo M, Mikami T, Sakakibara K, and Tsukagoshi S., 1995: Synthesis and antitumor activity of novel dolastatin 10; Chem Pharm Bull, 43(10): 1706-1718

179. Ebbinghaus, S., Hersh, E., Cunningham, C. C., O'Day, S., McDermott, D., Stephenson, J., Richards, D. A., Eckardt, J., Haider, O. L., and Hammon, L. A., 2004: Phase II study of synthadotin (SYN-D; ILX651) administered daily for 5 consecutive days once every 3 weeks (qdx5q3w) in patients (Pts) with inoperable locally advanced or metastatic melanoma; Journal of Clinical Oncology, 22(14S): suppl.-

180. Joanne, P., Nicolas, P., and El Amri, C., 2009: Antimicrobial Peptides and Viral Fusion Peptides : How Different They Are?; Protein and Peptide Letters, 16(7): 743-750

181. Wimley, W. C., Creamer, T. P., and White, S. H., 1996: Solvation Energies of Amino Acid Side Chains and Backbone in a Family of Host-Guest Pentapeptides; Biochemistry, 35(16): 5109-5124

182. Dennison, S., Harris, F., Bhatt, T., Singh, J., and Phoenix, D., 2010: A theoretical analysis of secondary structural characteristics of anticancer peptides; Molecular and Cellular Biochemistry, 333(1): 129-135

183. Dennison, S. R., Whittaker, M., Harris, F., and Phoenix, D. A., 2006: Anticancer alpha-helical peptides and structure/function relationships underpinning their interactions with tumour cell membranes; Curr.Protein Pept.Sci., 7(6): 487-499

184. Blondelle, S. E. and Lohner, K., 2010: Optimization and High-Throughput Screening of Antimicrobial Peptides; Current Pharmaceutical Design, 16(28): 3204-3211

185. Papo, N. and Shai, Y., 2003: New lytic peptides based on the D,L-amphipathic helix motif preferentially kill tumor cells compared to normal cells; Biochemistry, 42(31): 9346-9354

186. Papo, N., Braunstein, A., Eshhar, Z., and Shai, Y., 2004: Suppression of human prostate tumor growth in mice by a cytolytic D-, L-amino Acid Peptide: membrane lysis, increased necrosis, and inhibition of prostate-specific antigen secretion; Cancer Res., 64(16): 5779-5786

187. Newman, D. J. and Cragg, G. M., 2007: Natural Products as Sources of New Drugs over the Last 25 Years; Journal of Natural Products, 70(3): 461-477

188. Wimley, W. C., Creamer, T. P., and White, S. H., 1996: Solvation Energies of Amino Acid Side Chains and Backbone in a Family of Host-Guest Pentapeptides; Biochemistry, 35(16): 5109-5124

Results

In the following chapter, three papers discuss the results of the present study, namely "In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy" (accepted as a regular paper in Biochimica et Biophysica Acta Biomembranes), "Killing of melanoma cells and metastases by human Lactoferricin derivatives correlates with effect on cancer models composed of phosphatidylserine" (manuscript prepared, will be submitted after patent application) and "Slow killing of cancer cells, phosphatidylserine interaction and formation of a ß-sheet structure by hLFcin derived anticancer peptides is the key for high cancer specificity" (manuscript prepared for submission, will be submitted after patent application).

In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy

Sabrina Riedl[1], Beate Rinner[2], Martin Asslaber[3], Helmut Schaider[2, 5], Sonja Walzer[4, 6], Alexandra Novak[2], Karl Lohner[1] and Dagmar Zweytick[1]

[1]Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstraße 6, A-8042 Graz, Austria

[2]Center for Medical Research, Medical University of Graz, Stiftingtalstraße 24, A-8010 Graz, Austria

[3]Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, A-8036 Graz, Austria

[4]Institute of Orthopedy, Medical University of Graz, Auenbruggerplatz 5, A-8036 Graz, Austria

[5]Cancer Biology Unit, Department of Dermatology, Medical University of Graz, Auenbruggerplatz 8, A-8036 Graz, Austria

[6]present address: Institute of Orthopedy, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria

Accepted for publication in Biochimica et Biophysica Acta, Biomembranes

Abstract

This study was performed in the aim to identify potential targets for the development of novel therapy to treat cancer with poor outcome or treatment efficacy. We show that the negatively charged phospholipid phosphatidylserine (PS) is exposed in the outer leaflet of their plasma membrane not only in tumor cell lines, but also in metastases and primary cultures thereof, which contrasts with a lack of PS exposure by differentiated non-tumorigenic counterparts. Studied tumor cell lines were derived from non-tumorigenic and malignant melanomas, prostate- and renal cancer, glioblastoma and a rhabdomyosarcoma. Importantly, also metastases of melanoma expose PS and there is a correlation between malignancy of melanoma cell lines from different stages of tumor progression and PS exposure. The PS exposure we found was neither of apoptotic nor of experimental artificial origin.

Finally potentially malignant and non-malignant cells could be differentiated by sorting of a primary cell culture derived from a glioblastoma based on PS exposure, which has so far not been possible within one culture due to lack of a specific marker.

Our data provide clear evidence that PS could serve as uniform marker of tumor cells and metastases as well as a target for novel therapeutic approaches based on e.g. PS specific host defense derived peptides.

Introduction

Cancer is the leading cause of death worldwide (http://www.who.int/mediacentre/factsheets/fs297/en/). In the last decades significant progress has been made in respect of diagnostics and therapeutic approaches. However, despite favorable advancements, state of the art chemotherapy is still not successful in many cancers. Potential toxicity and many side effects of chemotherapeutics can mainly be explained by a lack of adequate specificity for tumor cells. Chemotherapy, due to resistance and other reasons, is essentially inefficient for prostate cancer, metastatic melanoma, and bladder, kidney and pancreatic cancer. Further, the benefit of chemotherapy in general is modest, since the increase of survival rate after 5 years for all adult cancers is only by 2% (1). Consistently, the search for more specific targets generally expressed within a certain tumor is a major issue in anti-cancer research. Cell membrane, growth factor receptors and proteins involved in cell-cell-signaling are of broad interest, but relatively little is known about changes in lipid composition of the cell membrane in carcinogenesis. Nevertheless, in recent years lipidomics in cancer research has gained more attention.

The outer leaflet of eukaryotic plasma membranes normally comprises neutral phospholipids, like phosphatidylcholine (PC) and sphingomyelin (SM), whereas the negatively charged phospholipid phosphatidylserine (PS) as well as phosphatidylethanolamine (PE) are located in the inner leaflet (2). It has been reported earlier that this asymmetry may be lost due to reduced activity of the ATP dependent phospholipid translocase, which specifically transports PS and PE between bilayer leaflets and is susceptible to oxidative injury, or due to the activation of a scramblase induced by increased intracellular Ca2+ levels which leads to non-specific movement of phospholipids (3). This loss of asymmetry results in exposure of the negatively charged PS on the surface of cancerous and other pathological cells (4, 5), as well as apoptotic cells (6, 7) and thus PS represents a promising target for cationic host defense peptides. The fact that platelets and erythrocytes also expose PS upon

activation (8) seems to cause minimal side effects compared to much higher side effects observed with other applied therapies since these peptides can act specifically within the organism.

Surface exposure of PS by malignant cells was shown in tumorigenic Friend erythroleukemic cells (9), other leukemia and neuroblastoma cells (10), malignant melanoma (11) and human gastric carcinoma cells (12). *In vivo* exposure of anionic phospholipids could be shown on the surface of endothelial cells in tumor blood vessels (13). In an attempt to correlate changes in the phospholipid bilayer of malignant melanoma (MM) cell membranes with progression of these tumors Cichorek et al. showed slightly higher exposure of PS in the more progressive amelanotic transplantable hamster cell lines than in melanotic ones (14). Lack of surface exposure of PS was demonstrated for non-cancer cells as normal human epidermal keratinocytes (11), 3T3-fibroblasts (15) and lymphocytes (10).

In general, apoptotic cells exposing PS in the outer leaflet of the plasma membrane are specifically recognized by macrophages (6, 7) and dendritic cells (16). However, tumor cells, although exposing PS, circumvent apoptosis and prevent the recognition by macrophages (17, 18). Therefore, membrane-active peptides have been proposed to constitute a promising approach in the development of anticancer drugs (10, 19-21). The positively charged small peptides are produced by the innate immune system and are able to discriminate between different cell membranes. These host defense peptides specifically interact with negatively charged lipids on their target cell membrane (22-25), which would minimize side effects upon treatment and could reach all cancer types exposing PS, even those resistant to conventional chemotherapy.

The discovery of a specific target generally expressed within all tumor types as well as by metastases would be a major success in cancer research. Therefore, the present study focuses on PS exposure of malignancies with poor outcome or treatment efficacy, which require new pharmaceutical therapies and would enormously benefit from cancer specific peptide drugs. This study has to be considered as an extension of the pioneering work by Utsugi and coworkers (9, 11) who proved PS expression in the

outer leaflet of three different tumor cell lines. We extended the study by increasing the number of cancer types and including metastases and primary cell cultures. We have therefore clearly demonstrated that PS-exposure is a general phenomenon not only in primary lesions of cancer but also in metastases, cancer cell lines and primary cultures and a by far underestimated Achilles' heel of cancer.

Materials and Methods

Cells and Culture

Cell lines

Glioblastoma (U87-mg) purchased from CLS (Cell Line Service Heidelberg, Germany) and rhabdomyosarcoma cell lines (TE671) purchased from ECAAC (Health Protection Agency Culture Collections Salisbury, UK) were cultured in Dulbecco´s Modified Eagle Medium (DMEM, Invitrogen, UK) with addition of 2 mM Glutamine, 10% FBS (fetal bovine serum; Lot A10109-0523, PAA Laboratory, Pasching, Austria) and 1% Pen/Strep (Penicillin/Streptomycin, PAA Laboratory, Austria). Kidney carcinoma (769-P) and prostate adenocarcinoma cell lines (LNCaP) purchased from CLS were cultured in RPMI1640 medium (Invitrogen, UK) with addition of 10% FBS, 2 mM L-Glutamine, 1% PenStrep and 1 mM sodium pyruvate (Invitrogen, UK).

The melanocytic cell lines were kindly provided by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). The primary human melanoma cell lines SBcl2, WM35 and metastatic melanoma cell lines WM9, WM164 were maintained in RPMI (Sigma, St. Louis, MO) supplemented with 2% FBS, 2% L-glutamine and 1% Pen/Strep.

All cell lines were purchased frozen and thawed freshly before the experiments. During culturing they were kept in a 5% CO_2 atmosphere at 37°C. At 90% confluence cell-cultures were passaged with

accutase (PAA, Pasching, Austria). All cell cultures were periodically checked for mycoplasma and passaged in the laboratory no longer than 1 month after first thawing.

Primary Cell Cultures

A surgical specimen of primary glioblastoma (GBM), histologically independently verified by two pathologists (GFAP positive, Ki67 index above 50%), was blended mechanically and transferred to culture flask containing growth medium (DMEM, 10% FBS, 1% L-Glutamine, 1% Pen/Strep, 1% Amphotericin B; PAA Laboratory, Austria) (26). Tumorigenicity has been shown in nude mice. A melanoma metastase to the brain (MMB), was blended mechanically and transferred to a culture flask containing growth medium (RPMI, 5% FBS, 1%L-Glutamine, 1%Pen/Strep, 1% Amphotericin). Human melanocytes (FOM101), derived from human foreskin were cultured in human melanocytes growth medium (PromoCell GmbH, Heidelberg, Germany). Normal human dermal fibroblasts (NHDF) purchased from PromoCell GmbH (Heidelberg, Germany) were cultured in fibroblast growth medium 2 (PromoCell GmbH, Heidelberg, Germany).

Synoviocytes were isolated from synovium of Osteoarthritis (OA) patients undergoing joint replacement surgery, who fulfilled the criteria of the 1986 American College of Rheumatology. Random biopsies of synovial membrane were obtained aseptically from joints of OA patients. Synovial membrane tissue was separated macroscopically from ligaments, fat and other non-synovial tissue, minced in 1 mm slices and washed with PBS (phosphate buffered saline; GIBCO Invitrogen) supplemented with Pen/Strep and 0.25 µg Amphotericin B (PAA Laboratory, Austria). Synovial membrane specimens were rinsed several times with 1x PBS, finely dissected and digested with 0.2% Collegenase B (Roche Diagnostics, Germany) in high glucose Dulbecco`s-modified Eagle`s medium (DMEM-HG; GIBCO Invitrogen), containing 10% FBS, 1% L-Glutamine, Pen/Strep and Amphotericin B (PAA Laboratory, Austria). Following overnight incubation at 37°C, the cell suspension was filtered on a nylon membrane (Cell Strainer 40 µm; BD Bioscience). Cells were collected by centrifugation,

washed twice, re-suspended in DMEM-HG growth medium, plated in culture flasks and allowed to attach for 3 days. OA cells were authenticated by flow cytometry.

Primary culture T22 was obtained after surgical excision of a Rhabdomyosarcoma, histologically specified by a pathologist. The tissue was minced mechanically, washed twice and transferred to Collagenase B and transferred to culture flask containing growth medium (DMEM-HG, 10% FBS, 1% L-Glutamine, 1% Pen/Strep).

Primary cell cultures were used between passage 2 and 6 (except for FOM101 and GBM, which were passaged up to 12). All cell cultures were periodically checked for mycoplasma. All patients were consented and ethics approval for the use of patient-derived material was given by the local research ethics committee.

Flow cytometry analysis

PS exposure

For Annexin V binding cultured cells with ~90% confluence were treated with accutase for 2-3 min at 37 °C. Cells were removed gently from the plate, dispensed into fresh media and washed once with media. 10^6 cells were re-suspended in 1 ml 1x binding buffer (Abcam, Cambridge, USA). 100 µl (10^5 cells) cell suspension were stained with 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) (Abcam, Cambridge, GB), i.e. incubated at room temperature for 5 min in the dark. Flow cytometry analysis of PS-surface content of the cells was performed with an LSRII Flow cytometer (Becton Dickinson, USA). 10.000 events were collected. Cells were identified in the side scatter and forward scatter with linear scale. Fluorescence signal was analyzed with logarithmic scale. Analysis was done with the FACS Diva software (Becton Dickinson, USA). The graphical analysis was performed in FCS-express (De Novo Software, Los Angeles, USA).

Cell Sorting

1.5 x 10^7 primary glioblastoma cells were stained with 50 µl Annexin V-FITC for 15 min at RT in the dark. Dead cells were discriminated by PI staining. Annexin negative cells and Annexin positive cells were analyzed and sorted by a live sterile cell sorting system (BD FACSAria Cell-Sorting System BD, Biosciences, San Jose, CA, USA).

Apoptosis

Cleavage of Caspase-3: 2 x 10^6 cells / ml were fixed with formaldehyde for 10 min at 37 °C and permeabilized with methanol. The pellet was re-suspended in incubation-buffer and then stained with FITC- conjugated monoclonal active caspase-3 antibody (Cell Signaling, USA). Cells were then analyzed by flow cytometry (FACS Calibur, BD, Biosciences, USA). Untreated cells were used as negative control. As positive control apoptosis was induced by treatment with 10µg/ml of a petrol ether extract of roots of the plant Onosma paniculatum (27) within up to 72 hours. Analysis was performed with CellQuest (BD, Biosciences, USA)

Immunocytochemistry

After cell sorting Annexin negative and Annexin positive cells were suspended in DMEM culture medium and seeded onto chamber slides (BD Falcon). After overnight incubation the cells were washed with PBS and fixed with 4% formaldehyde/PBS. Staining with Ki67 (Mib-1) antibody purchased from Ventana was performed using standardized automated procedures with CC1 predilute antigen retrieval and Ventana iView detection system (Ventana Medical Systems, Tucson, AZ, USA and Dako Cytomation, Glostrup, Denmark). Counterstaining was performed with haematoxylin.

Electron Microscopy

After cell sorting Annexin negative and Annexin positive cells were separately suspended in complete DMEM culture medium, washed with PBS, and replaced with fixatives (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer/pH 7.4) for 30 min at RT. Fixatives were removed and replaced with cacodylate buffer/pH 7.4 and the tissue was post fixed in 2% osmium tetraoxide solution, dehydrated in a series of graded ethanol and embedded in TAAB epoxy resin. Sections were cut at app. 70 nm thickness, counter stained with uranyl acetate and lead citrate, and visualized using a Zeiss EM 902 transmission electron microscope.

Fluorescence Microscopy

Cells (2×10^4) were seeded onto 8 wells chamber slides (ibidy GmbH, Martinsried, Germany) and grown in 300 µl medium for 2-3 days in a confluent layer. Medium was removed and cells were washed twice with 1x Annexin binding buffer (ABB) of Vybrant Apoptosis Assay Kit #2 (Molecular ProbesTM, Invitrogen). 5 µl Annexin V-Alexa Fluor 488 (staining PS exposing cells) and 5 µl PI (staining DNA of necrotic cells) in 300 µl ABB were added and incubated at room temperature in the dark. After incubation cells were washed twice with ABB to remove unbound Annexin V-Alexa Fluor 488 and covered with ABB. A Leica DMI6000 B with IMC in connection with a Leica DFC360 FX camera and AF 6000 software was used for imaging.

Brightfield transmission, green (Annexin V-Alexa Fluor 488, λ_{ex} = 488 nm and λ_{em} = 530 nm) and red fluorescence (PI, λ_{ex} = 536 nm and λ_{em} = 617 nm) were measured in the respective channels. Exposure time, intensity and gain were fixed for measurement of all cell lines and primary cell cultures studied.

Fluorescence Spectroscopy

Cells were cultured to ~90% confluence and then detached by accutase/EDTA. Accutase activity was stopped with media containing 10% FBS. Cells were washed with 1x Annexin binding buffer (ABB) of an Annexin V-FITC Apoptosis Detection Kit (abcam, Cambridge, UK). 10^5 cells per experiment were re-

suspended in 500 µl ABB and incubated with 5 µl Annexin V-FITC and 5 µl PI for 5min at RT in the dark. Cells were washed with ABB and re-suspended in 100 µl ABB and measured with a fluorescence spectrometer (Fluoromax 3) with a λ_{ex} = 488 nm and λ_{em} = 530 nm. Five independent experiments were performed.

Results

In the present study, we evaluated anionic phospholipids exposed by cancer cells as targets for cationic host defense peptides and thereof derived antitumor peptides. In particular, the specificity and significance of the presence of negatively charged PS in the outer leaflet of the plasma membrane of tumor cells and metastases of cell lines and primary cultures were investigated.

Cancer cells and metastases specifically and significantly expose PS

A standard Annexin V affinity assay, frequently used for detection of apoptosis, has been applied to specifically elucidate the exposure of PS by malignant melanoma, soft tissue, prostate, kidney and brain cancer cell lines. However, to avoid membrane stress, adherent cells were detached before Annexin V-FITC binding with accutase/low EDTA instead of the standard trypsin/EDTA detachment, since trypsinization or scrapping of the cells was shown to lead to increase of Annexin V binding (PS exposure) (PhD thesis Maximilian Martin Gemeinhardt, Munich, Faculty of Medicine, 2008). This is probably due to non-repairable membrane damage.

As demonstrated by flow cytometry the different cancer cell lines of rhabdomyosarcoma, a malignant soft tissue tumor of mesenchymal origin (TE671), prostate cancer (LNCaP), renal cell cancer (769-P) (Figure 1), melanoma of primary (SBcl-2, WM35) and metastatic lesions (WM9 and WM164) and glioblastoma (U-87 mg) (Figure 1 and 2) exposed significant amounts of PS compared to non-tumorigenic cells (Figure 1 and supplementary Figure 1S). This is indicated by selective binding of Annexin V-FITC to PS on the surface of tumor cells, revealed by a strong shift in fluorescence intensity

of Annexin V-FITC labeled tumor cells in contrast to unlabeled tumor cells compared to a negligible shift of the labeled corresponding “non-tumor” control cells like differentiated synoviocyte cells of OA patients (non-tumor counterpart to TE671), melanocytes derived of foreskin (FOM101) (Figure 1) and normal human dermal fibroblasts (NHDF) (supplements Figure 1S).

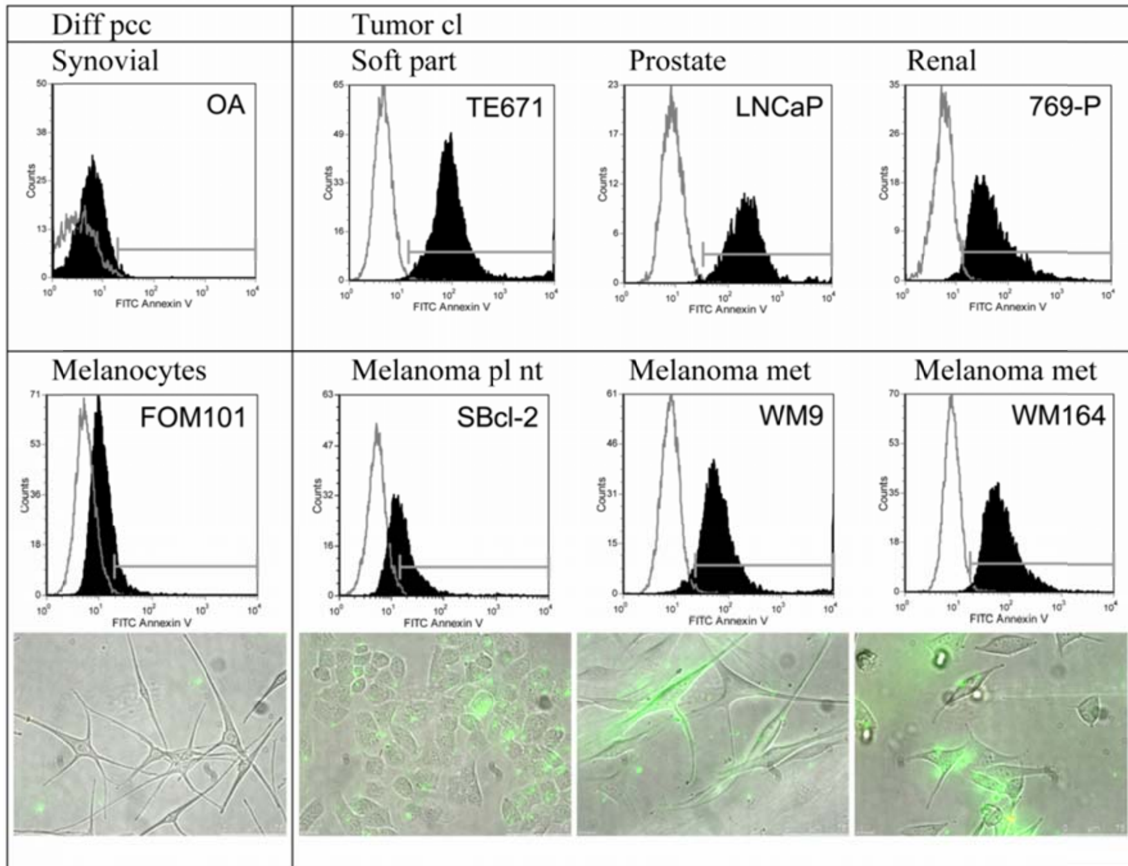

Figure 1: Top: Flow cytometry plots of Annexin V-FITC/PI labeled (black filled) and unlabeled cells (grey line) comprising differentiated control cells of primary cell cultures (diff pcc) of synoviocytes, isolated from synovium of Osteoarthritis (OA) patients and tumor cell lines (cl) of different cancer types, as rhabdomyosarcoma, a malignant soft tissue tumor of mesenchymal origin (TE671), prostate cancer (LNCaP) and renal cell cancer (769-P). Plots indicate a strong binding of Annexin V to PS on tumor cell lines. Second panel: Flow cytometry plots of Annexin V-FITC/PI labeled (black filled) and unlabeled cells (grey line) comprising differentiated control cells of primary cultures (diff pcc) of melanocytes (FOM101) and melanoma cell lines (cl) of primary lesions (pl) (nt non tumorigenic) (SBcl-2) and more highly malignant melanoma metastases (met) (WM9 and WM164). Annexin V

binds significantly to melanoma cell lines of primary and metastatic lesions. Fluorescence microscopy pictures (overlay of bright field and fluorescence channels) of melanocytes and melanoma cell lines indicate specific binding of Annexin V-Alexa Fluor 488 (green) to PS on the outside of melanoma cells. No PI (red) is integrated assuring integrity of cells studied.

To exclude false positive results by Annexin V labeled necrotic cells, the assays were always performed in the presence of propidium iodide (PI) staining necrotic cells that exhibit membrane damage and therefore also internalize Annexin V labels. Thus, PS levels referred to are derived from Annexin positive, but PI negative cells. In all cell types studied, PI positive necrotic cells (Q2, supplements, Figure 2S) constituted only a minor population of less than 5%, whereas cells in Annexin positive cells (Q4, supplements, Figure 2S) have to be ascribed to cells exposing PS. Thus, cells studied were not undergoing necrosis. In summary, results confirmed a cancer cell specific high exposure of PS by all the cancer cell lines investigated.

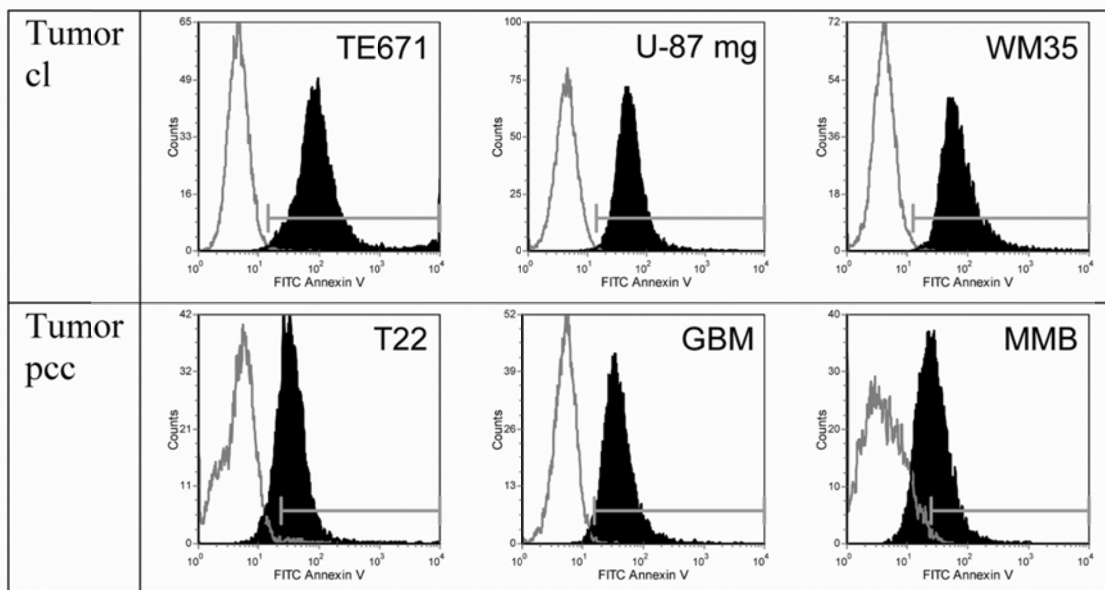

Figure 2: Comparison of Annexin V binding by tumor cell lines and correlating primary tumor cell cultures of a rhabdomyosarcoma (T22) (correlating to rhabdomyosarcoma cell line TE671), a glioblastoma (GBM) (correlating to glioblastoma cell line U-87 mg) and primary cell cultures of a malignant melanoma metastasis to

the brain (MMB) (correlating to melanoma cell line WM35). Flow cytometry plots of Annexin V-FITC/PI labeled cells (black filled) and unlabeled cells (grey line) comprising tumor cell lines (top) and primary cell cultures of tumor tissue (bottom) (cl cell line; pcc primary cell culture). Tumor primary cell culture, as well significantly expose PS indicated by Annexin V binding.

Correlation of level of PS exposure with malignancy and cancer type

To determine a possible correlation between PS exposure and tumor progression, four human malignant melanoma (MM) cell lines isolated from different stages were compared to melanocytes (Table1).

Table 1: PS exposure (Annexin V binding) of different tumor cell lines and tumor primary cell cultures related to PS exposure of melanocytes (fluorescence spectroscopy; 4 independent experiments of each cell line).

cells	Annexin V binding	absolute intensity /10^5 cells
Melanocytes pcc [a]	1.0 ± 0.4	$0.31 \cdot 10^5$
SBcl-2 cl [b]	4.1 ± 0.6	$1.28 \cdot 10^5$
WM35 cl	7.0 ± 1.1	$2.20 \cdot 10^5$
WM9 cl	7.6 ± 2.1	$2.38 \cdot 10^5$
WM164 cl	11.0 ± 3.9	$3.44 \cdot 10^5$
769-P cl	6.7± 0.3	$2.10 \cdot 10^5$
TE671 cl	14.9 ± 4.1	$4.65 \cdot 10^5$
GBM pcc	4.7± 0.5	$1.46 \cdot 10^5$

[a] pcc primary cell culture; [b] cl cell line

Fluorescence spectroscopy experiments enabled a comparison of the PS levels (Annexin V-FITC bound) exposed by different MM cell lines (Table 1). Results revealed a correlation between tumor malignancy and PS levels exposed. For instance, a nearly doubling in PS exposure was observed by WM35 (28) compared to SBcl-2, a non-tumorigenic cell line in SCID mice (29). In SBcl-2 cells PS exposure was increased 4-fold compared to melanocytes whereas in cancer cells of higher malignancy, WM9 and WM164 of metastatic lesions, exposure was increased by 8-11 fold. Thus, a clear correlation between malignant potential within a tumor entity and the level of PS exposed was found. These results are in agreement with data obtained by flow cytometry (Figure 1) (see first results section) (median deviation, supplements, Figure 3S).

Some striking differences were observed in terms of levels of PS exposed by other cancer types. In the case of the rhabdomyosarcoma cell line TE671, the amount of PS exposed was significantly higher in comparison to malignant melanoma or 769-P cells (Table 1), which suggests that this cancer type may be an even better candidate for efficient treatment by PS-specific peptides.

The PS level exposed by the primary cell culture of a glioblastoma (GBM) (see also next section) is lower than that of melanoma cell lines WM9 or WM164, which is quite feasible, since it is not yet a homogenous tumor cell line. Nevertheless, the PS level is still increased compared to melanocytes.

Fluorescence labeling of vital cells (Figure 1) grown on slides proved that PS (Annexin V-Alexa Fluor 488 bound, green fluorescence) is exposed by MM cells, even when not being detached before labeling (like for flow cytometry) and therefore “not stressed”, whereas non neoplastic melanocytes showed no Annexin V binding, thus no PS exposure. Furthermore, the quite interesting observation could be made that PS on the surface of MM cells appeared to be clustered and not distributed homogeneously over the plasma membrane, as would be the case if Annexin V stained PS in the inner leaflet of necrotic cells. Thus, to exclude false positive results by necrotic cells, cells were always co-labeled with PI, but only a negligible number of PI-stained necrotic cells were detected.

PS exposure – not an artifact of cell lines!

Cell culturing, which comprises several passages of the cells by repeated detachment and seeding of cells, is a standard procedure for cancer cells. The studied non-tumor cells however were primary cultures that can only be passaged a few times. Therefore tumor cell lines could suffer membrane damage through cell culturing procedures. In order to exclude this experimentally artificial reason for PS exposure, in addition to studies on cancer cell lines we also investigated primary cancer cell cultures of a glioblastoma (GBM) (correlating to glioblastoma cell line U-87 mg), primary cells of a rhabdomyosarcoma (T22) (correlating to rhabdomyosarcoma cell line TE671) and a malignant melanoma metastasis to the brain (MMB) (correlating to melanoma cell line WM35) (Figure 2). The studied primary tumor cells, like the cell lines showed significant binding of Annexin V-FITC. These results clearly indicate that not only cancer cell lines but also primary cell cultures of malignant tumors and even metastases expose significant levels of PS. This further demonstrates that PS exposure to the outer lipid leaflet of cancer cells is not caused by cell culturing procedures but is an original and permanent characteristic of cancer cells. As mentioned, it has to be taken into account that primary cell cultures of tumors also include non-neoplastic cells with a certain extent of stromal cells and therefore the PS level exposed can be lower compared to homogenous cancer cell lines. This is for instance nicely demonstrated by the primary cell culture of the melanoma metastases cells (MMB), which shows less shift of the Annexin V labeled cells (Figure 2) meaning slightly reduced PS exposure compared to melanoma cell line WM35, but still significantly increased levels compared to differentiated melanocytes (Figure 1).

PS - a potential common marker of cancer cells, but "no reliable eat me" signal.

There is no universal tumor marker known so far. To prove that PS could really be an accurate marker for all cancer cells, we tried to distinguish between tumor and non-tumor cells just by the characteristic of exposure or non-exposure of PS, respectively.

Therefore, a primary cell culture of a histologically verified glioblastoma (WHO grade IV) was evaluated. Even when the specimen for the culture is obtained from the center of the tumor, it is very likely to contain non neoplastic cells as well as glial tumor cells, like microglia and stromal cells, macrophages and maybe reactive glial cells. A fact being of advantage in this case, since we should be able to observe malignant and to some extent benign cell types in one culture. Microscopic inspection of the cell culture was insufficient to distinguish between the different cell types.

After Annexin V labeling and exclusion of necrotic factors by PI, strongly increased PS levels on the cell surface of a part of the cells were detected. In a second step the cells were sorted using a FACS ARIA II TM cell sorter according to their Annexin V binding events. It was possible to obtain Annexin positive (A+) and Annexin negative (A–) cells. These different cell types were returned to culture to let the cells recover. The two different cultures were then investigated by electron microscopy (Figure 3) and immunocytochemistry using the proliferation marker Mib-1 (Figure 4).

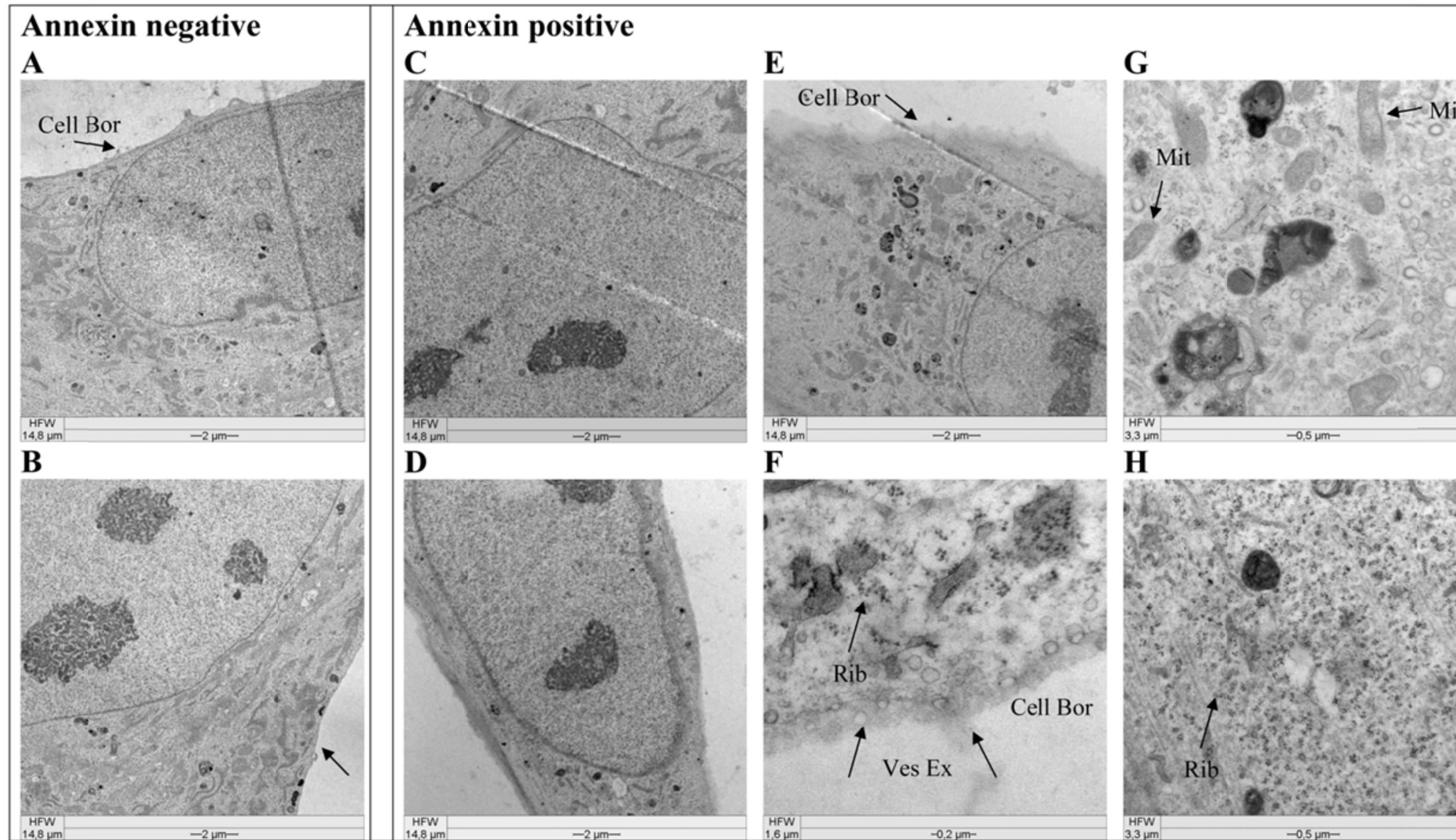

Figure 3: Comparison of morphology of Annexin V negative and positive cells. Pictures are taken by a Zeiss EM 902 transmission electron microscope after sorting of an Annexin V labeled primary culture of glioblastoma cells (GBM) by a BD FACSAria Cell-Sorting System. Annexin negative (A, B) and Annexin positive (C-H) cells are non-PS exposing and PS exposing cells, respectively. Arrows indicate diffuse cellular border (Cell Bor), Ribosomes (Rib), (exocytosed) vesicles (Ves Ex), normal mitochondria (Mit).

Annexin positive cells show characteristics of glioblastoma (tumor) cells, but not of apoptosis.

The electron microscopy pictures revealed that the Annexin positive cells (Figure 3 C-H) (potential glial tumor cells carrying the PS marker) showed some homogenous morphological characteristics indicating that these cells were tumor cells. Size and shape of nuclei of Annexin positive cells (Figure 3 C and D) were partially changed compared to those of Annexin negative cells (Figure 3 A and B), indicated by an elongated or polymorphic shape and sizes up to 25µm, which is twice the normal size. Increased nucleus/plasma ratios, as well as polymorphic nuclei are characteristics of tumor cells. Nevertheless, also the few Annexin negative cells exhibited quite large nuclei (10-15µm), potentially representing reactive astrocytes. Furthermore, a structural change of the plasma membrane of the Annexin positive cells could be seen, characterized by a quite diffuse cellular border (D, E, F, arrows Cell Bor) compared to a sharp border exhibited by Annexin negative cells (A and B). This membrane feature is probably reflecting a difference between tumor and stromal cells. Another characteristic of the Annexin positive cells was a strong increase in formation of ribosomes and vesicles (F and H, arrow Rib) and an increase in exocytosis of vesicles (F, arrow Ves Ex), a feature of GBM cells described in detail recently by Skog et al. (30). For better representation of intracellular changes only parts of cells are shown, whole cell images before sorting are presented in the supplements (Figure 4S).

The above findings were supported by slowly growing Annexin negative cells in contrast to well proliferating Annexin positive cells and the fact that proliferation marker Mib-1 (Figure 4) was slightly higher expressed in A+ than in A– cells, a feature of glioblastoma cells. However, it has to be noted that the amount of Annexin negative cells within the primary cell culture was quite low.

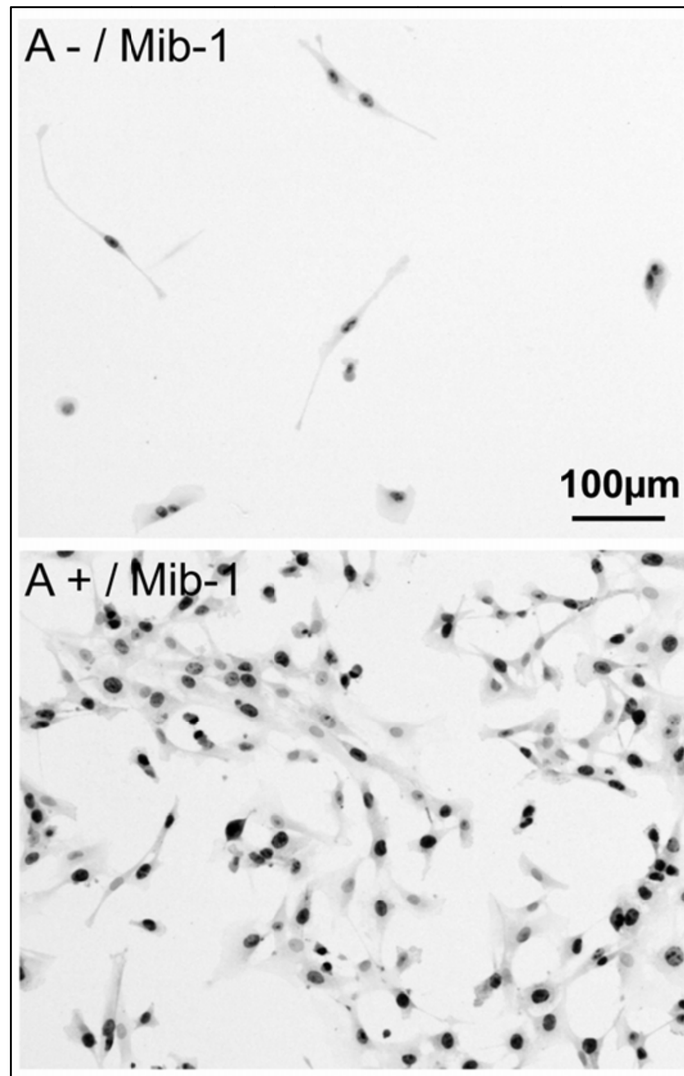

Figure 4: Morphology and Mib-1 immunocytochemistry of a FACS ARIA II TM cell sorted Annexin V labeled primary cell culture of glioblastoma. Top: Annexin negative cells (100x); bottom: Annexin positive cells (100x). Annexin positive cells express higher levels of Mib-1.

Further the Annexin positive cells of glioblastoma were checked for morphological changes due to apoptosis, like swelling of mitochondria, typical apoptotic blebbing or condensation of chromatin (Figure 3 G, D, E, F, arrow Mit (normal mitochondria)). The Annexin positive cells however were lacking all these signs of apoptosis. This was also supported by caspase-3 cleavage assays with the melanoma cancer cell lines used within this study (Figure 5) revealing no significant caspase-3 cleavage (below 5%), thus excluding PS exposure of studied cancer cells to be a sign of cells

undergoing apoptosis. This is further confirmed by a positive control, where apoptosis was induced in the melanoma cell line WM35 by 72h treatment with a plant extract of *Onosma paniculatum* resulting in significant increase of caspase-3 cleavage from 2.3% by untreated up to 38% by treated cells, indicated by a strong shift in fluorescence intensity. Inducibility of cell death in a caspase dependent manner by treatment with the plant extract was also reported for the studied melanoma cell lines SBcl-2, WM35, WM9 and WM164 recently by Rinner et al. (27).

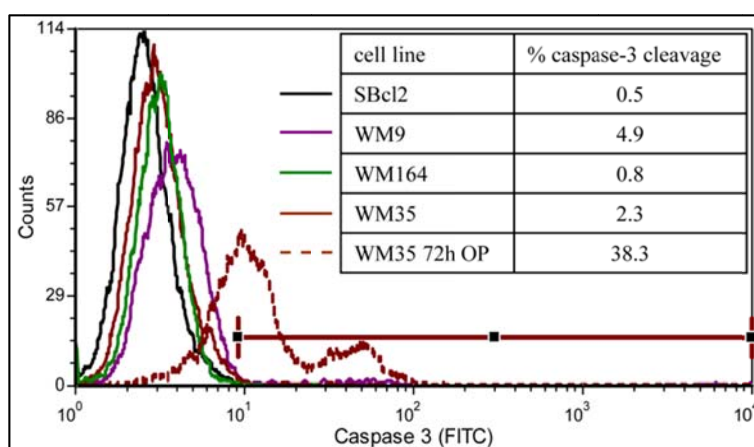

Figure 5: Minor caspase cleavage of melanoma cell lines.

Flow cytometry plots of melanoma cell lines were stained with FITC-conjugated monoclonal active caspase-3 antibody. Untreated cells were used as negative control. Analysis was performed with CellQuest (BD, Biosciences, USA). No significant cleavage of caspase-3 was detected for untreated melanoma cell lines represented by solid lines, indicating no apoptosis. As a positive control, WM35was treated with 10µg/ml petrol ether extract of roots of the plant *Onosma paniculatum* (OP) for 72 hours (brown dashed line) (27). Treatment caused clear apoptosis indicated by 38% of caspase-3 cleavage.

Discussion

After several decades of cancer research no common marker for malignant cells has been identified so far, which would offer many possibilities, not only for therapy, but also for fast and accurate diagnosis. Already in 1991 Utsugi et al. (11) reported about elevated expression of PS in the outer leaflet of human tumor cells. In 2002 Ran et al. (13) showed that the monoclonal antibody 9D2 and Annexin V, the natural ligand to anionic phospholipids, specifically localized to tumor vessels and tumor cells in and around necrotic regions after i.v. injection into tumor-bearing mice, whereas none of the blood vessels in normal tissues had detectable externalized anionic phospholipids. Our study strongly supports these findings and more importantly clearly demonstrates that exposure of PS to the outer leaflet of membranes is not restricted to blood vessels but is a general phenomenon for cancer plasma membranes independently on cancer type and is also valid for metastases and many cancer types including those with poor outcome or treatability, like glioblastoma or malignant melanoma. Thus, the anionic phospholipid PS could serve as a common cancer marker as well as a therapeutic target. Since the standard labeling technique with Annexin V requires an intact plasma membrane and apoptotic or necrobiotic cells also show increased levels of PS in their outer leaflet, there may be some limitations. Therefore in our study “false positive” results by increased Annexin V binding to PS by cells undergoing apoptosis had to be excluded. In fact, the cancer cells tested showed elevated PS levels, but without any sign of apoptosis. This is an important aspect, since it is mainly assumed that cancer cells expose higher levels of PS due to their higher rate of cell division and apoptosis, as e.g. described by Boersma et al. (31). On the other hand PS exposed could mean that cancer cells should be fated to die but are clever enough to avoid the last step of being killed. As mentioned above there are several ways for cancer cells to prevent apoptosis like melanoma cells inhibiting the expression of the gene encoding Apaf-1, the apoptotic protease activating factor-1 (17).

It is not fully understood how and why cancer cells expose PS to the outer plasma membrane leaflet. When separating phospholipids of a total cell lipid extract of human melanocytes (FOM101) and two melanoma cell lines (SBcl-2 and WM164) by two-dimensional thin-layer chromatography and quantification by the method of Broekhuyse (32), no increase in the total amount of PS was found in the cancer cells (data not shown). Therefore, not the overall PS content but only the asymmetric PS distribution in the plasma membrane seems to get lost. It has been reported that the combined action of an amino phospholipid translocase, responsible for the specific movement of PS and PE to the inner leaflet of plasma membranes, and a less specific floppase, responsible for the movement of PS and PC to the outside, both dependent on ATP, seems to equip the cell with a mechanism that corrects for alterations in lipid distribution to avoid potential pathological consequences (5). Nevertheless, as reported, inactivation of these activities *in vitro* did not result in loss of asymmetry. However, activation of a third protein, the lipid scramblase, by influx of Ca^{2+} into the cytoplasm can cause a rapid transbilayer phospholipid mixing leading to a nearly symmetric distribution across the membrane bilayer (5). Thus, cellular changes like depletion of ATP and/or influx of Ca^{2+} into the cytoplasm seem to be important factors accounting for exposure of PS. Furthermore, it has been proposed by Ran et al. (13) that injury and activation of tumor endothelium by cytokines and reactive oxygen species might induce PS exposure of tumor vessels. As mentioned above, we were able to observe by fluorescence microscopy that PS, visualized by Annexin V-Alexa Fluor 488, was localized in clusters on the plasma membrane of cancer cells. Interestingly some of these PS exposing clusters budded off the cells were quite mobile and even moved from one cell to the other in the form of small PS exposing blebs (unpublished observation). In this context it is of interest to note that loss of membrane asymmetry is often accompanied by blebbing and subsequent shedding of lipid-symmetric microvesicles from the cell surface (33, 34). It is known that eukaryotic cells are able to perform intercellular communication by e.g., exosomes (35), if these vesicles still carry the PS marker. One could then assume that host defense peptides, which can target PS would also be able to prevent possible intercellular communication between cancer cells.

Definitely, PS constitutes an Achilles' heel of cancer cells, because the anionic lipid represents a cancer type independent target for host defense peptides that rapidly kill cells exposing negatively charged lipids. A number of studies revealed that cationic amphipathic peptides specifically interact with PS containing membranes (10, 36-38). So far highly active antimicrobial peptides derived from host defense could be designed to selectively target bacterial membranes (39-43), whereas antitumor peptides to target cancer cell membranes are in development (20, 24, 37, 44, 45). The high levels of exposed PS found in our study for cancer cell lines, derived from metastatic lesions, suggest that these peptides can even be beneficial as a therapeutic intervention in the metastatic cascade, which is difficult so far. Furthermore, all cancer types including cancer with poor prognosis and treatability, like glioblastoma and melanoma, amongst others, would highly benefit using the PS target for novel approaches in diagnosis and therapy. Successful sorting of a primary culture of a glioblastoma within this study gives hope for possible differentiation between tumor and non-tumor cells and selective targeted therapy.

Conclusions

Our study constitutes an extension of previous work (11) regarding cancer types with a focus on cancer with poor outcome and treatment efficacy, including metastases and primary cell cultures using another assay. We show that PS exposure is also a characteristic of metastases, a very important aspect regarding therapy. Furthermore, we compared several primary cancer cell cultures with correlating cell lines to rule out cell culturing effects on the membrane and to prove originality of PS exposure. Cancer specific PS exposure was not a sign of apoptosis. By using PS as a marker it was possible to distinguish within a primary culture of glioblastoma between potential malign and benign cells.

The study aimed at supplying evidence for a uniform and novel target for cancer therapy in the form of the negatively charged lipid phosphatidylserine. We prove that PS is specifically exposed by cancer cells and metastases, which delivers the important basis and target for the further development of cationic antitumor peptide drugs, to one day support or even partially substitute chemotherapy.

Acknowledgement

We want to thank Dr. R. Hofmann-Wellenhof from the Department of Dermatology at the Medical University of Graz and Dr. Gerd Leitinger from the Core Facility Ultrastructure Analysis at the Center of Medical Research in Graz for their help regarding melanocytes and melanoma cell cultures and performance of electron microscopy experiments, respectively. We thank S. Tumer for technical support. Further we thank Dr. Sylvie Blondelle from Sanford-Burnham Medical Research Institute in La Jolla, USA, for carefully reading the manuscript. Financial support from the Austrian Science Foundation FWF (grant no. P20760-B11 to D.Z) is acknowledged.

Supplements

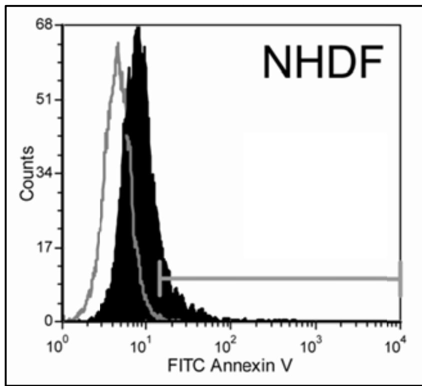

Figure 1S: Flow cytometry plots of Annexin V-FITC/PI labeled (black filled) and unlabeled cells (grey line) comprising differentiated control cells of primary cultures of normal human dermal fibroblasts (NHDF). Plot indicates a lack of binding of Annexin V to PS on non-tumor cells meaning lack of PS exposure by these cells (supplement to Figure 1).

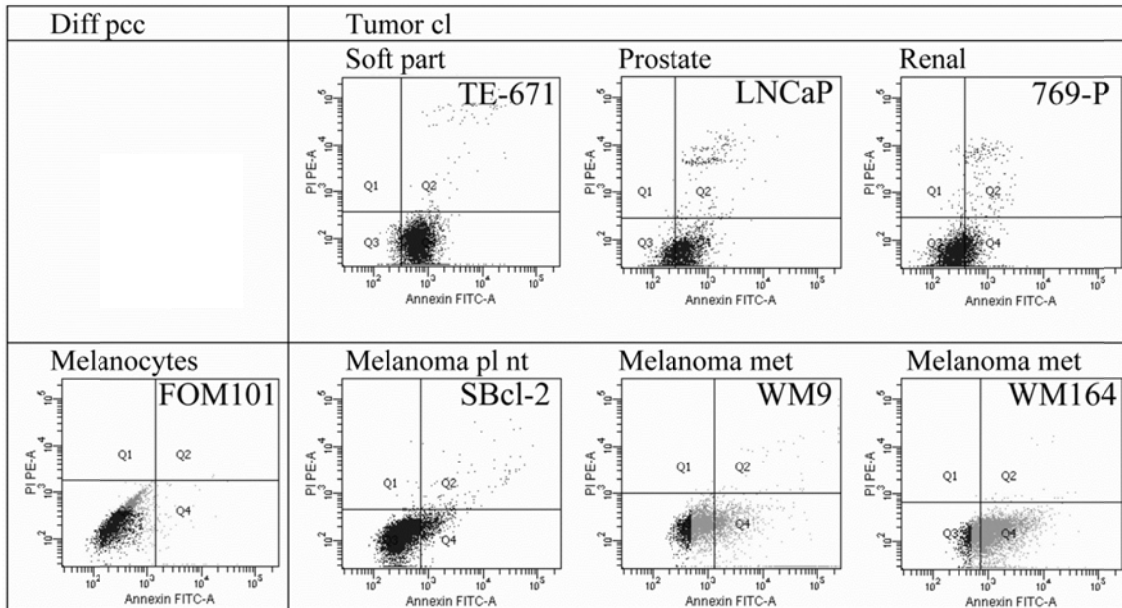

Figure 2S: 2D-flow cytometry plots of AnnexinV-FITC/PI labeled differentiated primary cell cultures (pcc) of melanocytes (FOM101) and tumor cell lines (cl) of melanoma (SBcl-2, WM9, WM164), prostate cancer (LNCaP), renal cancer (769-P) and a rhabdomyosarcoma (TE671). Events in section Q4 indicate binding of Annexin V-FITC, events in section Q2 indicate PI-positive cells. Plots demonstrate that amount of PI-positive cells is minor than 5%, thus Annexin V binding of tumor cells is due to PS exposure (supplement to Figure 1).

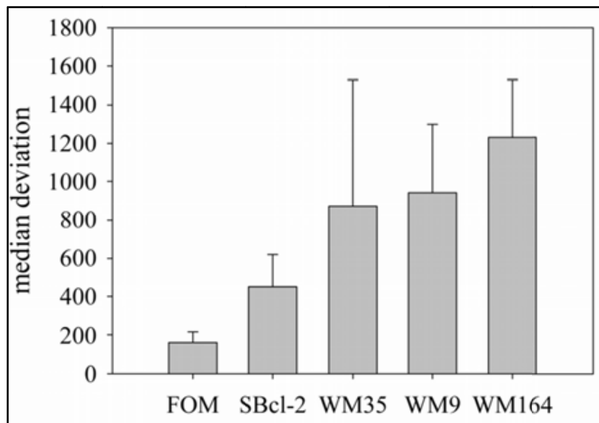

Figure 3S: Median deviation of flow cytometry experiments (mean value of 4 independent experiments) with Annexin V-FITC/PI labeled melanocytes (FOM101) and melanoma cells (SBcl-2, WM35, WM9, WM164) (supplement to Figure 1 and Table 1). Annexin V-FITC binding is increased in tumor cell lines and is higher in higher malign cell lines of metastases like WM9 and WM164.

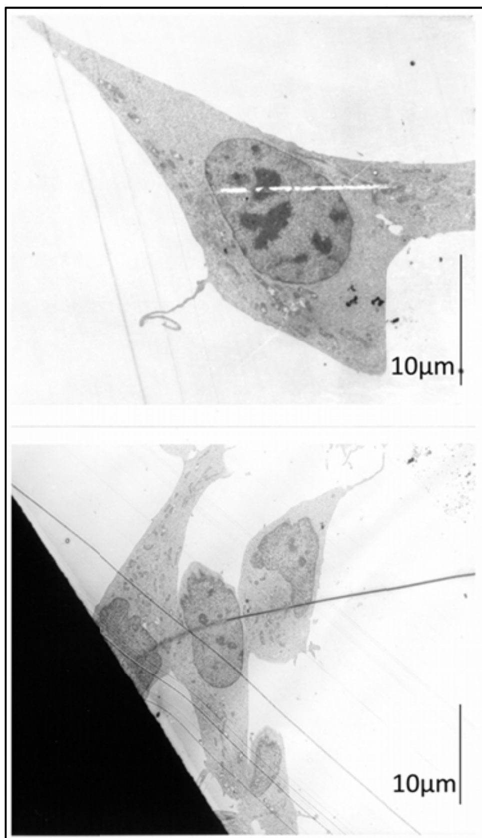

Figure 4S: Pictures of whole cells of a primary culture of glioblastoma cells (GBM) before sorting taken by a Zeiss EM 902 transmission electron microscope (supplement to Figure 3).

Reference List

1. Morgan, G., Ward, R., and Barton, M., 2004: The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies; Clin.Oncol.(R.Coll.Radiol.), 16(8): 549-560

2. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 1996: Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications; Lupus, 5(5): 480-487

3. Seigneuret, M. and Devaux, P. F., 1984: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes; Proc.Natl.Acad.Sci.U.S.A, 81(12): 3751-3755

4. Zwaal, R. F., Comfurius, P., and Bevers, E. M., 2005: Surface exposure of phosphatidylserine in pathological cells; Cell Mol.Life Sci., 62(9): 971-988

5. Zwaal, R. F. and Schroit, A. J., 1997: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells; Blood, 89(4): 1121-1132

6. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M., 1992: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages; J.Immunol., 148(7): 2207-2216

7. Martin, S., Reutelingsperger, C. P., McGahon, A., Rader, J., van Schie, R., La Face, D., and Green, D. R., 1995: Early redistribution of plasma membrane phophatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Ab1.; J.Exp.Med., 182: 1545-1556

8. Williamson, P., Bevers, E. M., Smeets, E. F., Comfurius, P., Schlegel, R. A., and Zwaal, R. F., 1995: Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets; Biochemistry, 34(33): 10448-10455

9. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J., 1989: Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation; Proc.Natl.Acad.Sci.U.S A, 86(9): 3184-3188

10. Schröder-Borm, H., Bakalova, R., and Andrä, J., 2005: The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine; FEBS Lett, 579(27): 6128-6134

11. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

12. Woehlecke, H., Pohl, A., Alder-Baerens, N., Lage, H., and Herrmann, A., 2003: Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2); Biochem.J., 376(Pt 2): 489-495

13. Ran, S., Downes, A., and Thorpe, P. E., 2002: Increased exposure of anionic phospholipids on the surface of tumor blood vessels; Cancer Res., 62(21): 6132-6140

14. Cichorek, M., Kozlowska, K., Witkowski, J. M., and Zarzeczna, M., 2000: Flow cytometric estimation of the plasma membrane diversity of transplantable melanomas, using annexin V; Folia Histochem.Cytobiol., 38(1): 41-43

15. Papo, N., Seger, D., Makovitzki, A., Kalchenko, V., Eshhar, Z., Degani, H., and Shai, Y., 2006: Inhibition of Tumor Growth and Elimination of Multiple Metastases in Human Prostate and Breast Xenografts by Systemic Inoculation of a Host Defense–Like Lytic Peptide; Cancer Res, 66(10): 5371-5378

16. Albert, M. L., Sauter, B., and Bhardwaj, N., 1998: Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs; Nature, 392(6671): 86-89

17. Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y. A., Cordon-Cardo, C., and Lowe, S. W., 2001: Inactivation of the apoptosis effector Apaf-1 in malignant melanoma; Nature, 409(6817): 207-211

18. Miyashita, T. and Reed, J. C., 1993: Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line; Blood, 81(1): 151-157

19. Papo, N., Braunstein, A., Eshhar, Z., and Shai, Y., 2004: Suppression of human prostate tumor growth in mice by a cytolytic D-, L-amino Acid Peptide: membrane lysis, increased necrosis, and inhibition of prostate-specific antigen secretion; Cancer Res., 64(16): 5779-5786

20. Gifford, J. L., Hunter, H. N., and Vogel, H. J., 2005: Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties; Cell Mol.Life Sci., 62(22): 2588-2598

21. Yang, N., Stensen, W., Svendsen, J. S., and Rekdal, O., 2002: Enhanced antitumor activity and selectivity of lactoferrin-derived peptides; J.Pept.Res., 60(4): 187-197

22. Lohner, K., Latal, A., Lehrer, R. I., and Ganz, T., 1997: Differential scanning microcalorimetry indicates that human defensin, HNP-2, interacts specifically with biomembrane mimetic systems; Biochemistry, 36(6): 1525-1531

23. Papo, N. and Shai, Y., 2005: Host defense peptides as new weapons in cancer treatment; Cell Mol.Life Sci., 62(7-8): 784-790

24. Vogel, H. J., Schibli, D. J., Jing, W., Lohmeier-Vogel, E. M., Epand, R. F., and Epand, R. M., 2002: Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides; Biochem.Cell Biol., 80(1): 49-63

25. Hoskin, D. W. and Ramamoorthy, A., 2008: Studies on anticancer activities of antimicrobial peptides; Biochim.Biophys.Acta, 1778(2): 357-375

26. Spiegl-Kreinecker, S., Buchroithner, J., Elbling, L., Steiner, E., Wurm, G., Bodenteich, A., Fischer, J., Micksche, M., and Berger, W., 2002: Expression and functional activity of the ABC-transporter proteins P-glycoprotein and multidrug-resistance protein 1 in human brain tumor cells and astrocytes; J.Neurooncol., 57(1): 27-36

27. Rinner, B., Kretschmer, N., Knausz, H., Mayer, A., Boechzelt, H., Hao, X. J., Heubl, G., Efferth, T., Schaider, H., and Bauer, R., 2010: A petrol ether extract of the roots of Onosma paniculatum induces cell death in a caspase dependent manner; Journal of Ethnopharmacology, 129(2): 182-188

28. Satyamoorthy, K., DeJesus, E., Linnenbach, A. J., Kraj, B., Kornreich, D. L., Rendle, S., Elder, D. E., and Herlyn, M., 1997: Melanoma cell lines from different stages of progression and their biological and molecular analyses; Melanoma Res., 7 Suppl 2(S35-S42

29. Nesbit, M., Schaider, H., Miller, T. H., and Herlyn, M., 2001: Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells; J.Immunol., 166(11): 6483-6490

30. Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr., Carter, B. S., Krichevsky, A. M., and Breakefield, X. O., 2008: Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers; Nat.Cell Biol., 10(12): 1470-1476

31. Boersma, H. H., Kietselaer, B. L., Stolk, L. M., Bennaghmouch, A., Hofstra, L., Narula, J., Heidendal, G. A., and Reutelingsperger, C. P., 2005: Past, present, and future of annexin A5: from protein discovery to clinical applications; J.Nucl.Med., 46(12): 2035-2050

32. Broekhuyse, R. M., 1968: Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids; Biochim.Biophys Acta, 152(2): 307-315

33. Comfurius, P., Senden, J. M., Tilly, R. H., Schroit, A. J., Bevers, E. M., and Zwaal, R. F., 2005: Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase; Biochim.Biophys.Acta, 1026(2): 153-160

34. Wiedmer, T., Shattil, S. J., Cunningham, M., and Sims, P. J., 1990: Role of calcium and calpain in complement-induced vesiculation of the platelet plasma membrane and in the exposure of the platelet factor Va receptor; Biochemistry, 29(3): 623-632

35. Belting, M. and Wittrup, A., 2008: Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease; J Cell Biol., 183(7): 1187-1191

36. Manavbasi, Y., Zweytick, D., Willumeit, R., and Lohner, K., 2009: Phosphatidylserine Selective Peptides As Novel Anti-cancer Agents; Biophysical Journal, 96(3, Supplement 1): 157a-157a

37. Riedl, S., Rinner, B., Tumer, S., Schaider, H., Lohner, K., and Zweytick, D., 2011: Targeting the cancer cell membrane specifically with human lactoferricin derivatives; Ann.Oncol., 22(Suppl 3): 33

38. Lohner, K. and Blondelle, S. E., 2005: Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics; Comb.Chem.High Throughput.Screen., 8(3): 241-256

39. Zasloff, M., 2002: Antimicrobial peptides of multicellular organisms; Nature, 415(6870): 389-395

40. Shai, Y. and Oren, Z., 2001: From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides; Peptides, 22(10): 1629-1641

41. Blondelle, S. E. and Lohner, K., 2010: Optimization and high-throughput screening of antimicrobial peptides; Curr.Pharm.Des, 16(28): 3204-3211

42. Hallock, K. J., Lee, D. K., and Ramamoorthy, A., 2005: MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain; Biophys.J., 84(5): 3052-3060

43. Papo, N. and Shai, Y., 2003: New lytic peptides based on the D,L-amphipathic helix motif preferentially kill tumor cells compared to normal cells; Biochemistry, 42(31): 9346-9354

44. Eliassen, L. T., Berge, G., Sveinbjornsson, B., Svendsen, J. S., Vorland, L. H., and Rekdal, O., 2002: Evidence for a direct antitumor mechanism of action of bovine lactoferricin; Anticancer Res., 22(5): 2703-2710

Killing of melanoma cells and metastases by human Lactoferricin derivatives correlates with effect on Cancer Models composed of Phosphatidylserine

Sabrina Riedl, Sabine Tumer, Karl Lohner and Dagmar Zweytick

[1]Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstraße 6, A-8042 Graz, Austria

Acknowledgement

We like to acknowledge Dr. Schaider and Dr. Rinner from the Department of Dermatology and the Center of Medical Research at the Medical University of Graz for supply with melanoma cell lines and primary culture of melanocytes.

Abstract

Despite favorable advancements in therapy, cancer is still not curative in many cases. Weak cancer toxicity or side effects can mainly be explained by inadequate specificity towards tumor cells. In this study derivatives of a short cationic peptide derived from the human host defense peptide Lactoferricin (hLFcin) were further optimized in their activity and selectivity towards cancer cells. The target of these peptides is the negatively charged membrane lipid phosphatidylserine (PS), specifically exposed by cancer cells.

We have studied the membrane interaction of three peptides namely LF11-322, 6-Methyloctanoyl-LF11-322 (6-MO-LF11-322) and a retro dimer with Pro-linker of LF11-322 (R-DIM-P-LF11-322) with liposomes mimicking cancerous and non-cancerous cell membranes composed of phosphatidylserine (PS) and phosphatidylcholine (PC), respectively. Calorimetric and permeability studies showed that N-acylation and even more dimerization of LF11-322 led to strongly improved interaction with PS-liposomes, whereas PC-liposomes were only affected by the lipopeptide though to minor extent than PS. Tryptophan fluorescence of selective peptides revealed peptide penetration selectively into the PS membrane interface and and CD spectra indicated β-sheet conformation in the presence of PS liposomes. Only 6-MO-LF11-322 exhibited α-helical conformation in presence of the cancer as well the non-cancer mimic. Data correlated with *in vitro* studies with melanomas and melanocytes, revealing R-DIM-P-LF11-322 to be the most active and selective peptide with up to 1000fold specificity for cancer cells, which indicates the need of high affinity to the target PS, a minimum peptide length, positive net charge, an adequate hydrophobicity and adoption of structural conformation in presence of the target membrane for high antitumor activity.

Introduction

In 2008 more than 12.7 million people worldwide were newly diagnosed with cancer accounting for 7.6 million deaths (http://www.who.int/mediacentre/factsheets/fs297/en/). Prospects of the World Health Organization (WHO) suggest a further increase of new cases in 2030 to 11 million deaths.

In the last decades, much progress has been achieved regarding chemotherapy, surgery, radiotherapy, targeted therapy and combinations thereof but there are still many disadvantages and problems that have to be handled. Severe side effects due to non-adequate specificity for tumor cells and drug resistance are major problems.

A strategy for the development of new cancer therapies is the use of host defense peptides (1) of various origins and derivatives thereof. Host defense peptides are mostly cationic amphipathic peptides, targeting the cell membrane by electrostatic interactions with anionic molecules at the cell surface. Various anionic molecules are provided by cancer cells that could account for the selectivity of these membrane active peptides towards cancer cells (2). One major target discussed is the negatively charged phospholipid phosphatidylserine (PS) which is exposed on the outer leaflet of the cancer cell membrane (3-7). In non-cancerous cells the outer leaflet of the plasma membrane exhibits an overall neutral charge due to its main components, the zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM). Phosphatidylserine together with phosphatidylethanolamine (PE) assembles the inner leaflet of eukaryotic non-cancerous plasma membranes (8). This regularly occurring asymmetric distribution of the major phospholipids between the two membrane leaflets is well documented (9,10) and is maintained by an ATP-dependent aminophospholipid translocase (11). The loss of membrane asymmetry was shown for several cancer types, which yields a promising overall marker for cancer (7,12) as well as a specific target for host defense peptides. In these recent studies, we could also show that not only cancer cell lines exhibit PS on the outer leaflet of the cell membrane but also cells from primary cultures and metastases expose significant amounts of PS confirming nativeness and maintenance of PS as a molecular target. Additionally, there is another

source of negative charges on the surface of human cells in form of sialic acid residues which can be linked to glycoproteins (e.g. mucins) and glycolipids, which are often overexpressed by tumor cells (13-18), nevertheless these are also present on healthy cell surfaces just at lower concentration, therefore not providing a selective target for host defense peptides.

In our study, we focused on the human host defense peptide lactoferricin (LFcin) which is reported to exhibit antimicrobial, antiviral, anti-inflammatory and antitumor activities (19-21). Human lactoferricin comprises amino acid residues 1-45 of the N-terminus of human Lactoferrin (hLF). LF11, an 11 amino acid fragment of hLFcin has already been optimized by our group regarding its activity against bacterial membranes (22,23). Since bacterial membranes like cancer membranes comprise negatively charged phospholipids on their surface, it was reasonable to test LF11 derivatives with cancer cell mimics. One peptide group was selected for thorough investigations: LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322. It has been shown in the antibacterial studies that N-acylation enhances the antibacterial activity due to the higher hydrophobic content (22,23). Since it was reported by Yang et al (24) that a minimum length and minimum net positive charge are needed for appropriate antitumor activity of an anticancer peptide, R-DIM-P-LF11-322 has been designed to fulfill these requirements. In this article an insight in the structural and mechanistic requirements for a selective antitumor peptide like R-DIM-P-LF11-322 is given.

Experimental procedures

Materials

1,2-Dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC), 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (POPC), 1,2-Dihexadecanoyl-sn-glycero-3-phospho-L-serine (Na-salt) (DPPS) and 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (Na-salt) (POPS) were purchased from Avanti Polar Lipids, Inc. (USA), and used without further purification. Stock solutions of DPPC and POPC were prepared in CHCl3/CH3OH (2:1, v/v), stock solutions of DPPS and POPS were prepared in CHCl3/CH3OH (9:1, v/v) and stored at -18°C.

The amidated peptides LF11-322 (PFWRIRIRR-NH_2, M=1298.6 g/mole), its N-6-Methyloctanoyl derivative 6-MO-LF11-322 (CH_3CH_2-$CH_2(CH_3)$-$(CH_2)_4$-CO-NH-PFWRIRIRR-NH_2, M=1438.9 g/mole) and R-DIM-P-LF11-322 (PFWRIRIRRPRRIRIRWFP-NH_2, M= 2677.4 g/mole) were purchased from NeoMPS, Inc. (San Diego, CA, USA). The purities were >96% as determined by RP-HPLC. Peptides were dissolved in Acetic acid (0.1%, v/v) at a concentration of 3 mg/ml. Peptide solutions were stored at 4°C and concentrations were determined photometrically at 280 nm.

ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and DPX (p-xylene-bis-pyridinium bromide) used for permeability studies were purchased from Molecular Probes (Eugene, OR).

Cell lines and culture

Melanoma cell lines from primary (SBcl-2) and metastatic (WM164) lesions were grown in RPMI 1640 medium with stable L-Glutamine (Invitrogen, UK), supplemented with 2% FBS. Human melanocytes used as healthy control cells: were isolated from foreskin. Foreskin was cut into small pieces and incubated with 0.3% trypsin (PAA) overnight at 4°C and for one hour at 37°C. Epidermis was separated. Cells were mechanically removed from the cell layer and centrifuged at 300g for 3 min. The pellet was re-suspended and cells further cultured in melanocyte growth medium (Biomedica, Vienna, Austria, PromoCell GmbH, Heidelberg, Germany). All cells were kept in a 5% CO_2 atmosphere

at 37°C. At 90% confluence cell-culture flasks were passaged with accutase (PAA, Pasching Austria). All cell cultures were periodically checked for mycoplasma.

Toxicity studies - PI-uptake assay

Cells were collected, re-suspended in media and diluted to a concentration of 10^6 cells/ml. Aliquots of 10^5 cells/100 µl media were incubated with peptides for 8 hours at 37°C and 5% CO_2. PI (2 µl/10^5cells of 50 µg/ml, Invitrogen, Camarillo, CA, USA) was added and cells were again incubated for 5 min at room temperature in the dark. Excitation and emission wavelengths were 536 nm and 617 nm, respectively.

Cytotoxicity was calculated from the percentage of PI positive cells in media alone (P_0) and in the presence of peptide (P_X). Triton-X was used to determine 100% of PI positive cells (P_{100}).

$$\% PI - uptake = \frac{100 * (P_X - P_0)}{(P_{100} - P_0)}$$

Preparation of liposomes

Appropriate amounts of respective phospholipid stock solution were dried under a stream of nitrogen and stored in vacuum overnight to completely remove organic solvents. The dry lipid film was then dispersed in phosphate buffered saline (PBS, 20 mM NaPi, 130 mM NaCl, pH 7.4) and hydrated at a temperature well above the gel to fluid phase transition of the respective phospholipid under intermittent vigorous vortex-mixing. The lipid concentration was 0.1 weight% for calorimetric experiments. Hydration was carried out in presence or absence of peptides at a lipid to peptide ratio of 25:1 and 12.5:1 using a protocol described for POPS (25), DPPS (26) and DPPC (27). The fully hydrated samples were stored at room temperature until measurement.

Differential scanning calorimetry (DSC)

DSC experiments were performed with a differential scanning calorimeter (VP-DSC) from MicroCal, Inc. (Northhampton, MA, USA). Heating scans were performed at a scan rate of 30°C/h (pre-scan thermostating 30 min) with a final temperature of approximately 10°C above the main transition temperature (T_m) and cooling scans at the same scan rate (pre-scan thermostating 1 min) with a final temperature approximately 20°C below T_m. The heating/cooling cycle was performed three times. Enthalpies were calculated by integration of the peak areas after normalization to phospholipid concentration and baseline adjustment using the MicroCal Origin software (VP-DSC version). The phase transition temperature was defined as the temperature at the peak maximum (28).

Fluorescence spectroscopy

Fluorescence spectroscopy experiments were performed using a SPEX Fluoro Max-3 spectrofluorimeter (Jobin-Yvon, Longjumeaum, France) and spectra were analyzed with Datamax software.

Tryptophan quenching

Tryptophan fluorescence spectra were obtained at room temperature using an excitation wavelength of 282 nm and a slit width of 5 nm for excitation and emission monochromators. Quenching of Tryptophan was carried out in the presence and absence of phospholipid liposomes (lipid to peptide ratio 25:1) using 0.1, 0.4 and 0.7 M acrylamide. The data were analyzed according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F represent the fluorescence emission intensities in the absence and presence of the quencher molecule (Q) and K_{SV} is the Stern-Volmer quenching constant, which is a quantitative measure for the accessibility of tryptophan to acrylamide (29).

ANTS/DPX Leakage Experiments

Leakage of aqueous contents from liposomes was determined using the 8-aminonaphthalene-1,3,6-trisulfonic acid / p-xylene-bis-pyridinium bromide (ANTS/DPX) assay (30). Lipid films (preparation see above) were hydrated with 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4 following a standard procedure.

Subsequently, the dispersions were extruded 20 times through a polycarbonate filter (Millipore - Isopore™) of 0.1 µm pore size to obtain LUVs. Unilamellarity and size were tested by X-ray and dynamic light scattering, respectively. The ANTS/DPX encapsulating vesicles were separated from free ANTS/DPX by exclusion chromatography using a column filled with Sephadex™ G-75 (Amesham Biosciences) fine gel swollen in an iso-smotic buffer (10 mM HEPES, 140 mM NaCl, pH 7.4). The void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis (31,32).

The fluorescence measurements were performed in 2 mL of the isosmotic buffer in a quartz cuvette at room temperature. Aliquots of LUVs were diluted with the iso-osmotic buffer to a final lipid concentration of 50 µM. Fluorescence spectra were obtained at 37°C using an excitation wavelength of 360 nm and an emission wavelength of 530 nm and a slit width of 5 nm for both excitation and emission monochromators.. Fluorescence emission was recorded as a function of time before and after the addition of incremental amounts of peptide. The fluorescence increase due to leakage and subsequent dilution of quenched dye was measured after addition of peptides. Peptides were added to final concentrations of 2, 4 and 8 µM, corresponding to peptide to lipid molar ratios of 1:25, 1:12.5 and 1:6.25, respectively.

Data are presented in terms of fluorescence intensity (I_F):

$$I_F = \frac{F - F_0}{F_{max} - F_0}$$

F is the measured fluorescence, F_0 the initial fluorescence without peptide and F_{max} the fluorescence corresponding to 100% leakage gained by addition of 1% Triton X-100.

Circular dichroism spectroscopy

Measurements were performed on a Jasco J 715 Spectropolarimeter (Jasco, Gross-Umstadt, Germany) at room temperature using quartz cuvettes with an optical path length of 0.02 cm. The CD spectra were measured between 260 nm and 180 nm with a 0.2 nm step resolution. To improve accuracy 5 scans were averaged. Peptides were dissolved in 10 mM Hepes (pH 7.4) to a final concentration of 100 µM. Spectra were measured in the absence and presence of 1 mM sodium dodecyl sulfate (SDS) and 1 mM dodecylphosphocholine (DPC) mimicking cancer and healthy mammalian membranes, respectively. The respective peptide to surfactant molar ratios were 1:25 and 1:100. Background signals were abstracted after measurements. Percentage secondary structure calculations were done using Dichroweb, CDSSR Convolution Program using reference set 4 (33,34).

Results

In this study, we report on activity, respectively selectivity of human Lactoferricin derivatives for cancer melanoma and/or non-cancer melanocyte cells *in vitro* and in model systems. The three peptides studied, LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322, derived from the membrane active peptide LF11, differed in several aspects: number of amino acids and positive charges, hydrophobicity and consequently structure.

Table 1: Overview of peptide sequences, net charge and hydrophobicity of hLFcin derivatives

	sequence	Net charge	ΔG_{woct}[a] [kcal/mol]
LF11-322	PFWRIRIRR-NH_2	+5	5.64
6-MO-LF11-322	6-Methyl-octanoyl-PFWRIRIRR-NH_2	+4	3.34*
R-DIM-P-LF11-322	PFWRIRIRRPRRIRIRWFP-NH_2	+9	7.12

[a] Peptide hydrophobicity expressed as transfer free energy of peptides from water to n-octanol (ΔG_{woct}) calculated from the whole-residue hydrophobicity scale taking into account the contribution of the C-terminal amide (35); * For calculation of ΔG_{woct} of 6-MO-LF11-322 instead of the 6-MO residue N-acetylation was taken as a first approximation.

Cancer toxicity and selectivity in vitro

Cytotoxic activity of the peptides towards melanoma cells of primary - (SBcl-2) and metastatic lesions (WM164) and differentiated non-tumorigenic melanocytes was determined by measurement of PI-uptake, which only occurs when integrity of the cell membrane is lost. Cells were incubated in media containing serum for 8h in the presence of peptides. Peptide concentrations were varied from 10 to 80 µM. Figure 1 illustrates that LF11-322 is only minor active against the melanoma cell line SBcl-2 with less than 5% killing at a peptide concentration of 80µM, as well as against melanocytes with a moderate twofold selectivity for WM164 cells at 20µM peptide concentration (Figure 1C). N-

acylation in the case of 6-MO-LF11-322 significantly improves cancer cell toxicity. Nevertheless at its ~IC_{50} of 20 µM peptide concentration it already kills ~15% of the non-cancer melanocytes again yielding a rather modest 3-4-fold specificity for cancer over non-cancer cells (Figure 1C). Interestingly the specificity of 6-MO-LF11-322 for cancer cells is 7-fold after one hour (data not shown) but subsequently decreases to 3-4-fold after 8 hours (Figure 1C). Surprisingly, the dimeric peptide R-DIM-P-LF11-322 is even more active against SBcl-2 than the N-acylated peptide 6-MO-LF11-322, but with very high specificity. Already at a peptide concentrations of 20 µM, R-DIM-P-LF11-322 yields more than 80% PI positive SBcl-2 cells, while only less than 1% of differentiated non-tumorigenic melanocytes are killed, exhibiting a specificity greater than 1000-fold for cancer cells (see Figure 1C). Sensitivity for the second melanoma cell line, WM164, tested at 20µM R-DIM-P-LF11-322 peptide concentration is also high with more than 500-fold.

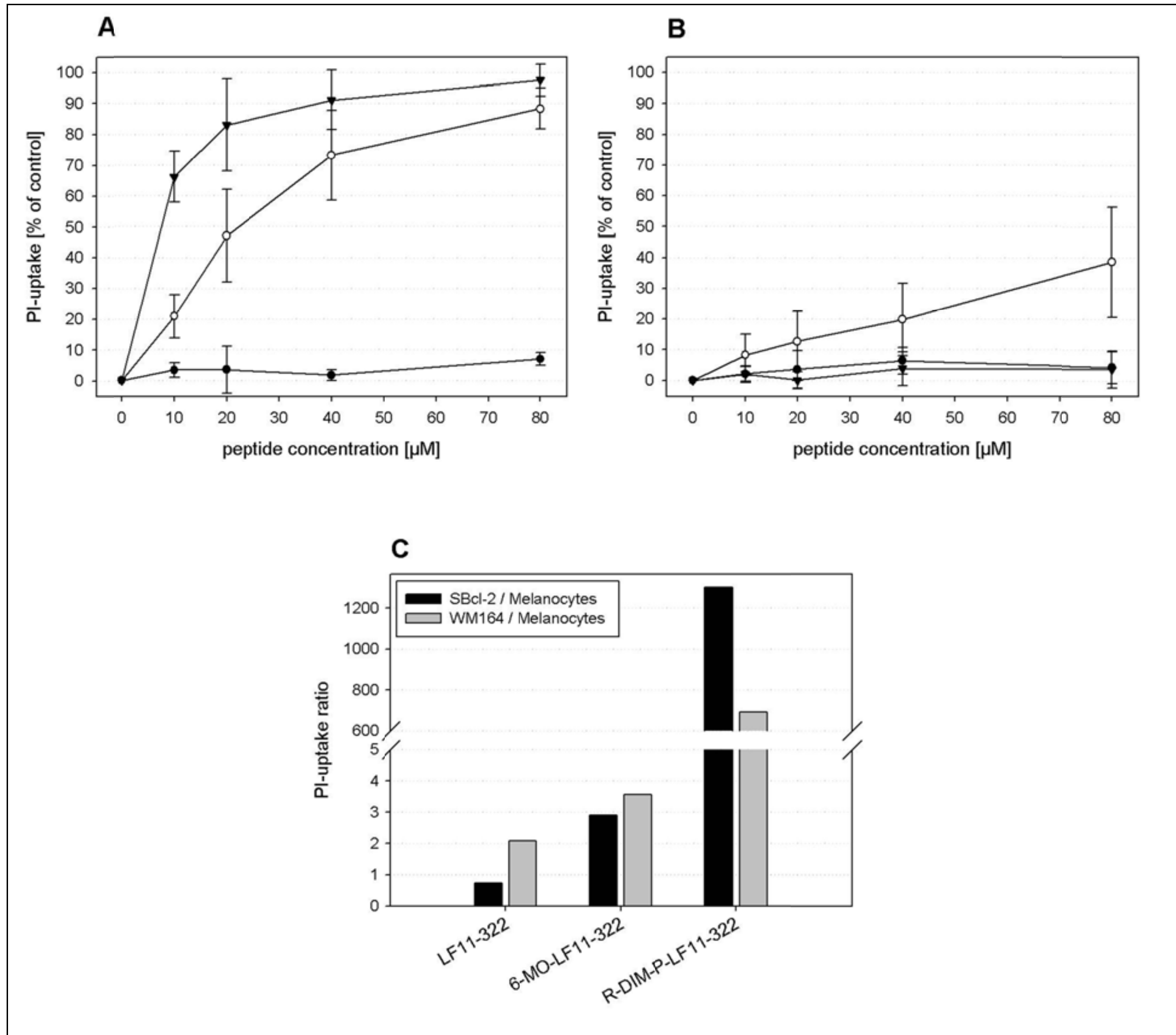

Figure 1: Peptide toxicity-PI-uptake of cancer and non-cancer cell lines: (A) Concentration dependent cytotoxic activity of LF11-322 (●), 6-MO-LF11-322 (○) and R-DIM-P-LF11-322 (▼) against melanoma cell line SBcl-2 after 8 hours of incubation with peptides; (B) Concentration dependent cytotoxic activity against primary cultures of differentiated non-tumorigenic melanocytes after 8 hour of incubation with peptides; (C) specificity of peptides at 20 µM peptide concentration after 8h of incubation displayed as PI-uptake ratio of SBcl-2 vs. melanocytes and WM164 vs. melanocytes.

Activity and selectivity in model systems-mechanistic and structural studies

Differential scanning calorimetry – destabilization of cancer model membranes

Different liposomal mimics of human cancerous and non-cancerous cell membranes were applied to study the respective membrane interaction of peptides: DPPS and POPS, negatively charged phospholipids exposed on the outer leaflet of plasma membranes of mammalian cancer cells (3;7), and DPPC, a zwitterionic phospholipid mimicking plasma membranes of non-neoplastic mammalian cells.

Consistently with published data (36), the excess heat capacity curve of pure DPPS shows one phase transition (Figure 2A) which corresponds to the main chain-melting from the lamellar-gel (L_β) to the fluid (L_α) phase at 52.6°C. Addition of LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322 (25:1 lipid to peptide molar ratio) results in a decrease of the main transition enthalpy (ΔH_m) of DPPS, but to different extent. DSC thermograms show that LF11-322 only slightly reduces the main transition enthalpy of PS by 10%. However, N-acylation of LF11-322 has a stronger effect leading to a reduction of the transition enthalpy by 35%. Finally dimerization, in form of R-DIM-P-LF11-322, leads to the most severe perturbance of the cancer mimic DPPS, indicated by a strong decrease of the transition enthalpy by 70% (see Table 1A). In the presence of all peptides the main transition temperature (T_m) shifts to lower values, due to a destabilization of the gel phase. Again the dimer exhibits the strongest effect by a decrease of the transition temperature by nearly 5°C and a broad peak over the whole transition range, which could indicate micellization of DPPS upon addition of the peptide. The peak of the main transition of the monomer is split into at least two peaks due to different peptide affected lipid domains, which was also observed with the non-acylated LF11-322 upon interaction with phosphatidylglycerol, a bacterial model system (23). Addition of the N-acylated peptide 6-MO-LF11-322 however causes a main peak at decreased transition temperature and a lower small transition at 40.6°C, the latter cannot clearly be related to the gel to liquid crystalline transition. The increase of the transition half-width ($\Delta T_{1/2}$) (Table 1A) shown for all 3 endotherms is due to severe

loss of cooperativity. Similar results were observed for POPS liposomes (data not shown). Interestingly, the effect of the monomer at doubled concentration (+ LF11-322; 12.5:1 lipid to peptide ratio; Figure 2A) does not reach that of the dimer. Though destabilization of the gel phase is enhanced it is minor than in the presence of R-DIM-P-LF11-322.

Unlike DPPS and POPS, thermograms of DPPC exhibit two transitions (Figure 2B), as well in agreement with published data (37). The transition at 34.6°C corresponds to the pre-transition, from the lamellar-gel (L_{β}’) to the ripple phase (P_{β}’). The second transition can be attributed to the main transition at 41.3°C (from the ripple (P_{β}’) to the fluid (L_{α}) phase). In contrast to DPPS and POPS, DPPC liposomes remain unperturbed by addition of LF11-322, even when the concentration of peptide is doubled (see Figure 2B and Tab.1B) (LF11-322; 12.5:1 lipid to peptide molar ratio) or upon addition of the dimeric form R-DIM-P-LF11-322, where only a slight loss of cooperativity occurs indicated by a minor increase of the half width of the transition. Addition of 6-MO-LF11-322 to DPPC liposomes (25:1 lipid to peptide molar ratio) however results in some perturbance of DPPC membranes displayed by a reduction of ΔH_{pre} and formation of a peptide affected domain exhibiting decreased transition temperature and increased cooperativity (Table 1B). Similar effects on DPPC were observed for the N-acylated derivative of LF11, namely, C12-LF11 (22).

Table 1: Thermodynamic parameters of DPPS (cancer cell mimic) (A) and DPPC (non-cancer cell mimic) (B) in the absence and presence of LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322 at a lipid to peptide molar ratio of 25:1 (unless otherwise noted).

A

	ΔH_m [kcal/mole]	T_m [°C]	$\Delta T_{1/2}$ [°C]
DPPS	9.9	52.6	0.65
+ LF11-322	4.7 / 4.5	49.3 / 50.9	1.7 / 1.4
+ LF11-322 (12.5:1)	2.1 / 4.9	47.8 / 50.1	n.d. / 2.48
+ 6-MO-LF11-322	0.7 / 5.9	40.6 / 51.7	n.d. / 2.24
+ R-DIM-P-LF11-322	3.1	47.9	1.53

B

	ΔH_{pre} [kcal/mole]	T_{pre} [°C]	ΔH_m [kcal/mole]	T_m [°C]	$\Delta T_{1/2,m}$ [°C]
DPPC	1.0	34.6	9.1	41.3	0.19
+ LF11-322	1.1	34.8	9.0	41.3	0.17
+ LF11-322 (12.5:1)	1.0	34.5	9.2	41.3	0.26
+ 6-MO-LF11-322	0.4	34.6	5.2 / 3.9	40.7 / 41.3	n.d. / 0.26
+ R-DIM-P-LF11-322	1.5	34.5	9.1	41.3	0.25

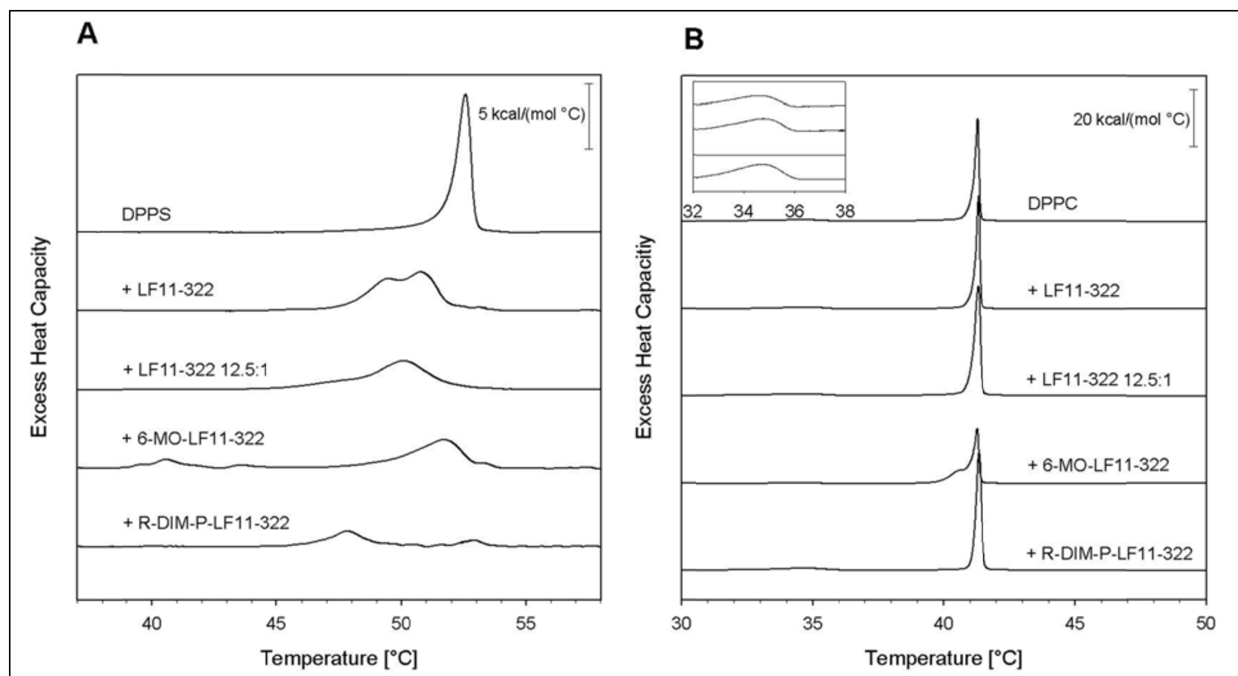

Figure 2: DSC thermograms of (A) DPPS and (B) DPPC in the absence and presence of LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322 (25:1, lipid to peptide molar ratio, unless otherwise noted). For clarity, the DSC curves were displayed on the ordinate by an arbitrary increment. The pre-transition of DPPC is shown enlarged in the inset (only for 25:1 lipid to peptide molar ratio).

ANTS/DPX leakage experiments – membrane permeabilization

The membrane permeabilizing effect of peptides (2-8 µM) on cancer and non-cancer mimics was also tested by induction of leakage of ANTS/DPX from large unilamellar vesicles composed of POPS and POPC, respectively.

As illustrated in Figure 3A, addition of the peptides to POPS vesicles leads to different release of ANTS/DPX. N-acylation increases the weak ANTS/DPX release induced by 4 and 8 µM LF11-322 (~20% at 8 µM) by a factor of three up to ~ 30 and 70%, respectively. Whereas the dimer R-DIM-P-LF11-322 is most effective in terms of permeability inducing leakage up to 90-100% at highest peptide concentration.

Figure 3B exhibits results of ANTS/DPX release from pure POPC liposomes, mimicking non-cancerous cell membranes. Obviously, addition of LF11-322 and R-DIM-P-LF11-322 does not result in any significant release of the fluorophore (less than 5%). Only the N-acylated peptide 6-MO-LF11-322 causes slight leakage from POPC vesicles of about 20% at a peptide-to-lipid molar ratio of 1:6.25, providing only 3-fold specificity for the cancer mimic. The dimer exhibits the highest activity for the cancer mimic with up to 20-fold selectivity for cancer over non-cancer mimics.

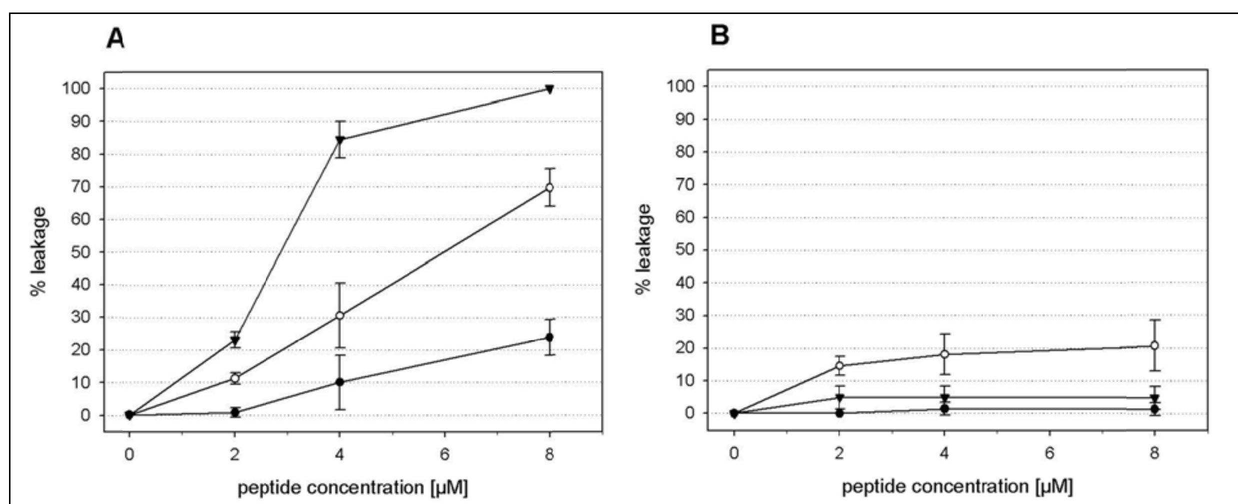

Figure 3: ANTS/DPX leakage of LUVs composed of POPS (A) and POPC (B) as a function of the concentration of LF11-322 (●), 6-MO-LF11-322 (○) and R-DIM-P-LF11-322 (▼). Concentration of LUVs was 50 µM and temperature was kept at 37°C during measurements. Complete lysis was determined by addition of Triton X-100 and zero levels correspond to fluorescence before peptide addition.

Tryptophan quenching – peptide solubility and penetration depth

The Trp-emission properties (emission wavelength and fluorescence quenching by acrylamide) can be used to study changes in solubility and localization of Trp of a peptide in different environments, as in solution or in the presence of membranes composed of different lipids. The emission spectra of the one Trp of LF11-322 and the two Trp of the dimer in buffer exhibit a maximum ($\lambda_{em,max}$) of 356 nm

(see Table 2). This is in agreement with the emission maximum of Trp exposed to polar environment as e.g. to aqueous solution. The N-acylated peptide 6-MO-LF11-322 however exhibits a maximum of 353 nm, indicating slightly less exposure of the Trp to the environment and probably shielding of the Trp by the N-acyl chain in solution. A similar observation was reported for the N-lauryl-derivative of LF11 (22). However together with the high Stern-Volmer constant of LF11-322 and 6-MO-LF11-322 (K_{SV}>20 M^{-1}) in solution an absence of peptide aggregation can be assumed. Very surprisingly, the Stern-Volmer constant of R-DIM-P-LF11-322 in buffer is only 0.7 M^{-1}. This indicates the Trp-residue of the peptide to be not exposed to the environment, which can only be due to peptide aggregation or the peptide structured in solution (probably shielding of the Trp residue by other and polar amino acids).

By comparing Trp fluorescence in solution with fluorescence in the presence of the cancer mimics POPS and DPPS (lipid to peptide ratio 25:1) it is obvious that the Stern-Volmer constant (K_{SV}) is dramatically decreased for LF11-322 and 6-MO-LF11-322. This observation gives evidence for a less polar environment of the Trp of the two peptides indicating localization at least at the interface of the PS bilayer. In the case of R-DIM-P-LF11-322, the K_{SV} is still as low in the presence of the anionic phospholipids DPPS and POPS as in solution and together with a strong decrease of the $\lambda_{em,max}$ by 20nm it can be suggested that the Trp in the presence of the cancer mimic is shielded by the hydrophobic fatty acyl chains of the cancer mimic lipids. Additionally, a significant blue shift of the emission wavelength could also be observed for LF11-322 and 6-MO-LF11-322 giving as well evidence of penetration of the Trp into the membrane interface. The K_{SV} for all peptides was lower in the presence of anionic phospholipids with unsaturated fatty acyl chains (POPS) than saturated (DPPS), which seems feasible since peptides can penetrate more easily in less tightly packed membranes. Besides acyl chains of POPS are fluid at the experimental temperature, whereas the acyl chains of DPPS are still in the gel phase.

Strikingly no significant blue shift by peptides LF11-322 and R-DIM-P-LF11-322 in the presence of DPPC, the non-cancerous cell mimic, implies any membrane penetration of Trp which is contrary to DPPS and POPS, the cancerous cell mimic. This indicates that these peptides do not penetrate into the DPPC membrane interface, but are specific for the cancer mimic. Only 6-MO-LF11-322 exhibits a blue shift in the presence of DPPC by 9nm though it is less than in the presence of the cancer model membranes, indicating less specific membrane interaction than by the other peptides.

Table 2: Stern-Volmer quenching constant (K_{SV}) and maxima of emission wavelength ($\lambda_{em,max}$)of LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322 in PBS, POPS, DPPS and DPPC membranes at a lipid to peptide ratio of 25:1.

Peptide	**K_{SV} in PBS [M^{-1}] ($\lambda_{em,max}$)**	**K_{SV} in POPS [M^{-1}] ($\lambda_{em,max}$)**	**K_{SV} in DPPS [M^{-1}] ($\lambda_{em,max}$)**	**K_{SV} in DPPC [M^{-1}] ($\lambda_{em,max}$)**
LF11-322	22.4 (356 nm)	5.4 (338 nm)	7.9 (334 nm)	8.8 (355 nm)
6-MO-LF11-322	24.5 (353 nm)	4.5 (342 nm)	6.6 (337 nm)	8.9 (344 nm)
R-DIM-P-LF11-322	0.7 (356 nm)	0.6 (336 nm)	1.7 (337 nm)	1.0 (354 nm)

Circular dichroism spectroscopy - secondary structure vs. activity and selectivity

Circular dichroism studies revealed significant differences in secondary structure of the three peptides in lipidic environment. Only in solution all peptides exhibit similar structural properties, being mainly unstructured with some proportion of β-sheet conformation with turns (see Figure 4A-C).

The N-acylated peptide (Figure 4B) exhibits the highest (15%) though still minor fraction of α-helical content in solution. SDS was taken as negatively charged cancer mimic and DPC served as neutral healthy mimic. Two different peptide to lipid ratios were used, 1:25 to define conditions comparable to model studies, 1:100 to ensure complete binding of whole peptide amount, rather comparable to conditions *in vitro*. Nevertheless only LF11-322 (Figure 4A) in the presence of SDS showed some enhanced effect at higher peptide concentration. The other peptides do not show any significant structural changes at different concentrations, therefore it can be assumed that also at high peptide to lipid ratios no or all peptide is bound. Strikingly the selective peptides LF11-322 (Figue 4A) and R-DIM-P-LF11-322 (Figure 4C) exhibit a significant increase of β-sheet conformation in the presence of the cancer mimic SDS, the α-helical content is even further decreased. Moreover the structure of these peptides in the presence of the healthy mimic DPC is the same as in solution, giving further hint for the cancer selective toxicity of these peptides. Only the less selective peptide 6-MO-LF11-322 exhibited a significant increase of the α-helical content to same extent in the presence of both cancer and non-cancer mimic.

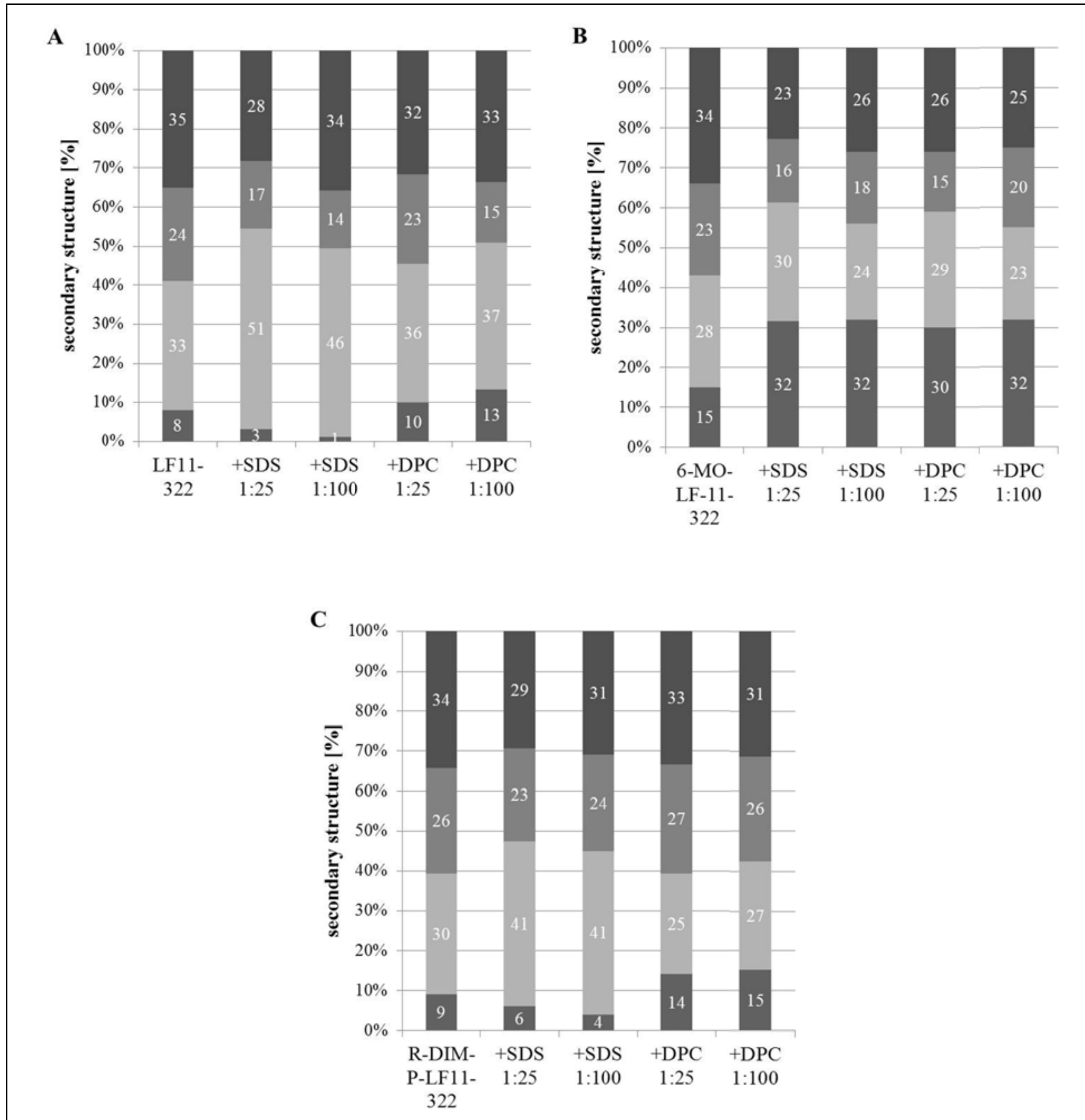

Figure 4: Secondary structures of LF11-322 (A), 6-MO-LF11-322 (B) and R-DIM-P-LF11-322 (C) in Hepes buffer (first bar) or presence of SDS and DPC at peptide to surfactant ratios of 1:25 and 1:10O, determined using CD spectroscopy. Percentage secondary structure calculations were done using Dichroweb, CDSSR Convolution Program using reference set 4 (33;34). The α-helical content is shown in gray at the bottom; β-turns in light grey; turns in middle grey; random coil structures in dark grey at the top.

Discussion

It has been shown before that plasma membranes of cancer cells selectively expose the negatively charged lipid PS (2;3;12), which offers a potent target for amphipathic cationic host defense peptides (1). In this study, we correlate the selective antitumor activity of human Lactoferricin derivatives with selective activity against the cancer cell mimic PS. The three peptides originally derived from the membrane active peptide LF11 (23;38), differed in several aspects: number of amino acids and positive charges, hydrophobicity and consequently interaction with model systems and structure.

The non-acylated LF11-322 exhibited only weak activity against melanoma cancer cell lines, the N-acylated peptide 6-MO-LF11-322 showed elevated activity, the dimeric form R-DIM-P-LF11-322 was the most active one. Only 6-MO-LF11-322 exhibited also toxicity for non-cancer melanocytes though at respective elevated concentrations. So, R-DIM-P-LF11-322 emerged to be a highly cancer specific peptide. PI-uptake of melanoma cells upon incubation with peptides 6-MO-LF11-322 and R-DIM-P-LF11-322 further nicely demonstrates that the peptides operate via a membrane mediated way, since PI can only be taken up by cells that suffer membrane disintegration. Actually host defense peptides are mainly described to act via a non-receptor mediated pathway against the target cell membrane by membranolytic effects, they can either trigger necrosis or apoptosis of cancer cells (1). For triggering necrosis they directly kill by disrupting the target plasma membrane, whereas for triggering apoptosis they have to selectively enter cancer cells and cause mitochondrial swelling with consequent release of cytochrome c (1) and consequent activation of the apoptotic cascade. Bovine lactoferricin, e.g. selectively induces apoptosis in human leukemia and carcinoma cell lines (39).

The correlation of activity against bacteria with activity on bacterial model systems for Lactoferricin derived peptides and their N-acylated derivatives was shown recently (23). However, as reported increase of activity of LF11-322 by N-acylation like 6-MO-LF11-322 on the bacterial model system did not completely correlate with increase in antimicrobial activity, which seems to be due to the fact that elevated binding of N-acylated peptides to lipopolysaccharides (LPS) of the outer membrane of

Gram-negative bacteria counteracts with the elevated membrane permeabilization (23). In this study however we demonstrate that antitumor activity as well as activity on the tumor model system PS is improved by N-acylation. Therefore it can be assumed that PS is the only target of the peptide on human cancer membranes and peptides are not reduced in their effective concentration by other membrane components, like described for 6-MO-LF11-322 and its bivalent interaction with LPS and lipids of the inner cytoplasmic membrane of *E. coli*. Interestingly, maximum toxicity of 6-MO-LF11-322 towards cancer cells is already reached after 15 minutes of incubation (data not shown) supporting the idea of killing via necrosis. This observation also sustains the idea of a lipid rather than a receptor target, a positive fact that would minimize the chance of generation of resistance upon application. Nevertheless unfortunately 6-MO-LF11-322 did not only exhibit elevated activity against the cancer model but also against the non-cancer model PC correlating with its toxicity against non-cancer melanocytes. A fact that goes conform to elevated hemolytic activity already described for N-acylated derivatives of LF11 as 6-MO-LF11-322 (20% lysis of 2.5% RBCs at 500µg peptide/ml, (23)) or C12LF11 (85% lysis of 0.25% RBCs at 100µg peptide/ml (38)). Low specificity might be due to increased peptide hydrophobicity (ΔG_{woct}) expressed as transfer free energy of peptides from water to n-octanol calculating an acetyl- instead of the 6-MO-group as a first approximation (35), which revealed an increase of the hydrophobicity by N-acylation from 5.64 to 3.34 kcal/mol (see Table 3).

However the dimer of LF11-322 exhibits less hydrophobicity 7.12 kcal/mol than the monomer, but highly improved activity against cancer cell lines without loss of selectivity. It has been reported by Yang et al. (24) that a prerequisite for antitumor but not for antibacterial peptides derived of bovine lactoferrin is a minimum net charge close to +7 and a size of 14 amino acids. Indeed LF11-322 exhibited respectable antibacterial activity with a minimal inhibitory concentration for *E. coli* of 8-16 (23) but negligible antitumor activity as shown in this study. We therefore designed R-DIM-P-LF11-322 (+9), a peptide composed of LF11-322 (+5) linked to its retro sequence via the amino acid Pro. Actually cancer-toxicity was highly increased. Improved interaction of the dimer with the cancer

mimic PS correlated with increased activity against the melanoma cancer cell line and non-interaction with the healthy mimic PC correlated with non-toxicity against non-cancer melanocytes. The dimer seems to exhibit a high membrane destabilization effect together with highly increased membrane permeability of PS bilayers. Besides, permeability studies show that a certain threshold concentration of the dimer is needed for induction of sufficient leakage of ANTS/DPX, differentiating it from highly lytic but mostly unspecific peptides like melittin (data not shown) (1). In agreement also the effect on neutral lipids is negligible. Moreover by calorimetric studies it could be demonstrated that the effect of the dimer is even much higher than that of the monomer at doubled concentration, rather suggesting a structural effect than a simple mass and charge effect.

Trp localization studies of the 3 peptides showed that if a peptide is active against a certain membrane, it exhibits a significant blue shift of Trp emission wavelength upon interaction with the membrane indicating a more hydrophobic environment of Trp due to interaction with the membrane interface. In the case of the dimer this blue shift is only present in presence of the target lipid PS exposed on the surface of cancer membranes, whereas in the presence of PC no blue shift appears, going in hand with a selective toxicity against cancer cells *in vitro*. The N-acylated peptide however reveals a blue shift in the presence of both model systems though the accessibility of Trp (K_{sv}) is more highly decreased in the presence of the cancer mimic, again resembling activity *in vitro*, where 6-MO-LF11-322 is also active against non-cancer cells but less than against cancer cells. Also membrane permeabilizing specificity by 6-MO-LF11-322 for the cancer model system, which is 3-fold, very nicely resembles the relatively low 3-fold specificity for melanoma cells *in vitro*, besides a very good demonstration of the relevance of the model studies for the biological activity (see Table 3). What is rather striking and surprising is that R-DIM-P-LF11-322 already exhibits an extremely low K_{sv} value in solution. Together with the unchanged emission wavelength of Trp of 356nm, it can only mean that Trp is not exposed to the solvent but to non-hydrophobic amino acids (like Arg) and therefore not accessible to acrylamide quenching. In presence of PS Trp accessibility is still low, but now it is in a

hydrophobic environment and seems to penetrate into the membrane interface, indicated by the strong blue shift in the presence of PS.

Further structural information on the studied peptides was obtained from CD experiments. Again structural changes for LF11-322 and the dimer appear only in the presence of the negatively charged cancer mimic (SDS). The N-acylated peptide changes its structure in environment of both models in agreement with its lower specificity. Although structural studies in the presence of SDS and DPC forming micelles cannot be absolutely compared to changes in the presence of a bilayer, the alterations can be correlated to changes induced by the outer monolayer of the cancer or non-cancer cells, which is the first and most important point of interaction. And the differences between the selective and non-selective peptides were quite obvious. Only the N-acylated less selective peptide shows an increase of the α-helical content in the presence of both model systems, differently LF11-322 and R-DIM-P-LF11-322 show an increase of the β-sheet content upon presence of the cancer model SDS.

From the differences in activity displayed by the monomer and the dimer it was however surprising that both peptides show quite similar structural characteristics in solution and model system. Considering the shortness of LF11-322, it is even questionable if a β-sheet conformation is possible. It is moreover reasonable that two monomer peptide stretches arrange on the surface like a dimer and thereby arrange in β-sheets. The dimer however is fixed in this conformation and will create stronger membrane perturbation and finally higher membrane permeabilization, which can explain its highly increased activity in model and cell system. Nevertheless from model studies, LF11-322 could be reasoned to be more active than *in vitro* studies show. Minor activity *in vitro* could be due to minor stability of the monomer considering that proteins with N-terminal Pro amongst others are described to exhibit short half-lives of half an hour (40). Proteolytic instability could on the one hand be the reason for the low activity of the monomer and proteolytic protection by structural conformation, also indicated by the extremely low Stern-Volmer constant in solution, on the other hand explain the

highly increased activity of the dimer R-DIM-P-LF11-322. We tested the activity of LF11-322 in the presence of buffer and absence of serum and could not detect any improvement of activity after 1 hour (data not shown). We chose such a short period to prevent membrane effects caused by growth deficiencies due to lack of serum components. However selective peptides that act over apoptosis rather than necrosis need longer time periods for killing.

Even though the synthesis of the dimer accounts for higher costs than the monomer it is still much more effective and cheaper in synthesis than the whole sequence of bovine Lactoferrin, which further requires 4 times higher protein amounts for 100% cell death (200µg/ml (39)) than described for R-DIM-P-LF11-322 in this study.

The results gained within this study suggest that the model described recently for the interaction of non-acylated and N-acylated peptides with bacterial membranes (23) can now be translated to the interaction of peptides with cancer membranes. Peptides first are attracted to the cancer membrane by electrostatic interaction with PS. Since activity on WM164 was slightly weaker than activity against SBcl-2, but SBcl-2 exposes lower levels of PS than WM164 (7;12), activity does not seem to be increased by higher PS levels. This reveals a mechanism of the peptides, which only requires an elevated (threshold) level of PS exposed compared to non-cancer cells for antitumor activity. However other negative charges on the cancer surface as targets as exhibited by sialic acid residues, which appear to be linked to some glycoproteins (e.g. mucins) and glycolipids present on membrane surfaces and partially overexpressed within certain cancer types, do not seem to interfere with activity of LF11 derived peptides, which was demonstrated by maintenance of activity after sialidase treatment (41). The peptides once attracted to the negatively charged PS on the cancer membrane seem to arrange in domains and penetrate with their hydrophobic amino acids like an anchor into the membrane. This can lead to direct membrane lysis, which in the case of the N-acylated peptide occurs due to the high hydrophobic moiety. The dimer however can due to its specific interaction with PS membranes and due to its larger volume selectively and potently perturb the cancer

membrane. Probably it enters the membrane selectively over the PS key and then interacts with negatively charged lipids of mitochondria, causing swelling and cytochrome c release. This idea is also supported by the fact that the dimer is acting more slowly than the N-acylated peptide.

Table 3: Correlation of activity exhibited by peptides LF11-322, 6-MO-LF11-322 and R-DIM-P-LF11-322 in model and *in vitro* studies.

Peptide	**LF11-322**	**6-MO-LF11-322**	**R-DIM-P-LF11-322**
amino acid sequence	PFWRIRIRR-(NH_2)	6-MO-	- PRRIRIRWFP-NH_2
net charge	+5	+4	+9
hydrophobicity - ΔG_{woct} [a]	5.64	3.34*	7.12
cancer- /healthy- mimic			
bilayer perturbation - DSC	+ / –	++ / +	+++ / –
permeability - ANTS/DPX leakage	+ / –	++ / +	+++ / –
bilayer affinity - quenching	+ + / –	++ / +	++ / –
structure – CD	β-sheet / as in solution	α-helical / α-helical	β-sheet / as in solution
cancer cells / healthy cells			
toxicity – PI uptake	– / –	++ / +	+++ / –
cancer specificity	(+)	+ –	++++

[a] ΔG_{woct} [kcal/mol] Peptide hydrophobicity expressed as transfer free energy of peptides from water to n-octanol calculated from the whole-residue hydrophobicity scale taking in account the contribution of the C-terminal amide (35). * For calculation of ΔG_{woct} of 6-MO-LF11-322 instead of the 6-MO residue N-acetylation was chosen.

In conclusion, we were able to demonstrate a significant correlation between the toxicity of the studied peptides against human cancer and non-cancer cells with model membrane systems proving PS as a molecular target for the peptides. Various characteristics of a peptide are important for antitumor activity. In our study we could prove that quite low hydrophobicity but a prolonged sequence combined with a higher positive net charge are sufficient or needed to improve the antitumor activity of hLFcin derivatives. Conformational changes to β-sheet in the presence of the target membrane seem to induce selective activity rather than induction of α-helices. As can be seen by the N-acylated peptide improvement of activity solely by increase of the hydrophobic moiety unfortunately also involves impact on neutral membrane components and therefore also yields toxicity against healthy non-cancer cells. Improvement of stability by proteolytic protection seems to occur within the dimer peptide resulting in high and selective toxicity on cancer cells *in vitro*.

Reference List

1. Papo, N. and Shai, Y., 2005: Host defense peptides as new weapons in cancer treatment; Cell Mol.Life Sci., 62(7-8): 784-790

2. Riedl, S., Zweytick, D., and Lohner, K., 2011: Membrane-active host defense peptides - challenges and perspectives for the development of novel anticancer drugs; Chem Phys Lipids, submitted

3. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

4. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J., 1989: Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation; Proc.Natl.Acad.Sci.U.S A, 86(9): 3184-3188

5. Ran, S., Downes, A., and Thorpe, P. E., 2002: Increased exposure of anionic phospholipids on the surface of tumor blood vessels; Cancer Res., 62(21): 6132-6140

6. Schröder-Borm, H., Bakalova, R., and Andrä, J., 2005: The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine; FEBS Lett, 579(27): 6128-6134

7. Zweytick, D., Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., and Lohner, K., 2011: In search of new targets - the membrane lipid phosphatidylserine - the underestimated Achilles' heel of cancer cells; Annals of Oncology, 22, suppl.3: 43

8. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 1996: Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications; Lupus, 5(5): 480-487

9. Zwaal, R. F. and Schroit, A. J.1997: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells; Blood, 89(4): 1121-1132

10. Bevers, E. M., Comfurius, P., Dekkers, D. W., Harmsma, M., and Zwaal, R. F., 1998: Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling; Lupus, 7 Suppl 2: S126-S131

11. Seigneuret, M. and Devaux, P. F., 1984: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes; Proc.Natl.Acad.Sci.U.S.A, 81(12): 3751-3755

12. Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., Lohner, K., and Zweytick, D., 2011: In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy; Biochim Biophys Acta, accepted

13. Kufe, D. W., 2009: Mucins in cancer: function, prognosis and therapy; Nature Reviews Cancer, 9(12): 874-885

14. Carraway, K. L., Price-Schiavi, S. A., Zhu, X., and Komatsu, M., 1999: Membrane Mucins and Breast Cancer; Cancer Control, 6(6): 613-614

15. Bafna, S., Kaur, S., and Batra, S. K., 2010: Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells; Oncogene, 29(20): 2893-2904

16. Wang, F. L., Cui, S. X., Sun, L. P., Qu, X. J., Xie, Y. Y., Zhou, L., Mu, Y. L., Tang, W., and Wang, Y. S., 2009: High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer; Cancer Detect.Prev., 32(5-6): 437-443

17. Litynska, A., Przybylo, M., Pochec, E., Hoja-Lukowicz, D., Ciolczyk, D., Laidler, P., and Gil, D., 2001: Comparison of the lectin-binding pattern in different human melanoma cell lines; Melanoma Res., 11(3): 205-212

18. Cazet, A., Julien, S., Bobowski, M., Krzewinski-Recchi, M. A., Harduin-Lepers, A., Groux-Degroote, S., and Delannoy, P., 2010: Consequences of the expression of sialylated antigens in breast cancer; Carbohydr.Res., 345(10): 1377-1383

19. Gifford, J. L., Hunter, H. N., and Vogel, H. J., 2005: Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties; Cell Mol.Life Sci., 62(22): 2588-2598

20. Bezault, J., Bhimani, R., Wiprovnick, J., and Furmanski, P., 1994: Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice; Cancer Research, 54(9): 2310-2312

21. Vogel, H. J., Schibli, D. J., Jing, W., Lohmeier-Vogel, E. M., Epand, R. F., and Epand, R. M., 2002: Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides; Biochem.Cell Biol., 80(1): 49-63

22. Zweytick, D., Pabst, G., Abuja, P. M., Jilek, A., Blondelle, S. E., Andrä, J., Jerala, R., Monreal, D., Martinez, d. T., and Lohner, K., 2006: Influence of N-acylation of a peptide derived from human lactoferricin on membrane selectivity; Biochim.Biophys.Acta, 1758(9): 1426-1435

23. Zweytick, D., Deutsch, G., Andrä, J., Blondelle, S. E., Vollmer, E., Jerala, R., and Lohner, K., 2011: Studies on lactoferricin derived E. coli membrane active peptides reveal differences in the mechanism of N-acylated versus non-acylated peptides; Journal of biological Chemistry, 24: 21266-21276

24. Yang, N., Strom, M. B., Mekonnen, S. M., Svendsen, J. S., and Rekdal, O., 2004: The effects of shortening lactoferrin derived peptides against tumour cells, bacteria and normal human cells; J.Pept.Sci., 10(1): 37-46

25. Jimenez-Monreal, A. M., Villalain, J., Aranda, F. J., and Gomez-Fernandez, J. C., 1998: The phase behavior of aqueous dispersions of unsaturated mixtures of diacylglycerols and phospholipids; Biochimica et Biophysica Acta (BBA) - Biomembranes, 1373(1): 209-219

26. Jing, W., Prenner, E. J., Vogel, H. J., Waring, A. J., Lehrer, R. I., and Lohner, K., 2005: Headgroup structure and fatty acid chain length of the acidic phospholipids modulate the interaction of membrane mimetic vesicles with the antimicrobial peptide protegrin-1; J.Peptide Sci., 11(11): 735-743

27. Sevcsik, E., Pabst, G., Jilek, A., and Lohner, K., 2007: How lipids influence the mode of action of membrane-active peptides; Biochim.Biophys.Acta, 1768: 2586-2893

28. McElhaney, R. N., 1982: The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes; Chem Phys Lipids, 30(2-3): 229-259

29. Tao, T. and Cho, J., 1979: Fluorescence lifetime quenching studies on the accessibilities of actin sulfhydryl sites; Biochemistry, 18(13): 2759-2765

30. Ellens, H., Bentz, J., and Szoka, F. C., 1985: H+- and Ca2+-induced fusion and destabilization of liposomes; Biochemistry, 24(13): 3099-3106

31. Bartlett, G. R., 1959: Colorimetric assay methods for free and phosphorylated glyceric acids; J.Biol.Chem., 234(3): 469-471

32. Broekhuyse, R. M., 2005: Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids; Biochim.Biophys.Acta, 152(2): 307-315

33. Whitmore, L. and Wallace, B. A., 2004: DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data; Nucleic Acids Research, 32(suppl 2): W668-W673

34. Whitmore, L. and Wallace, B. A., 2008: Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases; Biopolymers, 89(5): 392-400

35. Wimley, W. C., Creamer, T. P., and White, S. H., 1996: Solvation Energies of Amino Acid Side Chains and Backbone in a Family of Host-Guest Pentapeptides; Biochemistry, 35(16): 5109-5124

36. Lewis, R. N. and McElhaney, R. N., 2000: Calorimetric and spectroscopic studies of the thermotropic phase behavior of lipid bilayer model membranes composed of a homologous series of linear saturated phosphatidylserines; Biophys.J., 79(4): 2043-2055

37. Koynova, R. and Caffrey, M., 1998: Phases and phase transitions of the phosphatidylcholines; Biochim.Biophys.Acta, 1376(1): 91-145

38. Zweytick, D., Tumer, S., Blondelle, S. E., and Lohner, K., 2008: Membrane curvature stress and antibacterial activity of lactoferricin derivatives; Biochem.Biophys.Res.Commun., 369(2): 395-400

39. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W., 2005: Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines; Mol Cancer Ther, 4(4): 612-624

40. Westphal, G., Gerber, G., Lipke, B., 2003: Stoffwechsel der Proteine und Aminosäuren, In: Proteine - nutritive und funktionelle Eigenschaften; Springer Verlag, Berlin: 239-270

41. Riedl, S., Rinner, B., Sanchez-Gomez, S., Tumer, S., Schaider, H., Lohner, K., and Zweytick, D., 2011: Slow killing of cancer cells, phosphatidylserine interaction and formation of a ß-sheet structure by hLFcin derived anticancer peptides is the key for high cancer specificity; manuscript in preparation

Slow killing of cancer cells, phosphatidylserine interaction and formation of a ß-sheet structure by hLFcin derived anticancer peptides is the key for high cancer specificity

Sabrina Riedl[1], Beate Rinner[2], Susana Sánchez-Gómez[3], Sabine Tumer[1], Helmut Schaider[2,4], Karl Lohner[1] and Dagmar Zweytick[1]

[1]Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstraße 6, A-8042 Graz, Austria

[2]Center for Medical Research, Medical University of Graz, Stiftingtalstraße 24, A-8010 Graz, Austria

[3]Departamento de Microbiología Universidad de Navarra, 31080 Pamplona, Spain

[4]Cancer Biology Unit, Department of Dermatology, Medical University of Graz, Auenbruggerplatz 8, A-8036 Graz, Austria

Abstract

Host defense-derived peptides have emerged as novel strategy for the development of alternative anticancer therapies. These cationic amphipathic peptides can discriminate between neoplastic and non-neoplastic cells interacting specifically with negatively charged membrane components such as phosphatidylserine (PS) or sialic acid, which discriminate cancer and non-cancer cells. Much effort has been made to improve peptide specificity towards cancer cells by variations of peptide length, net positive charge and hydrophobicity, consequently influencing the secondary structure and the mechanism by which peptides kill the target cell.

In this study we report on characteristic features of hLFcin derivatives which facilitate specific killing of cancer cells. Time dependent studies clearly reveal that the highly active but nonselective peptide, namely DIM-LF11-318, acts through a direct membranolytic effect whereas the most selective peptide, namely R-DIM-P-LF11-322, presumably triggers apoptosis which is also membrane-mediated by primary interactions with the negatively charged PS exposed by cancer cells. In order to prove PS as a specific target, membrane permeability is studied using membrane-mimetic systems. This data gives strong evidence that PS is the main target for hLFcin derivatives. Sialic acid moieties as additional negative charges on the cell surface only play a minor role for the interactions of peptides with cancer cell lines, since cleavage of sialic acid from the cell surface of a rhabdomyosarcoma cell line does not affect the anticancer activity of the parent peptide LF11-322 and its N-acyl-derivative 6-MO-LF11-322.

Surprisingly, only the nonselective peptide DIM-LF11-318 adopts a predominant α-helical structure, in the presence of the cancer as well as the non-cancer mimic. In contrast, structural analysis by CD spectroscopy of R-DIM-P-LF11-322 reveals induction of mostly β-sheet structure in the presence of the target and no structural change in presence of the non-cancer mimic which seems to be favored regarding selectivity.

Summarizing, slow killing and adoption of mostly β-sheet structure together with PS as specific target seem to be important key characteristics of peptides that account for high cancer cell specificity. Contrariwise, predominant α-helical structure yields highly active but nonselective peptides.

Introduction

Cancer is still a leading cause of death worldwide. In the year 2008, more than 12.7 million people all over the world were diagnosed with cancer (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900) and there were 7.6 million deaths which accounts for 13% of all deaths (http://www.who.int/mediacentre/factsheets/fs297/en/). The WHO projects a further rise of deaths owing to cancer to over 11 million in 2030.

Since the 1940s and the development of the first chemotherapeutics (1), much progress has been made regarding cancer treatment using chemotherapy, surgery, radiation, targeted therapy and combinations thereof. But still, cancer is not curative in many cases and therapy is usually accompanied by severe side effects due to non-adequate specificity for tumor cells. Commonly, chemotherapeutics act on cells that divide rapidly, one of the main properties of cancer cells. Therefore, damage of in particular rapidly dividing normal cells, like bone marrow, gastrointestinal mucosa and hair follicles, can be expected resulting in the most common side effects of chemotherapy like myelosuppression (decreased production of blood cells), mucositis (inflammation of the lining of the digestive tract) and alopecia (hair loss). Besides cytotoxicity, resistance to treatment with anticancer drugs is another huge problem. Resistance can be intrinsic and result from individual variations in patients and somatic cell genetic differences in tumors (2). In addition acquired resistance has become common, by e.g. expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells before interacting with intracellular targets, or acquired insensitivity to drug induced apoptosis and induction of drug detoxifying mechanism (2-4). Some cancer types, e.g. malignant melanoma, exhibit only weak sensitivity to

chemotherapeutics (10-15%), therefore supplementation by other drugs is a matter of interest. Furthermore, many tumors tend to metastasize, complicating the treatment even more.

From 1940 to 2006 175 new anticancer drugs have been approved (5). More than 50% thereof are either biological (10%, usually large peptides and proteins), natural (14%) or naturally derived products (28%, semisynthetic modifications) (5).

In search of novel anticancer agents host defense peptides (1) and derivatives thereof have emerged as potential alternative anticancer therapeutics offering many advantages over other therapies. Because of their mode of action and specificity – the cell membrane being the major target – resistance and cytotoxicity is less likely to occur (6-9) and thus, in addition they are expected to cause fewer side effects. Host defense peptides which are part of the innate immune system of many diverse species (e.g. mammals, insects, amphibians) are mostly cationic but amphipathic, targeting the cell membrane by electrostatic interactions with anionic molecules at the cell surface. Because of their mode of action and specificity – the cell membrane being the major target – resistance and cytotoxicity is less likely to occur (6;9;10) and thus, in addition they are expected to cause fewer side effects. Moreover, these peptides mostly damage cell membranes within minutes (11), which would hinder formation of resistance (7).

Regarding the mode of action of the specific killing by anticancer peptides two general mechanisms of anticancer peptides, triggering necrosis or apoptosis as a consequence of their membrane related mode of killing of cancer cells, have been discussed (7;9;12). Both killing based on necrosis via cell membrane lysis and apoptosis via the mitochondrial lytic effect seem to be dependent on the presence of anionic lipids. The negatively charged phospholipid phosphatidylserine (PS) located in the outer leaflet of cancer cell plasma membranes can represent such a specific target (13), as can be cardiolipin, a major anionic phospholipid of the mitochondrial membrane. Other sources of negatively charged molecules, which are also exposed by non-cancer plasma membranes such as sialic acid, may play a minor role, since they are present on neoplastic and non-neoplastic cells, though to different extent (14).

As mentioned above a specific target is provided by cancer cells in the form of the negatively charged phosphatidylserine (PS) which is exposed on the outer leaflet of the cancer cell membrane during malignant transformation (15-20). In non-cancerous cells the cell membrane comprises an overall neutral charge due to its main components, the zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM). Phosphatidylserine together with the major part of phosphatidylethanolamine (PE) assembles the inner leaflet of eukaryotic non-cancerous plasma membranes (21). This asymmetry of the major phospholipids between the two membrane leaflets is well documented (22;23) and is maintained by an ATP-dependent aminophospholipid translocase (24). The loss of membrane asymmetry was shown for several cancer cells, promising a general marker and target for cancer (13;15-20;25,26).

In our study, we focused on the human host defense peptide lactoferricin (hLFcin) which is known to exhibit antimicrobial, antiviral, anti-inflammatory and anticancer activities (for a review see (27)). Human lactoferricin comprises amino acid residues 1-45 of the N-terminus of human Lactoferrin (hLF). LF11, an 11 amino acid fragment of hLFcin has already been optimized regarding its activity against bacterial membranes (28-30) and cancer cell mimics (31). One peptide group, already studied with cancer cell mimics (see previous chapter), was now selected for further studies with cancer cells: LF11-322, 6-MO-LF11-322 (6-Methyloctanoyl-LF11-322) and R-DIM-P-LF11-322 (LF11-322-Pro-LF11-322retro). Additionally, a second peptide group was chosen to investigate the differences correlation in specificity and killing mechanism: LF11-318 and DIM-LF11-318 (LF11-318-LF11-318). In our previous study we could demonstrate that peptides of the LF11-322 family are highly active on liposomes containing PS, mimicking cancerous cells, whereas they hardly affected the non-cancerous cell mimic composed of PC (13) In the present study we elucidate some important prerequisites of the hLFcin derived peptides for high and cancer specific activity.

Materials and Methods

Peptides

The peptides LF11-322 (PFWRIRIRR-NH_2, M=1298.6 g/mol), its N-6-Methyloctanoyl derivative 6-MO-LF11-322 (CH_3CH_2-$CH_2(CH_3)$-$(CH_2)_4$-CO-NH-PFWRIRIRR-NH_2, M=1438.9 g/mol), its dimer R-DIM-P-LF11-322 (PFWRIRIRRPRRIRIRWFP-NH_2, M= 2677.4 g/mol) and LF11-318 (FWQRRIRRWRR-NH_2, M= 1715.0 g/mol) and its dimer DIM-LF11-318 (FWQRRIRRWRRFWQRRIRRWRR-NH_2, M= 3413.1 g/mol) were purchased from NeoMPS, Inc. (San Diego, CA, USA). The purities were >96% as determined by RP-HPLC. Peptides were dissolved in Acetic acid (0.1%, v/v) at concentrations of 3 mg/ml. Peptide solutions were stored at 4°C and concentrations were determined photometrically at 280 nm.

Cell lines and Culture

Melanoma cell lines from primary (SBcl-2) and metastatic (WM164) lesions are grown in RPMI 1640 medium with stable L-Glutamine (Invitrogen, UK), supplemented with 2% FBS. Rhabdomyosarcoma cell lines (TE671) purchased from ECAAC (Health Protection Agency Culture Collections Salisbury, UK) are cultured in Dulbecco´s Modified Eagle Medium (DMEM, Invitrogen, UK) with addition of 2 mM Glutamine, 10% FBS (fetal bovine serum; Lot A10109-0523, PAA Laboratory, Pasching, Austria). Human melanocytes used as healthy control cells were isolated from the foreskin. The foreskin was cut into small pieces and incubated with 0.3% trypsin (PAA) overnight at 4°C and for one hour at 37°C. Epidermis was separated. Cells were mechanically removed from the cell layer and centrifuged at 300g for 3 min. The pellet was resuspended in melanocyte growth media (Biomedica, Vienna, Austria) Melanocytes were further cultured in human melanocytes growth medium (PromoCell GmbH, Heidelberg, Germany). Normal human dermal fibroblasts purchased from PromoCell GmbH (Heidelberg, Germany) were cultured in fibroblast growth medium 2 (PromoCell GmbH, Heidelberg, Germany). All cells are kept in a 5% CO_2 atmosphere at 37°C. At 90% confluency cell-culture flasks

were passaged with accutase (PAA, Pasching Austria). All cell cultures were periodically checked for mycoplasma.

Fluorescence spectroscopy

Fluorescence spectroscopy experiments were performed using a SPEX Fluoro Max-3 spectrofluorimeter (Jobin-Yvon, Longjumeaum, France) and spectra were analyzed with Datamax software.

PI-uptake assay

Cells were collected, resuspended in media and diluted to a concentration of 10^6 cells/ml. Aliquots of 10^5 cells were incubated with peptides for up to 8 hours at 37°C and 5% CO_2. PI was added and cells were again incubated for 5 min at room temperature in the dark. Excitation and emission wavelengths were 536 nm and 617 nm, respectively.

Cytotoxicity was calculated from the percentage of PI positive cells in media alone (P_0) and in the presence of peptide (P_X). Triton-X was used to determine 100% of PI positive cells (P_{100}).

$$\% PI - uptake = \frac{100 * (P_X - P_0)}{(P_{100} - P_0)}$$

Sialic acid cleavage

To remove sialic acid moieties from the cell surface (33;34), cells were incubated for 45 min at 37°C in PBS with 0.1 U/ml neuraminidase from *Clostridium perfringens* purchased from Sigma (Deisenhofen, Germany). Cells were washed twice with buffer, resuspended in media and then incubated with peptides as described above. PI-uptake was determined the same way as without sialic acid cleavage.

MTS viability assay

Cell proliferation was measured by using a CellTiter 96 AQ nonradioactive cell proliferation assay (Promega, Madison, WI). Cells were plated in 96-well plates and grown until confluency. Peptides were added to a final concentration of 5-100µM. After incubation for 24 h at 37°C (5% CO_2) MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-phenozine methosulfate solution (20 µl/well) was added and cells were again incubated for 2 h at 37°C (5% CO_2). The MTS compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of the formazan product as measured photometrically by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. Data are calculated as a percentage of the control (untreated) samples and represent the average of three wells in one experiment which was repeated three times per cell line.

Hemolysis

The hemolytic activity towards human red blood cells (RBCs), which were obtained from heparinized human blood, was determined by the release of hemoglobin following one hour incubation at 37°C in MHNA (Mueller Hinton cation Non Adjusted). Percentage of hemolysis of RBCs was calculated using 1% Triton as 100% lysis and PBS as 0% lysis, peptide concentration was 500µg/ml.

Fluorescence microscopy

Experiments were performed on a Leica DMI6000 B with IMC in connection with a Leica DFC360 FX camera and AF 6000 software.

PI-uptake assay

Cells (1-5x10^4) were seeded on Ibidi µ-Slide 8 wells and grown in 300µl media for 2-3 days to a confluent layer. Propidium iodide (PI, 2 µl of 50 µg/ml in PBS, Biosource, Camarillo, CA, USA) was added to the well and cell status was checked after 5 min of incubation in the dark at room

temperature. Then, peptides were added to the desired concentration and peptide effect was followed immediately. Pictures were taken every 5 or 15 min for up to 8 h from the same section of cells. Excitation and emission wavelength were as follows: PI excitation, 535 nm and emission, 617 nm.

Circular dichroism spectroscopy

Measurements were performed on a Jasco J 715 Spectropolarimeter (Jasco, Gross-Umstadt, Germany) at room temperature using quartz cuvettes with an optical path length of 0.02 cm. The CD spectra were measured between 260 nm and 180 nm with a 0.2 nm step resolution. To improve accuracy 5 scans were averaged. Peptides were dissolved in 10 mM Hepes (pH 7.4) to a final concentration of 100 µM. Spectra were measured in the absence and presence of 1 mM sodium dodecyl sulfate (SDS) and 1 mM dodecylphosphocholine (DPC) mimicking cancer and healthy mammalian membranes, respectively. The respective peptide to surfactant molar ratios were 1:25 and 1:100. Background signals were abstracted after measurements. Percentage secondary structure calculations were done using Dichroweb, CDSSR Convolution Program using reference set 4 (34;35).

ANTS/DPX leakage

Leakage of aqueous contents from liposomes was determined using the 8-aminonaphthalene-1,3,6-trisulfonic acid / p-xylene-bis-pyridinium bromide (ANTS/DPX) assay (36). Lipid films (preparation: as previously described (31)) were hydrated with 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4 following a standard procedure.

Subsequently, the dispersions were extruded 20 times through a polycarbonate filter (Millipore - Isopore™) of 0.1 µm pore size to obtain LUVs. Unilamellarity and size were tested by X-ray and dynamic light scattering, respectively. The ANTS/DPX encapsulating vesicles were separated from free ANTS/DPX by exclusion chromatography using a column filled with Sephadex™ G-75 (Amesham

Biosciences) fine gel swollen in an iso-smotic buffer (10 mM HEPES, 140 mM NaCl, pH 7.4). The void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis (37;38).

The fluorescence measurements were performed in 2 mL of the isosmotic buffer in a quartz cuvette at room temperature. Aliquots of LUVs were diluted with the iso-osmotic buffer to a final lipid concentration of 50 µM. Fluorescence spectra were obtained at 37°C using an excitation wavelength of 360 nm and an emission wavelength of 530 nm and a slit width of 5 nm for both excitation and emission monochromators.. Fluorescence emission was recorded as a function of time before and after the addition of incremental amounts of peptide. The fluorescence increase due to leakage and subsequent dilution of quenched dye was measured after addition of peptides. Peptides were added to final concentrations of 2, 4 and 8 µM, corresponding to peptide to lipid molar ratios of 1:25, 1:12.5 and 1:6.25, respectively.

Data are presented in terms of fluorescence intensity (I_F):

$$I_F = \frac{F - F_0}{F_{max} - F_0}$$

F is the measured fluorescence, F_0 the initial fluorescence without peptide and F_{max} the fluorescence corresponding to 100% leakage gained by addition of 1% Triton X-100.

Results

Recently we demonstrated that human lactoferricin derivatives are highly active against cancer cell mimics as well as against melanoma *in vitro* (31). In this study we now report on the different modes of action of hLFcin derivatives against cancer and non-cancer cells. The five peptides studied can be divided in 2 groups. The first group is directly derived from LF11-322 and comprises, additionally to the mother peptide LF11-322, the N-acylated derivative 6-MO-LF11-322 and the dimeric derivative R-DIM-P-LF11-322. The second group is derived from LF11-318 and is complemented by the dimer DIM-LF11-318. All peptides are originally derived from the membrane active peptide LF11 and differed in several characteristics, as length, number of positive charges, hydrophobicity and consequently secondary structure (Table 1).

Table 1: Overview of peptide sequences, net charge and hydrophobicity of hLFcin derivatives

	sequence	Net charge	ΔG_{woct}[a] [kcal/mol]
LF11-322	PFWRIRIRR-NH_2	+5	5.64
6-MO-LF11-322	6-Methyl-octanoyl-PFWRIRIRR-NH_2	+4	3.34*
R-DIM-P-LF11-322	PFWRIRIRRPRRIRIRWFP-NH_2	+9	7.12
LF11-318	FWQRRIRRWRR-NH_2	+7	8.92
DIM-LF11-318	FWQRRIRRWRRFWQRRIRRWRR-NH_2	+13	13.54

[a] Peptide hydrophobicity expressed as transfer free energy of peptides from water to n-octanol (ΔG_{woct}) calculated from the whole-residue hydrophobicity scale taking into account the contribution of the C-terminal amide (39); * For calculation of ΔG_{woct} of 6-MO-LF11-322 instead of the 6-MO residue N-acetylation was taken as a first approximation.

Toxicity and mechanistic studies

Membrane disintegration and time dependence – PI-uptake

Cytotoxic activity of the peptides towards melanoma cells of primary (SBcl-2) and metastatic lesions (WM164), a rhabdomyosarcoma cell line (TE671) and their healthy counterparts differentiated non-tumorigenic melanocytes and normal human dermal fibroblasts (NHDF) was determined by

measurement of PI-uptake, which indicates potential loss of cell membrane integrity. Cells were incubated in media containing serum for up to 8h in the presence of 20µM peptide. Both non-acylated short peptides LF11-322 as well as LF11-318 are only minor active against cancer cells (< 30%, see Figure 1A-C). The acylated peptide 6-MO-LF11-322 exhibits increased activity against human cancer cells (~60%) and kills within 20 min (short time data not shown). LF11-318 is also slightly active against non-cancer cells, killing up to ~30% of HNDF (see Figure 1D-E). Interestingly, the dimer R-DIM-P-LF11-322 shows higher cancer toxicity (up to 80%, see Figure 1A, 1C) than the acylated peptide but with less (negligible) non-cancer toxicity (see Figure 1D/E, 3D/E). In contrast to the other peptides, R-DIM-P-LF11-322 kills quite slowly reaching its highest activity not before 4-8 h. Additionally, apoptotic like blebbing of the cell membrane is observed during incubation of the rhabdomayosarcoma cell line TE671 (Figure 5) in the presence of the peptide. The second dimer, namely DIM-LF11-318, possesses the highest and fastest anticancer activity with up to 90% killing within minutes (short time data not shown) (Figure 1A-C, Figure 3C). Nevertheless, DIM-LF11-318 reveals to be quite unspecific since it is also highly active against differentiated non-tumorigenic melanocytes as well as against normal human dermal fibroblasts (Figure 1D-E, Figure 3F). Cytotoxic activity of the peptides after 8 h of incubation is listed in Figure 2.

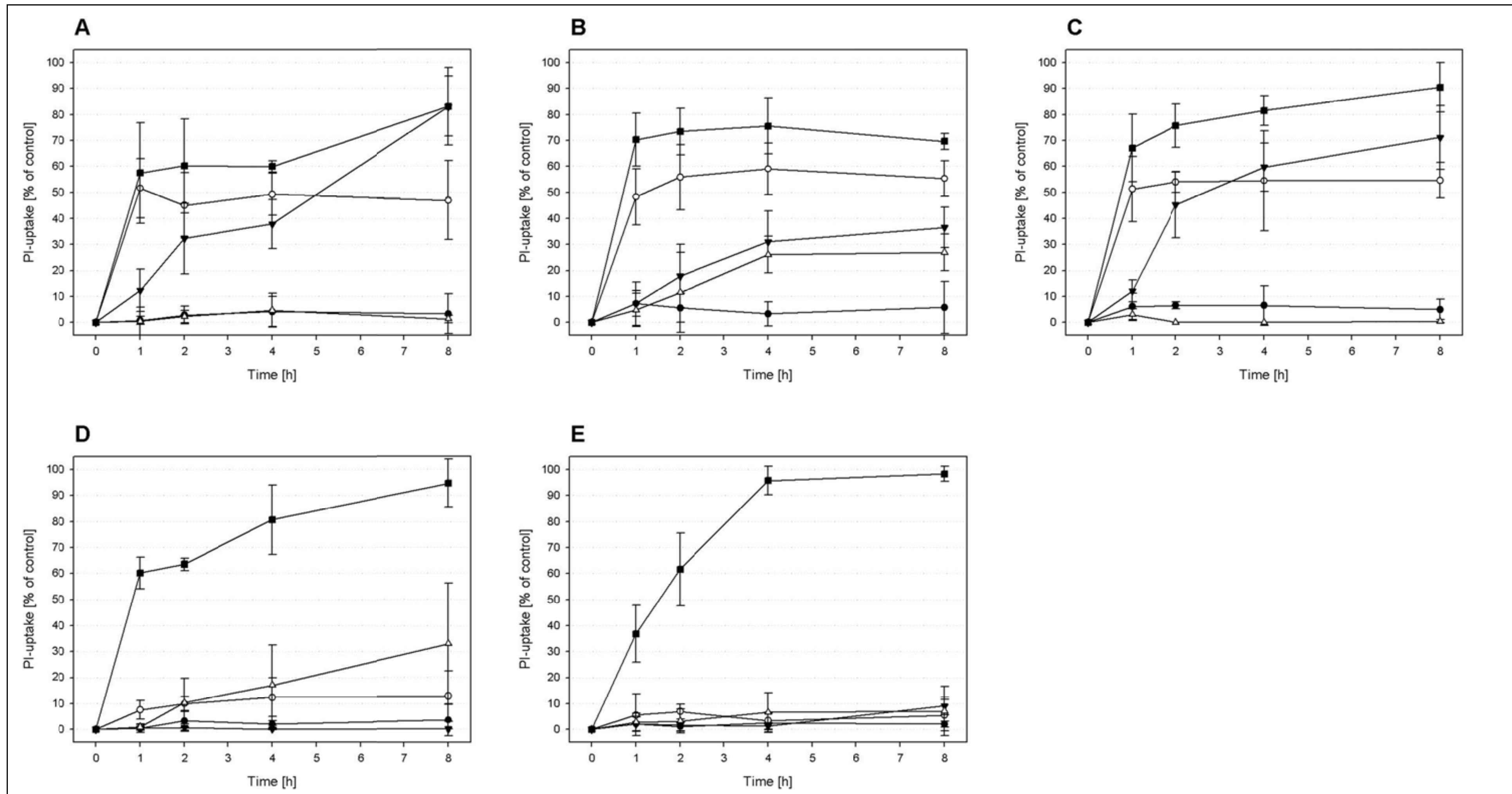

Figure 1: PI-uptake of various cancerous and non-cancerous cell lines upon incubation with 20μM peptide: Time dependent cytotoxic activity of LF11-322 (●), 6-MO-LF11-322 (○), R-DIM-P-LF11-322 (▼), LF11-318 (Δ) and DIM-LF11-318 (■) against cell lines of melanoma of primary lesions SBcl-2 (A), metastatic lesions WM164 (B), Rhabdomyosarcoma TE671 (C), differentiated non-tumorigenic melanocytes (D) and normal human dermal fibroblasts NHDF (E) at 20 μM peptide concentration is shown.

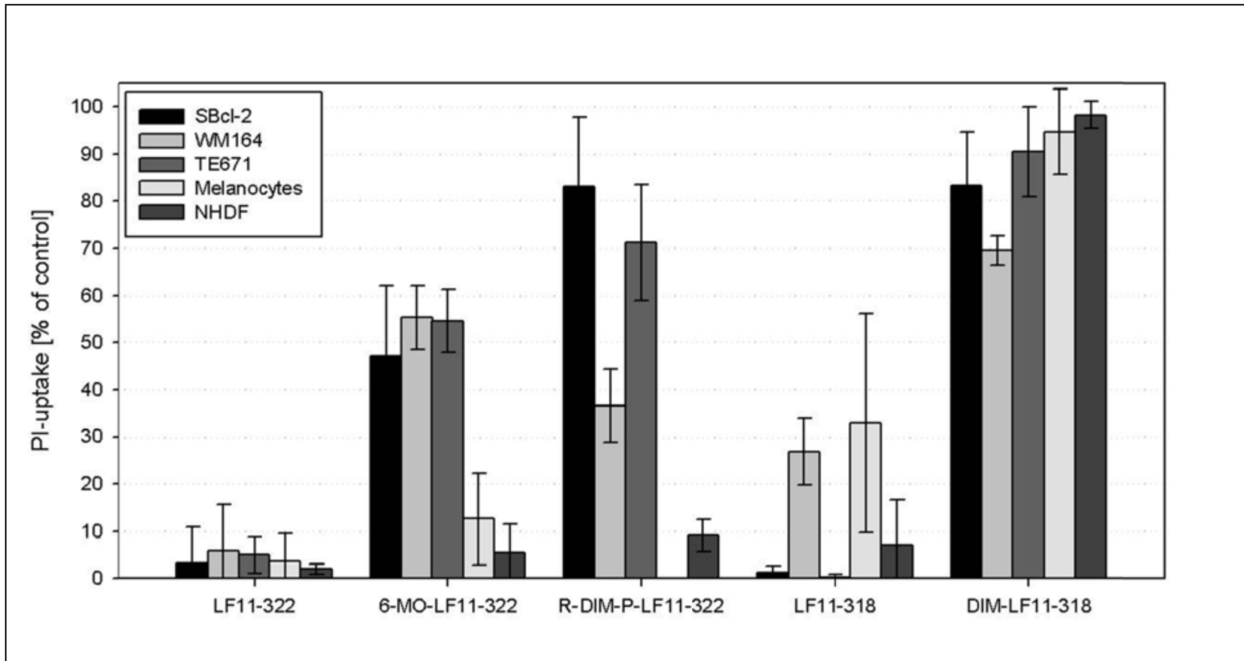

Figure 2: Cytotoxicity of peptides against cell lines of melanoma SBcl-2, melanoma metastases WM164, rhabdomyosarcoma TE671, differentiated non-tumorigenic melanocytes and normal human dermal fibroblasts NHDF at 20 µM peptide concentration after 8 h of incubation.

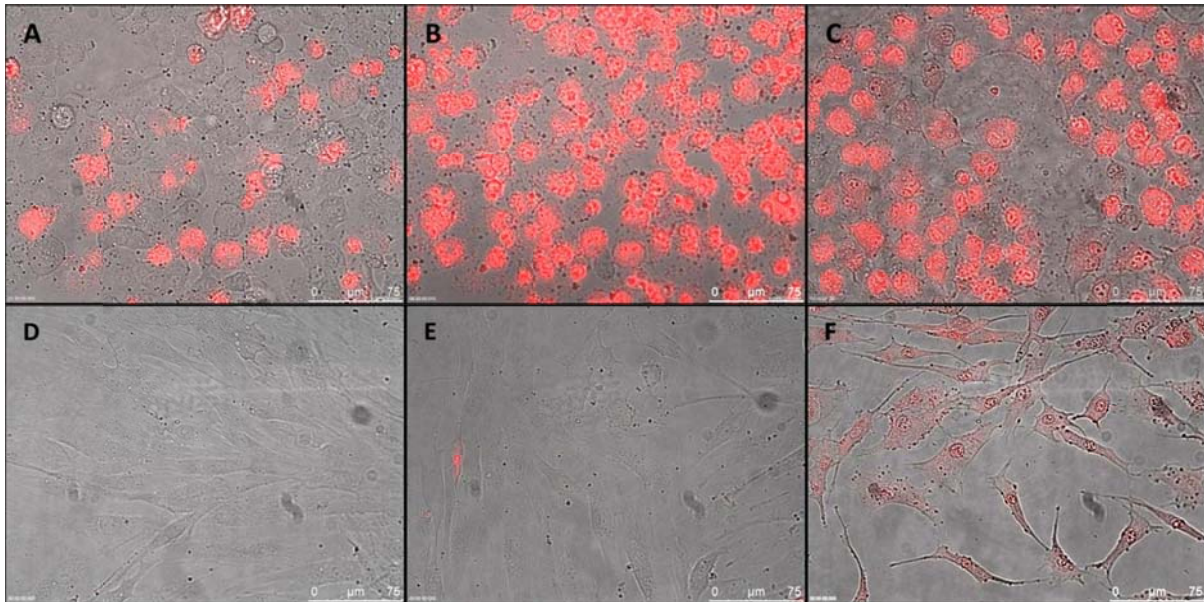

Figure 3: Fluorescence microscopy pictures of PI-uptake of SBcl-2 melanoma cells (A–C) and normal human dermal fibroblasts (D-F) at 20 µM peptide concentration: (A) 1 h R-DIM-LF11-322, (B) 8 h R-DIM-LF11-322, (C) 1 h DIM-LF11-318, (D) 1 h R-DIM-LF11-322, (E) 8 h R-DIM-LF11-322, (F) 1 h DIM-LF11-318. Red cells indicate dead cells after successful membrane disintegration by the respective peptide.

Cell viability – MTS cell proliferation

To determine long-time toxicity of peptides, a MTS cell proliferation assay was used to elucidate cell viability upon 24 h incubation with variant peptide concentration. As shown in Figure 3, LF11-322 affects cancer cells only marginally even at high peptide concentrations of 100 µM sustaining more than 70% cell viability (IC_{50} > 100 µM). 6-MO-LF11-322 possesses increased cancer toxicity providing an IC_{50} ~ 35 µM. Dimerization could further improve anticancer activity. R-DIM-P-LF11-322 exhibits a decreased IC_{50} value of 10 µM and 15 µM for melanoma cell line SBcl-2 (Figure 4A) and melanoma metastasis WM164 (Figure 4B), respectively. DIM-LF11-318 again shows highest activity against cancer cells resulting in an IC_{50} < 10 µM. As indicated already by PI uptake studies this peptide is also toxic for non-cancer cells, revealed by a very low IC_{50} for NHDF, as well. Results of MTS and PI assays with cancer cells correlated well, however the Dimer R-DIM-P-LF11-322 shows slightly higher toxicity towards non cancer NHDF in the MTS (Figure 4C) than in the PI study. An overview of IC_{50} values is listed in Table 2.

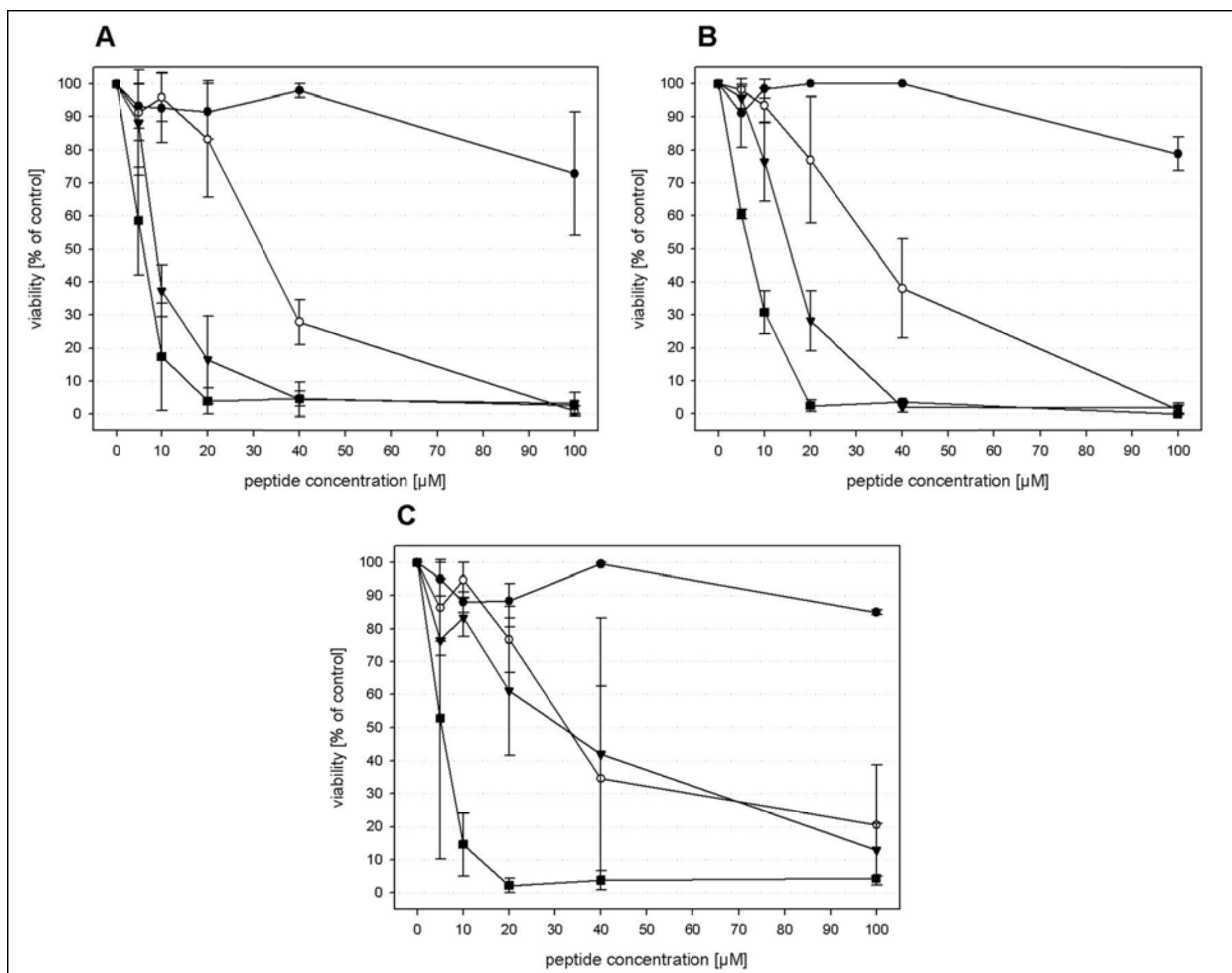

Figure 4: Cytotoxic activity of LF11-322 (●), 6-MO-LF11-322 (○), R-DIM-P-LF11-322 (▼), LF11-318 (Δ) and DIM-LF11-318 (■) determined by MTS cell proliferation assay against cell lines of melanoma SBcl-2 (A), melanoma metastases WM164 (B) and normal human dermal fibroblast NHDF (C). Cells were kept in the appropriate medium during incubation time of 24 h.

Table 2: Comparison of IC_{50} values determined through PI-uptake (8 h) and MTS cell viability assay (24 h)

	SBcl-2 (PI / MTS)	**Fibroblasts** (PI / MTS)
LF11-322	>80 µM / >100µM	>80 µM / >100 µM
6-MO-LF11-322	20 µM / 32 µM	>80 µM / 33 µM
R-DIM-P-LF11-322	8 µM / 9 µM	>80 µM / 32 µM
DIM-LF11-318	<<20µM / 6 µM	<<20µM / 5 µM

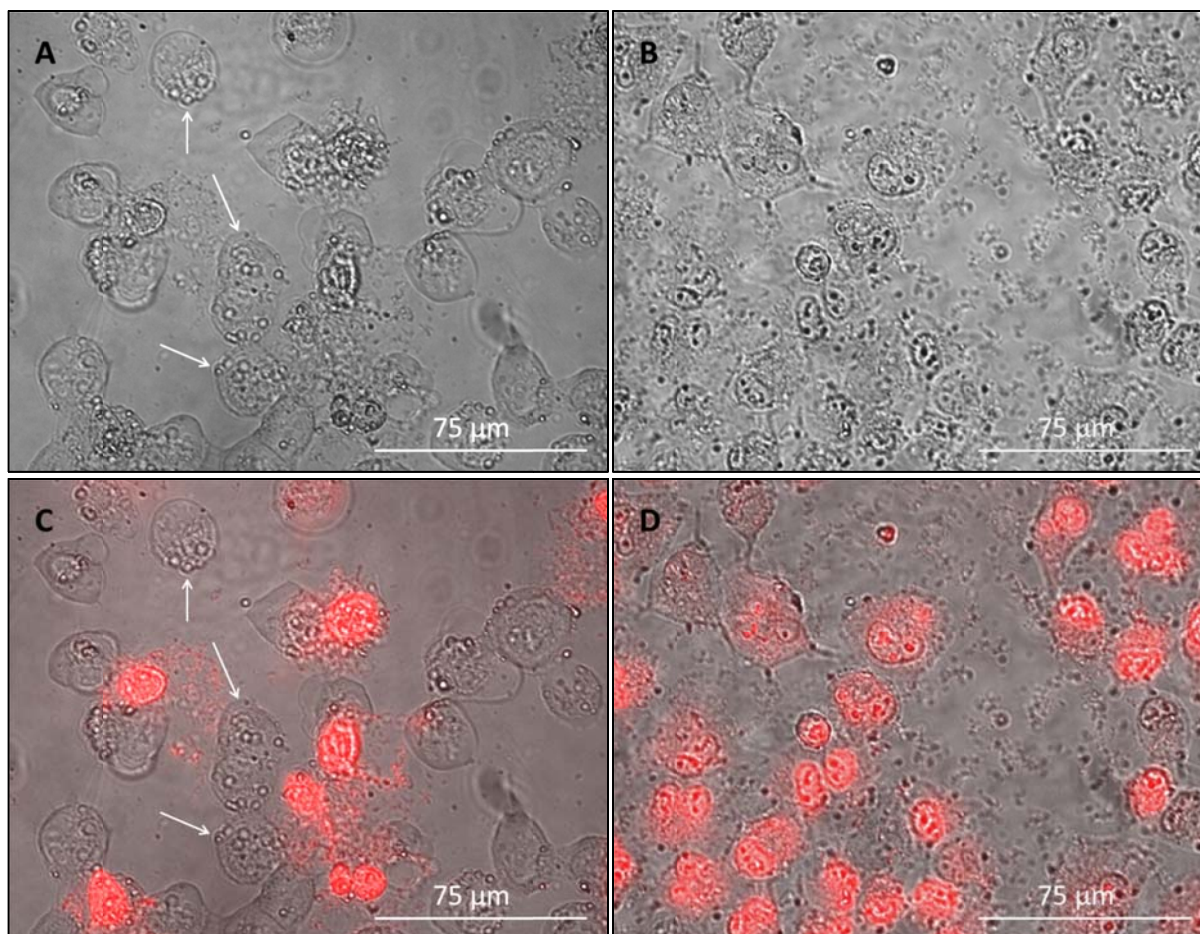

Figure 5: Fluorescence microscopy pictures of PI-uptake: Potential apoptotic blebbing (arrows) of rhabdomyosarcoma cells (TE671) during incubation with 20 µM R-DIM-LF11-322 (A) bright field and (C) PI-uptake and potential necrotic membrane lysis of SBcl-2 melanoma cells during incubation with 20 µM DIM-LF11-318 (B) bright field and (D) PI-uptake. Red cells in C and D indicate dead cells that have integrated PI upon membrane disintegration by peptides.

Hemolytic activity against red blood cells – specificity

Hemolytic activity of peptides against red blood cells was tested at 500 µg/ml peptide and 2.5% red blood cell concentration. Very interestingly, only an increase in the hydrophobic content by N-acylation, 6-MO-LF11-322, leads to a slightly elevated hemolytic activity of approximately 10-fold cell lysis compared to non-acylated peptides (see Table 3). Further it was very surprising that DIM-LF11-318 was not hemolytic, considering the high toxicity towards melanocytes and fibroblasts.

Table 3: Hemolytic activity of peptides against human red blood cells

	% lysis of 2.5% RBCs[a]	IC_{50} [µg/ml]
LF11-322	2[b]	>500[b]
6-MO-LF11-322	20[b]	>500[b]
R-DIM-P-LF11-322	0.84+ 0.63	>500
LF11-318	n.d.	n.d.
DIM-LF11-318	2.87+ 0.57	>500

[a] Percentage of hemolysis of human red blood cells (RBCs) was calculated following one hour incubation at 37ºC in PBS using 1% Triton as 100% lysis and PBS as 0% lysis, peptide concentration was 500µg/ml.
[b] from Zweytick et al., 2011 (30)
n.d. not determined

Apoptosis or necrosis – TUNEL-assay and caspase-3 cleavage

To differentiate between a necrotic and an apoptotic killing mechanism of the peptides direct in situ DNA fragmentation- (TUNEL-) and Caspase-3 cleavage assays were used to detect emergence of apoptosis. The experiments were under progress during composition of the thesis.

Secondary structure – selectivity

Circular dichroism spectroscopy analysis of the LF11-322 peptide group was already described in our previous study (31). Figure 6 now compares the results for DIM-LF11-318 and R-DIM-P-LF11-322. In contrast to the R-DIM-P-LF11-322 , peptide DIM-LF11-318 possesses a highly increased α-helical content in the presence of the cancer mimic SDS as well as in the presence of the non-cancer mimic DPC. DIM-LF11-318 adopts up to 75% α-helical structure without discrimination between cancer and non-cancer cell mimic. This is in line with un-specificity displayed by this peptide in vitro (Figure 1, 2). As demonstrated in Figure 6 in the presence of excess peptide (1:25) the α-helical content further increases indicating that more peptide is bound or probably the peptide starts to aggregate. Whereas R-DIM-P-LF11322 only changes its structure in the presence of the cancer mimic SDS by predominant adoption of β-sheet conformation. Excess of peptides does not further alter the structural

conformation of the peptide. LF11-318 (data not shown) changes its structure in the presence of SDS and DPC though the content of about 30% α-helical conformation is lower than of DIM-LF11-318, going in hand with slight toxicity against non-cancer cells exhibited by the monomeric form (Figure 1, 2).

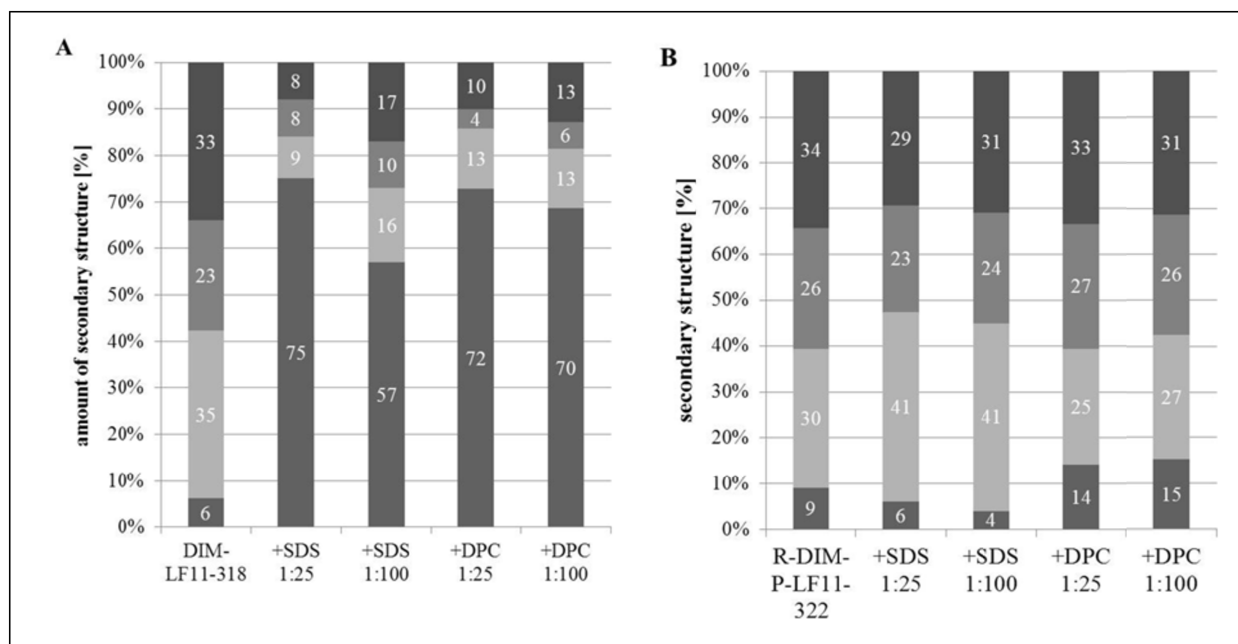

Figure 6: Secondary structures of DIM-LF11-318 (A) and R-DIM-P-LF11-322 (B) in Hepes buffer (first bar) or presence of SDS and DPC at peptide to surfactant ratios of 1:25 and 1:100, determined using CD spectroscopy. The α-helical content is shown in gray at the bottom; β-turns are shown in light grey; turns are shown in middle grey; random coil structures are shown in dark grey at the top.

Phosphatidylserine – a potential peptide target

A potent target of anticancer peptides is described to be the negatively charged lipid phosphatidylserine (7;14) specifically exposed by cancer cells (13;15). As reported in a previous study of our group (31) the dimer of LF11-322 R-DIM-P-LF11-322 induces specific permeabilization of the cancer mimic POPS whereas the non-cancer mimic POPC is not affected. As demonstrated in Figure 7 ANTS/DPX leakage of liposomes composed of the cancer mimic POPS is also induced by DIM-LF11-318, however in contrast to R-DIM-P-LF11-322 membrane permeabilization does not seem to be

specific for PS since also POPC liposomes are permeabilized though to some lower extent. Induced leakage by LF11-318 is comparable minor as by LF11-322.

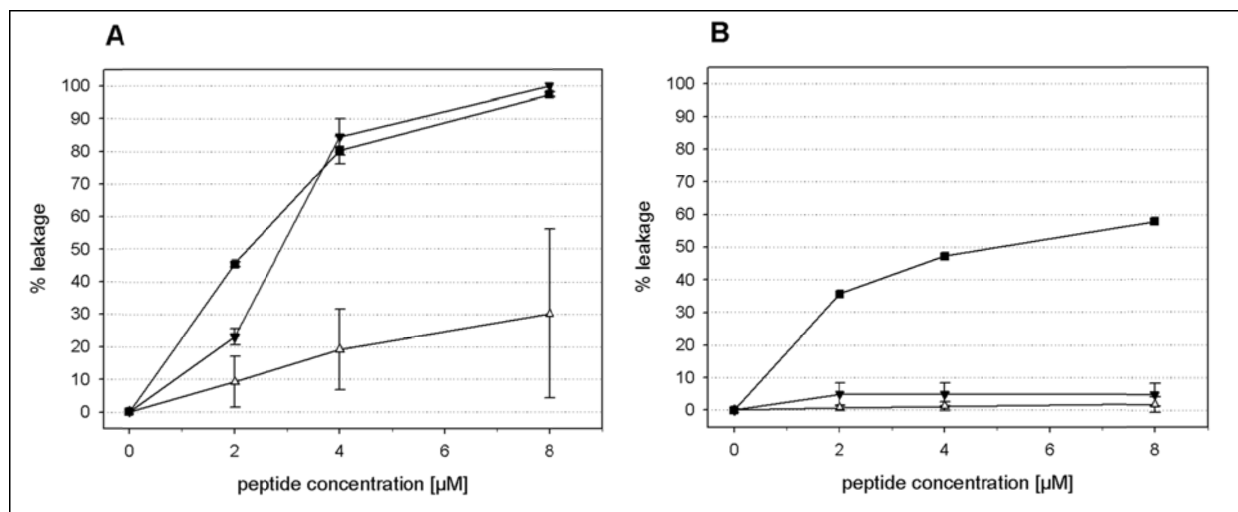

Figure 7: ANTS/DPX leakage of LUVs composed of POPS (A) and POPC (B) as a function of the concentration of R-DIM-P-LF11-322 (▼), LF11-318 (Δ) and DIM-LF11-318 (■). Concentration of LUVs was 50 µM and temperature was kept at 37°C during measurements. Complete lysis was determined by addition of Triton X-100 and zero levels correspond to fluorescence before peptide addition.

Sialic acid as target

To test other negatively charged membrane components as potential peptide target as e.g., sialic acid, being part of glycoproteins (mucins), which are partially overexpressed by some cancer types (40-42), we applied neuraminidase from *C. perfrigens* to the Rhabdomyosarcoma cell line TE671 to cleave off terminal sialic acid residues which are α-2,3- α-2,6- or α-2-8- linked to Gal, GlcNAc, AcNeu GlcNeu, oligosaccharides, glycolipids or glycoproteins. Figure 8 and Table 3 clearly demonstrate that cleavage of sialic acid residues from the rhabdomyosarcoma cells does not affect activity of the studied peptides LF11-322 and 6-MO-LF11-322 determined by PI-uptake after 1 h incubation.

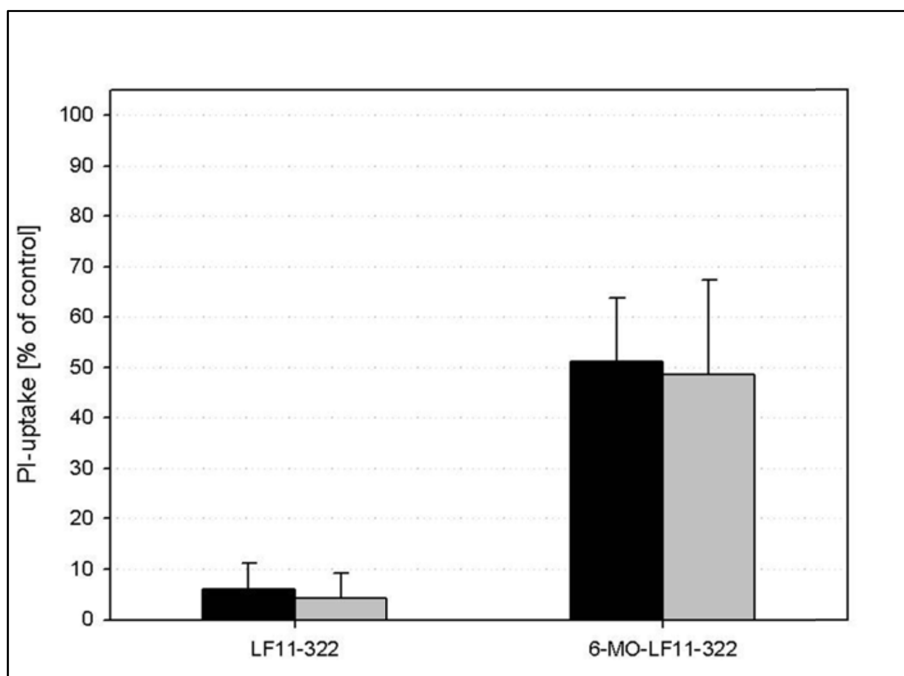

Figure 8: Influence of sialic acid cleavage from Rhabdomyosarcoma cell line TE671 on cytotoxic activity of LF11-322 and 6-MO-LF11-322 after 1 h of incubation with 20 µM peptides. Untreated cells and sialidase treated cells are shown in black and grey, respectively.

Table 3: Influence of sialic acid cleavage from rhabdomyosarcoma cell line TE671 on cytotoxic activity of LF11-322 and 6-MO-LF11-322 after 1 h of incubation with 20 µM peptides.

	PI-uptake [%] untreated TE671	**PI-uptake [%] Sialidase treated TE671**
LF11-322	6.1 ± 5.1	4.3 ± 5.0
6-MO-LF11-322	51.2 ± 12.5	48.6 ± 18.7

Discussion

Recently it has been shown that hLFcin derivatives can target the negatively charged phospholipid phosphatidylserine (31) which is specifically exposed by cancer cells (13;15). In this previous study we demonstrated that high membrane interaction of peptides derived from the membrane active peptide LF11 (29;30) with the anionic PS correlates with high activity against melanoma cells. We could also illustrate that increasing interaction with the healthy mimic neutral PC correlates with decreased specificity indicated by increased interaction with non-cancer cells like melanocytes and fibroblasts. This was in this study confirmed by two other peptides derived from LF11, namely LF11-318 and the dimer DIM-LF11-318. In the present study we further investigated the mechanism of cytotoxicity of the two peptide groups derived from LF11-322 and LF11-318. It has already been reported for bLFcin that a minimum length and net positive charge are required for appropriate anticancer activity (43). Expectedly, the short peptides LF11-322 and LF11-318 are only minor active against cancer cells even if incubation time is extended to 8 h. Partially the low activity might be also due to less defined structure and lower serum stability (31). In contrast, the acylated peptide 6-MO-LF11-322 as well as the dimeric peptides DIM-LF11-322 and DIM-LF11-318 exhibit increased anticancer activity. However the different peptides seem to operate by different mechanism, since peptides 6-MO-LF11-322 and DIM-LF11-318 reach their maximum toxicity against cancer cells already after 20 minutes (short time data not shown), whereas in contrast the dimer R-DIM-LF11-322 kills more slowly reaching its maximum activity not before 8 hours. Nevertheless it shows similar or even increased cancer toxicity compared to DIM-LF11-318 and 6-MO-LF11-322, after long time period. The different time dependence of cell killing by the peptides indicates 2 different killing mechanism as described for other host defense peptides which mainly trigger cell death by necrosis or by induction of apoptosis (7;14). For triggering necrosis peptides directly kill by disrupting the target plasma membrane, whereas for triggering apoptosis they have to reach a mitochondrial target (7). The very fast killing kinetic of 6-MO-LF11-322 and DIM-LF11-318 gives strong evidence for a direct membranolytic effect caused by necrosis. For 6-MO-LF11-322 it can be assumed that the

higher hydrophobic content due to N-acylation facilitates insertion into the bilayer followed by lysis of the cell membrane. Structural analysis through CD spectroscopy of DIM-LF11-318 and 6-MO-LF11-322 (31) reveals induction of a mostly α-helical structure in the presence of the cancer as well as the non-cancer mimic resulting in non-selective lysis of cells. The most selective peptide R-DIM-P-LF11-322 obviously acts via a different mechanism. The relatively slow action together with the observation of membrane blebbing is an indication for membrane-mediated apoptosis. However, this assumption has yet to be proven by e.g. TUNEL or caspase-3 cleavage studies, which are under progress. For induction of apoptosis the peptide has to enter the cell specifically over probably the PS domains on the surface and further reach another negatively charged target on the surface of cancer cell mitochondria, like cardiolipin, leading to successive swelling of mitochondria and release of cytochrome-C activating the caspase dependent pathway of the programmed cell death. Induction of apoptosis in human leukemia was also observed for bovine LFcin by Mader et al. (44) and is probably one of the keys for specific anticancer activity of host defense peptides. Interestingly R-DIM-P-LF11-322 shows induction of an increase in the predominant β-sheet structure and no changes in structure in the presence of the non-cancer cell mimic. So far most studies report about the necessity of formation of an α-helical structure (45;46). Though it was reported before by Hoskin et al. (6) that tilted helical peptide structure was more commonly found for peptides being toxic to both cancer and non-cancer cells.

Interestingly, the dimeric peptide R-DIM-P-LF11-322 appears to be less active against the metastasis WM164 compared to non-metastatic cancer cells even though the surface PS level of WM164 is higher (13). Since PS is not the only negatively charged interaction partner on the cell surface (for a review see Riedl et al. (14)), also other cancer cell characteristics like sialic acid moieties have to be studied. As the extent of surface sialylation seems to be directly correlated with the metastatic potential of different cancer cells exhibiting a protective function against the host immune system and facilitating migration and invasion via modulation of adhesiveness (47-49) it might also hinder anticancer drugs from accessing cancer cells of metastatic lesion like WM164 melanoma cells. Due to

its structure with high β-sheet content (31) R-DIM-P-LF11-322 seems to be partially prevented from interacting with PS whereas others, especially the shorter and more hydrophobic peptide 6-MO-LF11-322 and the unspecific peptide DIM-LF11-318, retain their activity. Additionally, we could show for the non-metastatic rhabdomyosarcoma cell line TE671 that cleavage of sialic acid moieties did not change the activity of LF11-322 and 6-MO-LF11-322.

Summarized our study provides evidence that specific activity of hLFcin derived peptides depends on a slow killing mechanism, as e.g. apoptosis, rather than killing by necrosis, specific interaction with PS exposed by cancer cells and not with PC present on all cell types and adoption of a β-sheet structure in the presence of cancer cells and no changes in structure in the presence of the non-cancer cell mimic.

Reference List

1. Shewach, D. S. and Kuchta, R. D., 2009: Introduction to Cancer Chemotherapeutics; Chem Rev., 109(7): 2859-2861

2. Gottesman, M. M., 2002: Mechanisms of cancer drug resistance; Annu.Rev.Med., 53(615-627

3. Gillet, J. P. and Gottesman, M. M., 2010: Mechanisms of multidrug resistance in cancer; Methods Mol.Biol., 596(47-76

4. Perez-Tomas, R., 2006: Multidrug Resistance: Retrospect and Prospects in Anti-Cancer Drug Treatment; Curr Med Chem., 13(16): 1859-1876

5. Newman, D. J. and Cragg, G. M., 2007: Natural Products as Sources of New Drugs over the Last 25 Years; Journal of Natural Products, 70(3): 461-477

6. Hoskin, D. W. and Ramamoorthy, A., 2008: Studies on anticancer activities of antimicrobial peptides; Biochim.Biophys.Acta, 1778(2): 357-375

7. Papo, N. and Shai, Y., 2005: Host defense peptides as new weapons in cancer treatment; Cell Mol.Life Sci., 62(7-8): 784-790

8. Mader, J. S. and Hoskin, D. W., 2006: Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment; Expert Opin.Investig.Drugs, 15(8): 933-946

9. Schweizer, F., 2009: Cationic amphiphilic peptides with cancer-selective toxicity; Eur J Pharmacol, 625(1-3): 190-194

10. Mader, J. S. and Hoskin, D. W., 2006: Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment; Expert Opin Investig Drugs, 15(8): 933-946

11. Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H. C., and Colamonici, O., 1991: Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation; Proc Natl Acad Sci U S A, 88(9): 3792-3796

12. Bhutia, S. K. and Maiti, T. K., 2008: Targeting tumors with peptides from natural sources; Trends Biotechnol., 26(4): 210-217

13. Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., Lohner, K., and Zweytick, D., 2011: In search of a novel target - Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy; Biochim Biophys Acta, accepted

14. Riedl, S., Zweytick, D., and Lohner, K., 2011: Membrane-active host defense peptides - challenges and perspectives for the development of novel anticancer drugs; Chem Phys Lipids, submitted

15. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

16. Rao, L. V., Tait, J. F., and Hoang, A. D., 1992: Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity; Thromb.Res., 67(5): 517-531

17. Woehlecke, H., Pohl, A., Alder-Baerens, N., Lage, H., and Herrmann, A., 2003: Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2); Biochem.J., 376(Pt 2): 489-495

18. Cichorek, M., Kozlowska, K., Witkowski, J. M., and Zarzeczna, M., 2000: Flow cytometric estimation of the plasma membrane diversity of transplantable melanomas, using annexin V; Folia Histochem.Cytobiol., 38(1): 41-43

19. Zweytick, D., Riedl, S., Rinner, B., Asslaber, M., Schaider, H., Walzer, S., Novak, A., and Lohner, K., 2011: In search of new targets - the membrane lipid phosphatidylserine - the underestimated Achilles' heel of cancer cells; Annals of Oncology, 22, suppl.3: 43

20. Schröder-Borm, H., Bakalova, R., and Andrä, J., 2005: The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine; FEBS Lett, 579(27): 6128-6134

21. Bevers, E. M., Comfurius, P., and Zwaal, R. F., 1996: Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications; Lupus, 5(5): 480-487

22. Zwaal, R. F. and Schroit, A. J., 1997: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells; Blood, 89(4): 1121-1132

23. Bevers, E. M., Comfurius, P., Dekkers, D. W., Harmsma, M., and Zwaal, R. F., 1998: Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling; Lupus, 7 Suppl 2: S126-S131

24. Seigneuret, M. and Devaux, P. F., 1984: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes; Proc.Natl.Acad.Sci.U.S.A, 81(12): 3751-3755

25. Kirszberg, C., Lima, L. G., Da Silva, d. O., Pickering, W., Gray, E., Barrowcliffe, T. W., Rumjanek, V. M., and Monteiro, R. Q., 2009: Simultaneous tissue factor expression and phosphatidylserine exposure account for the highly procoagulant pattern of melanoma cell lines; Melanoma Res., 19(5): 301-308

26. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J., 1989: Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation; Proc.Natl.Acad.Sci.U.S A, 86(9): 3184-3188

27. Gifford, J. L., Hunter, H. N., and Vogel, H. J., 2005: Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties; Cell Mol.Life Sci., 62(22): 2588-2598

28. Zweytick, D., Pabst, G., Abuja, P. M., Jilek, A., Blondelle, S. E., Andrä, J., Jerala, R., Monreal, D., Martinez, d. T., and Lohner, K., 2006: Influence of N-acylation of a peptide derived from human lactoferricin on membrane selectivity; Biochim.Biophys.Acta, 1758(9): 1426-1435

29. Zweytick, D., Tumer, S., Blondelle, S. E., and Lohner, K., 2008: Membrane curvature stress and antibacterial activity of lactoferricin derivatives; Biochem.Biophys.Res.Commun., 369(2): 395-400

30. Zweytick, D., Deutsch, G., Andrä, J., Blondelle, S. E., Vollmer, E., Jerala, R., and Lohner, K., 2011: Studies on lactoferricin derived E. coli membrane active peptides reveal differences in the mechanism of N-acylated versus non-acylated peptides; Journal of biological Chemistry, 24(21266-21276

31. Riedl, S., Tumer, S., Lohner, K., and Zweytick, D., 2011: Killing of melanoma cells and metastasis by human Lactoferricin derivatives correlates with effect on Cancer Models composed of Phosphatidylserine; Manuscript in preparation,

32. Rosenberg, S. A. and Einstein, A. B., Jr., 1972: Sialic acids on the plasma membrane of cultured human lymphoid cells. Chemical aspects and biosynthesis; J.Cell Biol., 53(2): 466-473

33. Risso, A., Zanetti, M., and Gennaro, R., 1998: Cytotoxicity and apoptosis mediated by two peptides of innate immunity; Cell Immunol., 189(2): 107-115

34. Whitmore, L. and Wallace, B. A., 2004: DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data; Nucleic Acids Research, 32(suppl 2): W668-W673

35. Whitmore, L. and Wallace, B. A., 2008: Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases; Biopolymers, 89(5): 392-400

36. Ellens, H., Bentz, J., and Szoka, F. C., 1985: H+- and Ca2+-induced fusion and destabilization of liposomes; Biochemistry, 24(13): 3099-3106

37. Bartlett, G. R., 1959: Colorimetric assay methods for free and phosphorylated glyceric acids; J.Biol.Chem., 234(3): 469-471

38. Broekhuyse, R. M., 2005: Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids; Biochim.Biophys.Acta, 152(2): 307-315

39. Wimley, W. C., Creamer, T. P., and White, S. H., 1996: Solvation Energies of Amino Acid Side Chains and Backbone in a Family of Host-Guest Pentapeptides; Biochemistry, 35(16): 5109-5124

40. Kufe, D. W., 2009: Mucins in cancer: function, prognosis and therapy; Nature Reviews Cancer, 9(12): 874-885

41. Carraway, K. L., Price-Schiavi, S. A., Zhu, X., and Komatsu, M., 1999: Membrane Mucins and Breast Cancer; Cancer Control, 6(6): 613-614

42. Bafna, S., Kaur, S., and Batra, S. K., 2010: Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells; Oncogene, 29(20): 2893-2904

43. Yang, N., Strom, M. B., Mekonnen, S. M., Svendsen, J. S., and Rekdal, O., 2004: The effects of shortening lactoferrin derived peptides against tumour cells, bacteria and normal human cells; J.Pept.Sci., 10(1): 37-46

44. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W., 2005: Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines; Mol Cancer Ther, 4(4): 612-624

45. Huang, Y. b., Wang, X. f., Wang, H. y., Liu, Y., and Chen, Y., 2011: Studies on Mechanism of Action of Anticancer Peptides by Modulation of Hydrophobicity Within a Defined Structural Framework; Molecular Cancer Therapeutics, 10(3): 416-426

46. Eliassen, L. T., Haug, B. E., Berge, G., and Rekdal, O., 2003: Enhanced antitumour activity of 15-residue bovine lactoferricin derivatives containing bulky aromatic amino acids and lipophilic N-terminal modifications; J Pept.Sci., 9(8): 510-517

47. Wang, F. L., Cui, S. X., Sun, L. P., Qu, X. J., Xie, Y. Y., Zhou, L., Mu, Y. L., Tang, W., and Wang, Y. S., 2009: High expression of alpha 2, 3-linked sialic acid residues is associated with the metastatic potential of human gastric cancer; Cancer Detect.Prev., 32(5-6): 437-443

48. Litynska, A., Przybylo, M., Pochec, E., Hoja-Lukowicz, D., Ciolczyk, D., Laidler, P., and Gil, D., 2001: Comparison of the lectin-binding pattern in different human melanoma cell lines; Melanoma Res., 11(3): 205-212

49. Cazet, A., Julien, S., Bobowski, M., Krzewinski-Recchi, M. A., Harduin-Lepers, A., Groux-Degroote, S., and Delannoy, P., 2010: Consequences of the expression of sialylated antigens in breast cancer; Carbohydr.Res., 345(10): 1377-1383

Summary and Outlook

Even at the beginning of the 21st century, cancer is still a leading cause of death (http://www.who.int/mediacentre/factsheets/fs297/en/). Besides conventional treatments, new therapies and strategies are strongly needed.

In a comprehensive study we could prove that the negatively charged phospholipid phosphatidylserine is exposed on the outer leaflet of the plasma membrane of human cancer cells, independently of cancer cell type. Additionally, PS exposure as a consequence of cell culture techniques was excluded by demonstration of surface PS on primary cancer cells. The result of the study that cancer cells from metastatic lesions still expose the anionic phospholipid PS on their cell surface to an even higher extent thus correlating with stage of malignity, might be further beneficial for cancer therapy. Formerly, it has been shown by Ran et al. (1) that PS is externalized by tumor blood vessels. Utsugi et al. (2) demonstrated that tumorigenic cells expose 3-7-fold more PS than normal keratinocytes. In extension to these previous studies (1;2), our data provide clear evidence that PS could serve as a uniform marker for cancer and be used as an independent target for new cancer therapy based on e.g. PS specific host defense peptides like human Lactoferrin and derivatives thereof.

Succeeding to studies of cancer specific PS exposure, we started to optimize peptide derivatives of hLFcin using membrane mimetic systems to enhance peptide / lipid interactions. Cancer cells were mimicked by liposomes composed of pure PS, yielding information about cancer toxicity of peptides. PC liposomes, mimicking non-cancerous cell membranes, were used to determine peptide selectivity. Especially ANTS/DPX leakage experiments were a useful tool to screen the hLFcin derived peptide library for potential anticancer active and selective candidates. To gain further information about details of lipid/peptide interactions, DSC, Trp-fluorescence and CD spectroscopy were applied. The model studies with approximately 50 peptides tested revealed that short peptides show only

moderate interaction with the cancer mimic PS and almost none with the non-cancer mimic PC. The 20 N-acylated peptides exhibited strongly increased activity towards the cancer mimic PS but to different extent also towards the non-cancer mimic composed of PC.

For *in vitro* experiments we selected several peptide groups for further investigations. This thesis only reports about 2 peptide families based on LF11-322 and LF11-318, comprising the most contrary two peptides in terms of specificity. In a first approach one mother peptide LF11-322 was acylated at the *N*-terminus with a 6-methyloctanoyl chain named 6-MO-LF11-322 characterized by increased hydrophobicity. In a second approach its retro dimer peptide R-DIM-P-LF11-322 exhibiting increased length and charge. The second peptide family was based upon LF11-318 which was modified by sole dimerization of the mother peptide, termed DIM-LF11-318.

Somewhat surprisingly, both parent peptides were only minor active against cancerous as well as against non-cancerous cells *in vitro*, though they exhibited slight activity on the cancer mimic in the model system. This phenomenon was observed for all non-acylated monomeric peptides, which were antimicrobial active (3) but probably due to lack of sufficient stability in serum or inability of adoption of a defined structure not active against cancer cells. Also Yang et al. (4) reported about requirement of a minimal length for a potent antitumor peptide. However, N-acylated peptides and dimers of parent peptides were able to exhibit the same high activity in model and *in vitro* system, probably due to serum stability and defined structure in the presence of the target membrane. The *N*-acylated peptide 6-MO-LF11-322 accordingly showed increased activity against all cancer cells and interestingly very fast killing within minutes. Nevertheless it was also slightly toxic to non-cancerous cells. The very fast killing rate is a clear evidence for killing through membrane lysis which is favored in respect of avoiding the development of drug resistance. R-DIM-P-LF11-322, however exhibited even higher cancer cell toxicity and almost no toxicity towards non-cancerous cells. Very surprisingly, R-DIM-P-LF11-322 killed more slowly than the other active peptides. Together with the microscopic observation of membrane blebbing the relatively slow action supposes a killing mechanism via

apoptosis. This assumption has yet to be proven by e.g. TUNEL or caspase-3 cleavage studies. For induction of apoptosis the peptide has to enter the cell, most likely over PS domains on the surface and further reach another negatively charged target, i.e. cardiolipin on the surface of cancer cell mitochondria. Successive swelling of mitochondria and release of cytochrome-C activate the caspase dependent pathway of the programmed cell death. Induction of apoptosis in human leukemia was also observed for bovine LFcin by Mader et al. (5) and is probably the key for specific antitumor activity. However, in our study the smaller human derived peptide R-DIM-LF11-322 exhibits much higher activity towards melanoma and rhabdomyosarcoma cells with an up to 1000fold specificity for these cancers compared to activity (specificity) of bovine LFcin reported against leukemia (5). Further the second dimer, namely DIM-LF11-318, was a very interesting candidate showing the highest cancer cell activity and an even faster killing than 6-MO-LF11-322. However, DIM-LF11-318 was completely unselective exhibiting nearly the same activity against non-cancerous cells. The unselective mode of action can probably be contributed to its high α-helical content in the presence of the cancer and non-cancer cell mimic whereas the most selective peptide R-DIM-P-LF11-322 exhibited an increase in β-sheet structure and no changes in structure in the presence of the non-cancer cell mimic. Figure 1 gives a schematic overview of the suggested modes of action.

Summarized, we could prove that PS is specifically and natively exposed on the cancer cell surface and that hLFcin derivatives have the potential as highly active cancer peptides. Comparing model with *in vitro* studies we can conclude the suitability of biophysical methods for screening of peptide activity and therefor for peptide optimization. The work of this thesis led to the discovery of some *in vitro* highly cancer specific novel peptides which will be applied for a patent and be part of further *in vitro* and *in vivo* studies in a follow up project, which will be submitted to the Austrian Science Foundation.

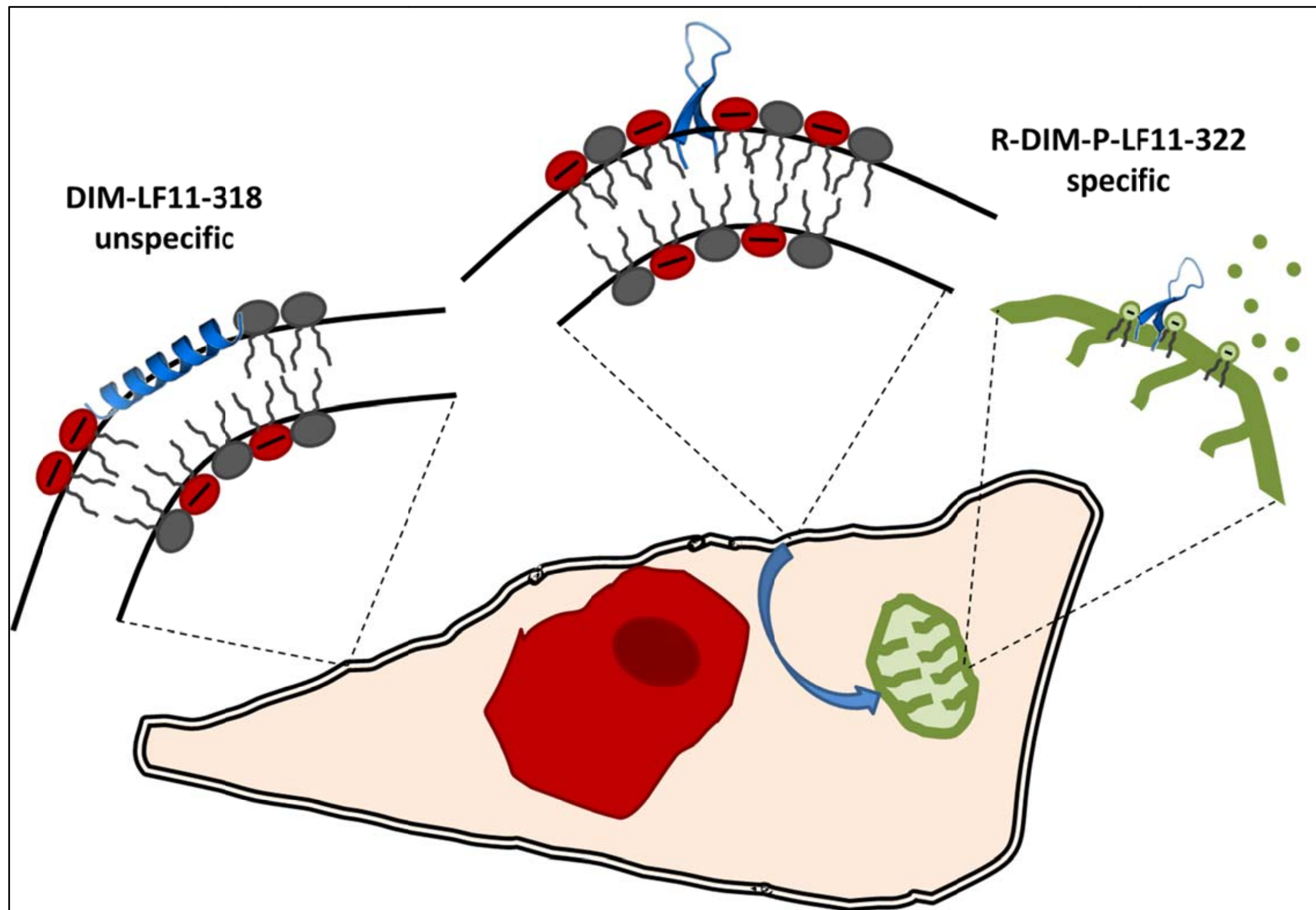

Figure 1: Scheme of suggested mode of action of the active but unspecific peptide DIM-LF11-318 and the active but cancer-specific peptide R-DIM-P-LF11-322: DIM-LF11-318 kills the cell within minutes by cell lysis via necrosis whereas R-DIM-P-LF11-322 kills more slowly through membrane-mediated apoptosis.

Reference List

1. Ran, S., Downes, A., and Thorpe, P. E., 2002: Increased exposure of anionic phospholipids on the surface of tumor blood vessels; Cancer Res., 62(21): 6132-6140

2. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J., 1991: Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes; Cancer Res., 51(11): 3062-3066

3. Zweytick, D., Deutsch, G., Andrä, J., Blondelle, S. E., Vollmer, E., Jerala, R., and Lohner, K., 2011: Studies on lactoferricin derived E. coli membrane active peptides reveal differences in the mechanism of N-acylated versus non-acylated peptides; Journal of biological Chemistry, 24(21266-21276

4. Yang, N., Strom, M. B., Mekonnen, S. M., Svendsen, J. S., and Rekdal, O., 2004: The effects of shortening lactoferrin derived peptides against tumour cells, bacteria and normal human cells; J.Pept.Sci., 10(1): 37-46

5. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W, 2005: Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines; Mol Cancer Ther, 4(4): 612-624