FEDERAL UNIVERSITY OF RIO GRANDE DO SUL

INSTITUTE FOR BIOTECHNOLOGY

POST-GRADUATION PROGRAM OF CELL AND MOLECULAR BIOLOGY

ELISABETH MARGARETHE DITMER

BACHELOR OF SCIENCE IN BIOLOGY

Evaluation of the anti-proliverative effect of the lipopeptides iturin A and fengycin of *Bacillus spp*. and the viral fusionprotein PTD4-VP3 on human cancer and normal cells

SUPERVISOR:

DR. ADRIANO BRANDELLI

Porto Alegre, Rio Grande do Sul, Brazil

October 2014

FEDERAL UNIVERSITY OF RIO GRANDE DO SUL

INSTITUTE FOR BIOTECHNOLOGY

POST-GRADUATION PROGRAM OF CELL AND MOLECULAR BIOLOGY

ELISABETH MARGARETHE DITMER

BACHELOR OF SCIENCE IN BIOLOGY

Evaluation of the anti-proliverative effect of the lipopeptides iturin A and fengycin of *Bacillus spp*. and the viral fusionprotein PTD4-VP3 on human cancer and normal cells

SUPERVISOR:

DR. ADRIANO BRANDELLI

Dissertation presented to the Post-Graduation Program as one of the requirements for the achievement of the Masters degree in Cell and Molecular Biology.

Porto Alegre, Rio Grande do Sul, Brazil

October 2014

Dedication

To my dearest sunshine Joana - miracle, love of my life and best friend.
Thank you for make me trying to get the best out of me every day.

In Memoriam:
To my beloved dad, Aristide Frederik Ditmer.
Your guidance and great example will always be part of all my decisions.
Thank you for everything you taught me.

In Memoriam:
To Cuba.

Acknowledgements

I would like to take this opportunity to express my profound gratitude and deep regards to everyone, who helped me to complete this task through various stages.

My special thanks to my supervisor Dr. Adriano Brandelli for your patient guidance, for all your support and every given advice, as I moved from ideas to a completed study. Thank your very much for being an outstanding example as creative tutor and composed person. I truly appreciate to have had the opportunity to develop this work under your guidance.

In a similar vein, I also would like to thank Dr. Ana Cláudia Franco for all her support and listening to me talking about endless ideas, concerning the work with the VP3. Also for providing the viral peptides and giving me the opportunity to perform decisive experiments in the Virology lab.
Further I truly be grateful for having found in you an encouraging and admirable friend, who I ever will look up to.

I would like to express my deep regards to Dr. Jeverson Frazzon, his wife Dr. Ana Paula Guedes Frazzon and Dr. Plinho Hertz, who helped me during completion of my dissertation with lots of valuable information provided by them in their respective fields. Also for every equipment, I borrowed and countless hours I could occupy SDS-PAGE cubes. It was a pleasure to share with you so many thought-provoking discussions.

To Dr. Carlos Termignoni, for the revision of my thesis. I appreciate your opinion, advices and effort so much.

I am obliged to my colleagues Stela, Juliana, Patricia, Géssica and Ana Paula at the lab Nr. 218 at ICTA, Campus do Vale ICTA. I am grateful for their cooperation and support over the past two years.

I want to thank my colleagues Helton, Fabricio, Esmaile, Juliana, Rhayssa, Raíssa, Rayra, and everybody of the Virology lab at the ICBS, „Campus do Centro“, for their support and help, company and willingness for constructive discussions. For lots of funny memories.

Especially I want to express my regards to my dear friend Juliana, who „borrowed“ me her student helper Livia, during the important months of final experiments.
To Livia, without your support and enthusiastic working methods, I would not have succeeded in performing the final experiments in that way. I count my self fortunate to got to know you.
To Jamile, although staying with her son at home during maternity leave, I am very grateful for every help you offered and for every convenient advice about how things (won ´t) work at the ICTA and the PPGBCM.
Thank you for being part of this.

Also I would like to thank Dr. Serena Schwenkert and Dr. Jörg Meurer at the LMU for their support during this work.
Also to all professors and colleagues, who participated in one way or the other during the development of this work.

To my dear friends Rhayssa and Andrei, I would like to express a deep sense of gratitude. Your constant encouragement, your friendship, your patience and your love made this work and also many other things possible. I am very glad to have met you two. You are very special to me, I´m very fond of you.

My „german girls“ Lisa, Loretta, Yvonne, Aline and Katja. The best friends on the „other side of the planet“. Your support and love you send me via skype and all other communications mediums, helped through times of homesickness and made me go on. I miss you so much.
Special thanks to Katja, for helping me out with some favors at the LMU.
Also special Thanks to Yvonne, who helped with „proofreading“.

Finally, I thank almighty my family: Rafael, our daughter Joana and our furry „kids“ Jamaica, Luke, Luna, Bella and Lucky for their strong support, their love and the innumerable sacrifices they made, while I pursued this degree.
Rafael, for often shouldering more than a fair part of the daily responsibilities of our every day life. Joana, for so many times you had to wait for Mama and all the moments I have missed to be with you. My furry kids, for tolerating the reduced attention I put them through.
My deep regards to my parents-in-law, Lisete and Paulo Ricardo, to my beloved brother in law Henrique and „our“ grandma Isis for their support during the last years.

Thank you all for having contributed to my work in various ways.

INDEX

List of Abbreviations

AGV2	*Avian Gyrovirus* II
ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
Apaf-1	Apoptotic Protease Activating Factor-1
Apo2L	Apo2 Ligand
Apo3L	Apo3 Ligand
Bad	Bcl-2-associated death promoter
BAG	molecular chaperone regulator protein
Bak	Bcl-2 homologous antagonist/killer protein
Bax,	Bcl-2-associated protein, Apoptosis regulator
Bcl-10	B-cell lymphoma 10
Bcl-2	B-cell lymphoma 2
Bcl-2	B-cell Lymphoma Protein 2
Bcl-w	Bcl-2-like protein 2, anti-apoptotic protein encoded by the *BCL2L2* gene

Bcl-x	anti-apoptotic interacting protein domain found in BAM and Bim
Bcl-x_L	anti-apoptotic protein of the Bcl-2 family
Bcl-X_S	anti-apoptotic protein of the Bcl-2 family
Bcl-XL	B-cell lymphoma extra-large
Bid	BH3 interacting-domain death agonist, homologous to Bcl-2
Bim	Bcl-2 like protein 11
Bik	Bcl-2-interacting killer
Blk	Tyrosine-protein kinase BLK, B-lymphocyte kinase
BSA	Bovine Serum Albumin
bp	base pairs
CAV	Chicken Anemia Virus
DEDAF	Death Effector Domain-Associated Factor
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate

FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FasR	Fas Receptoreracting protein 1 protein interactor
IMPβ1	Importin β1
INK4	Anti-oncogene
IPTG	β-D-1-Thiogalactopyranoside
kDa	Kilodalton
LB	Luria-Bertani
LRS	Leucine-Rich Stretch
NCBI	National Center for Biotechnology Information
NES	Nuclear Export Signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells, Transcription control protein
NLS	Nuclear Localization Signal
NPC	Nuclear Pore Complex
nt	Nucleotides
ORF	Open Reading Frame

PAGE	Polyacrylamidgelelectrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PP2A	Protein Phosphatase 2A
PTD4	Protein Transduction Domain 4
PUMA	p53 Upregulated Modulator of Apoptosis
RNA	Ribonucleic Acid
mRNA	messenger RNA
RPMI	Roswell Park Memorial Institute Medium
FBS	Fetal bovine serum
SDS	Sodiumdodecylsulfate
Smac/DIABLO	Second Mitochondria-derived Activator of Caspases
SV40	Simian Vacuolating Virus 40
TAp73	Transactivation-proficient p73
Taq	Thermus aquaticus
TEMED	N,N,N',N',-Tetramethylethlyendiamin

Thr	Threonine
TNF	Tumor Necrosis Factor
TNFR1	Tumoral Necrosis Factor Receptor 1
UV	Ultraviolet Radiation
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3
ΔNp73	Dominant-Negative Inhibitor of p53 and TAp73

List of Figures

The horizontal bar represents the VP3 gene from 577nt to 951nt. The hatched regions within the bars indicate the positions of conserved motifs. Within VP3: 3I = isoleucine stretch, 3II = NLS1, 3III = NES and 3IV = NLS2.
(adapted from the original figure of Rijsewijk et al., 2011)

Figure 6: **BSA equiation curve for protein quantity determination** -
blue graph: Avarage values of 3 samples, drawn against the aborbance value at 280nm
red graph: linear regression line of the BSA graph

Figure 7: **Charts for medium dependent cell growth and the medium dependent lipopeptide quantity for *B. amyloliquefaciens* LBM5006 and *Bacillus spp.* P34**.
Graphs for the BHI reference medium are displayed in blue, the composed ferrous ion medium is displayed in green.
The charts for the medium dependent cell growth are plotted for the timecourse against the mean values of the measured optic densities for every culture.
The data for the lipopeptide quantities are plotted for the timecourse against the mean values of the determined lipopeptide contents.
All demonstrated results are obtained via the calculated mean values of three replicates.
The asterisks indicate the measuring points, where a significant difference between the used composed culture medium and the reference medium was detectable.

Figure 8: **PCR amplicons of AGV II VP3 (T) and AGV II VP3 (SM)** - PCR amplification of the VP3 coding regions of AGV2-T (lane 1) and AGV2-SM (lane 2) producing a fragment of 405bp (Adapted from Knack et al., 2011).

Figure 9: **Alingment of AGV II VP3 (T) and AGV II VP3 (SM)** - Alingment of the coding regions of the nucleotide sequences for the AGV II VP3 isomers VP3 Thais (violet line) and VP3 Santa Maria (pink line).
The end of the fused SUMO protein is marked in orange (nt 301 - 357). Marked in red: the N-terminally to the VP3 peptide fused PTD4 sequence (nt 358 - 390). The sequence differences of the Thais isomer and Santa Maria isomer are marked with rhombos #.

Figure 10: **PCR products for pET SUMO PTD4 VP3 constructs** - the pET SUMO PTD4 VP3 (T) construct and MiniPrep, Colony PCR products of transformed *E. coli* DH5α and BL21(DE3) pLysS. Amplicon has a calculated size of 476bp.
1: 1kb Marker (NEB)
2: PCR product of the pET SUMO PTD4 VP3 (T) construct
3: Amplicon of *E. coli* DH5α transformed with pET SUMO PTD4 VP3 (T)
4: Amplicon of the plasmid extraction of *E. coli* DH5α pET SUMO PTD4 VP3 (T)
5: *E. coli* BL21(DE3) pLysS transformed with pET SUMO PTD4 VP3 (T)

Figure 11**: SDS PAGE of the 6xHIS SUMO PTD4 VP3 IMAC purification** - SDS PAGE analysis of the 6xHIS SUMO PTD4 VP3 (T) protein expressed in recombinant *E. coli*, purified with Ni^{2+}-columns under native conditions

Analysis of the pellet after cell lysis (1) and the lysate (2). Lane 3 represents the sixth (last) flowthrough fraction. Lane 4 and 5 show the first and last (6th) fractions of the 1st washing step with 50mM Imidazole. Lanes 6 tp 11 display the fractions 1 to 6 of the 2nd washing step with 100mM Imidazole. The protein elution fractions 1 to 4 with 250mM Imidazole are shown in lane 12 to 15.

M: BenchMark™ Protein Ladder (Invitrogen)

12% SDS PAGE; yellow line indicates 30 kDa size

Figure 12**: SDS PAGE of the final 6xHIS-SUMO-PTD4-VP3 isoforms** - SDS PAGE of the enzymatic reaction with SUMO Protease (cleavage) to obtain the final 6xHIS-SUMO-PTD4-VP3 isoforms.

Left side: samples for the VP3 (T) isomer;

right side: samples for the (SM) isomer;

M: BenchMark Protein Ladder (Invitrogen)

1:purified and pooled HIS-tagged fusion protein

2: sample after filtration

3: final PTD4-VP3 fusionprotein after 6xHIS-SUMO cleavage

Figure 13: **Anti-proliverative effect of iturin for 24h (1 day)** - the anti-proliferative activity of iturin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (50 - 500 µg/mL) of iturin for 24h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.

Bars and graph in red: A549

Bars and graphs in yellow: SiHa

Bars and graphs in blue AS405

Figure 14: **Anti-proliverative effect of iturin for 120h (5 day)** - the anti-proliferative activity of iturin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (100 - 1000 µg/mL) of iturin for 24h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.

Bars and graph in red: A549

Bars and graphs in yellow: SiHa

Bars and graphs in blue AS405

Figure 15: **Anti-proliverative effect of fengycin for 24h (1 day)** - the anti-proliferative activity of fengycin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (50 - 500 µg/mL) of iturin for 24h. Cell viability was determined by MTT and

expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

Figure 16: **Anti-proliverative effect of fengycin for 120h (5 day)** - the anti-proliferative activity of fengycin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (100 - 1000 µg/mL) of iturin for 24h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

Figure 17: **Anti-proliferative effect of 200 µg/mL (lines) and 500 µg/mL (broken lines) iturin for max. 120h (5 days)** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (200 and 500 µg/mL) of iturin for max. 120h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

Figure 18: **Anti-proliverative effect of PTD4-VP3 (T) for 24h** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (100 - 1000 µg/mL) of PTD4-VP3 (T) for 24h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in blue: A549
Bars and graphs in yellow: SiHa
Bars and graphs in violet AS405

Figure 19: **Anti-proliferative effect of PTD4-VP3 (SM) for 24h** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (100 - 1000 µg/mL) of PTD4-VP3 (SM) for 24h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in blue: A549
Bars and graphs in yellow: SiHa
Bars and graphs in violet AS405

Table 1 - selected lipopeptides produced under different cultivation conditions with the calculated peptide quantity (total sample protein content) and determinated arbitrary units

ABSTRACT

In cancer cells the apoptotic pathway is damaged by deletion or mutation of important genes, e.g. tumor-suppressor gene p53 or Check2. This causes a loss in cancer cells to undergo controlled cell death resulting in unstopped cell division and tumor growth. Also, many tumors show resistances against the traditional applied treatments like chemo or radiation therapy. Therefore, accompanied or independent cancer treatments based on induced apoptosis or strengthened growth inhibition in cancer cells are under development.

The objective of this work was to investigate the anti-proliferative effect on human cancer cells of the lipopeptides iturin A and fengycin obtained from strains of *Bacillus spp.* as well as of the *Avian Gyrovirus* II (AGVII) protein -VP3.

The VP3 protein of the AGV II was discovered in 2011 and its amino acid sequence showed 32.2% homology and similar functional domains to the chicken anemia virus apoptin (CAV-apoptin), a protein that was proven to induce apoptosis in cancer cells but not in normal cells. Initially, to obtain the VP3 protein for further analysis, the PTD4 sequence, known as a transmission sequence into the cell, was N-terminally added to the VP3 sequence via PCR. After sequencing, the PCR product was cloned into the expression vector pET-SUMO and the final construct transformed into *E. coli* BL21(DE3)pLyS. After induced protein overexpression and subsequent purification, the PTD4-VP3 protein was quantified and incubated with human cancer-cell cultures. The lipopeptides iturin A and fengycin were produced by *Bacillus amyloliquefaciens* LBM 5006 and *Bacillus* sp. P34*,* respectively. The lipopeptides were purified and added to the cell cultures of human tumor cells.

The human non-cancer cell line AS405 (skin fibroblasts) was chosen as control. The protein effect on cell viability was determined via MTT assays. The results showed that the lipopeptides iturin and fengycin as well as the viral peptides PTD4-VP3 (T) and PTD4-VP3 (SM) demonstrated dose-dependent anti-proliferative activity on cancer cells. For the lipopeptides also time-dependent growth-inhibition effect could be detected.

A anti-proliferation effect on normal human cells was not excludable but could not clearly be demonstrated.

This is the first study validating the anti-proliferative potential of the lipopeptides iturin and fengycin and the viral fusion proteins PTD4-VP3 (T) and PTD4-VP3 (SM) to inhibit cell growth mainly in human cancer cells.

RESUMO

Nas células cancerosas, a via apoptótica é danificada pela supressão ou mutação de genes importantes, como genes supressores de tumor p53 ou Check2. Isto faz com que as células cancerígenas percam a habilidade de executar a morte celular controlada, resultando na desobstrução da divisão celular e crescimento do tumor. Muitos tumores também mostram resistências aos tratamentos tradicionais como quimioterapia ou radioterapia. Tratamentos de câncer baseados na apoptose induzida ou em aumento na inibição das células cancerosas estão em desenvolvimento. O objetivo deste trabalho é investigar nas células cancerígenas humanas o efeito anti-proliferativo dos lipopeptídeos iturin A e fengycin obtidos das estirpes de *Bacillus* spp. bem como dos da proteina VP3 de *Avian gyrovirus* II (AGVII). A proteína VP3 do AGV II foi descoberta em 2011 e sua seqüência de aminoácido mostra 32.2% de homologia e domínios funcionas similares à proteina apoptina do vírus da anemia infecciosa das galinhas (CAV - apoptin), uma proteína que induz apoptose nas células cancerígenas mas que não afeta células normais. Inicialmente, para obter a proteína VP3 em uma análise adicional, a sequência PTD4, conhecida como uma sequência de transmissão dentro da célula, foi adicionada à seqüência VP3 através de PCR. Após a determinação da sequencia de nucleotideos, o produto de PCR foi clonado dentro do vetor de expressão PET-SUMO e a construção final foi transformada em *E. coli* BL21 (DE3) pLyS. Após a super expressão da proteína e a purificação subsequente, a proteína PTD4-VP3 foi incubada com culturas de células de câncer humano. Os lipopeptídeos iturin A e fengycin foram produzidos pelo *Bacillus amyloliquefaciens* LBM 5006 e pelo *Bacillus sp*. P34, respectivamente. Os lipopeptídeos foram purificados e adicionados às culturas de células de câncer humano. A linhagem de célula não-cancerígena humana AS405 (fibroblasto) foi escolhida como o controle. O efeito da proteína na viabilidade celular foi determinado através de testes de MTT. Os resultados mostraram que o efeito antiproliferativo dos lipopeptídeos utilizados iturin e fengycin, bem como os peptídeos virais PTD4-VP3 (T) e PTD4-VP3 (SM) dependem da dose. Para os lipopeptídeos o efeito de inibição do crescimento dependente do tempo, também pode ser detectada. Um efeito anti-proliferativo em células humanas normais não pode ser excluído, embora não tenha sido claramente demonstrado. Este é o primeiro estudo validando o

potencial anti-proliferativo dos lipopeptideos, iturin e fengycin e das proteínas de fusão viral PTD4-VP3 (T) e PTD4-VP3 (SM) em inibir o crescimento de células, principalmente células cancerígenas humanas.

1. Introduction

One of the most common diseases nowadays is cancer. Responsible for 8.2 million deaths in 2012, it is thought to be the prime cause for death in economically evolved countries and the second in undedeveloped countries. About 60% of the world´s total new annual cases occur in Africa, Asia and Central and South America, whereas these regions account for 70% of the global cancer deaths. (WHO, 2008; Ferlay, 2008; International Agency for Research on Cancer; de Martel, Ferlay, Franceschi et al., 2012). Behind the word cancer stands a large group of various diseases which can affect any part of the body. To name only some of the cancers variety: lung cancer, the most common cancer, breast cancer, which is the most common cancer in women, and colorectal cancer.

The characteristics of cancer is the unstopped cell growth and division into daughter cells beginning with a certain single cell. As a result of the persons genetic factors in combination with common carcinogens, the result is often a malignant tumor. This behavior of abnormal cell growth can also invade neighbor tissues as well as migrate and spread through the body, building metastasis.

Comparable to the huge number of different cancer types, there exists also a huge number of therapy possibilities. Chemotherapy, radiotherapy and also surgery are the most common therapies nowadays, aiming for the elimination of the cancerous cells, or at least to help to reduce the patients´ pain.

Notwithstanding all the numerous therapies, the fight against cancer is still very difficult and in some special cases the disease cannot be cured.

The applied anti-cancer therapies are directed against the cell proliferation process, and current therapies often made no distinction between normal and abnormal cancer cells. While cancer cells proliferate faster than normal cells, usual anti-cancer agents attack more likely abnormal cancer cells but also, as one of the unfortunate side effects, normal cells are affected too. Other problems of these anti-cancer therapies are the developing resistances of the cancers against drugs and the toxic side effects which are entailed by anti-cancer agents (Gottesmann et al., 2002).

While these side effects persist, the research increases among natural agents and other efficient anti-cancer treatments which are capable to distinguish between normal and abnormal cells and therefore do not affect the healthy cells and further the organisms health.

In 1994, a small viral protein, codified by the chicken anemia virus (CAV), was discovered by Noteborn and coworkers (Noteborn et al., 1994). As the protein showed to induce apoptosis in infected cells, later it was demonstrated that this protein has also an apoptotic function in cancer cells and therefore it was called apoptin. Tests in human cell lines show that the induction of cell death is effective only on cancerous cell lines, while human normal cells stay alive under the same treatment conditions. (Danen van Oorschot et al., 1997). Until 2009 over 70 human cancerous cell lines were tested and were sensitive to apoptin induced apoptosis (Noteborn et al.,2009). Further *in vivo* tests in mice also show that apoptin has a pro-apoptotic effect on implanted human tumors. Moreover, it was proven that apoptin is capable of inducing cell death independently from the tumor suppressor gene *TP*53 which is a prime agent for the initiation of the apoptotic pathway in normal cells and is found to be mutated in most types of cancer. Besides this, proto-oncogenes like Bcl-2, also known as anti-apoptotic agents, showed to have no effect on apoptin induced apoptosis (Danen van Oorschot, Van der Eb, Noteborn, 1999; Zhuang et al., 1995). All these results lead to the assumption that the CAV apoptin might be an efficient anti-tumor/anti-cancer agent, also for cases, where usual chemo- and radiotherapies do not show any effect on cancer.
Later in 2011, Rijsewijk and his coworkers discovered a new gyrovirus, the *avian gyrovirus* II (AGV II). This virus presents a genome of a single-stranded circular DNA consisting of 2383 nucleotides. During the analysis of the viral genome, the virus presented a similarity (40%) to the CAV virus comparing the general organization and the codified proteins. The homology between CAV apoptin and AGV II VP3 is 32.2% homology (Rijsewijk et al., 2011), whereas the similarities are found in the functional domains of the proteins. Further analysis presented results concerning the variability of the AGV II VP2-VP3 codifying region. Alterations within the amino acid sequences of the AGV II VP3

from different samples, isolated from different diseased chicken in different locations, could be shown (dos Santos et al., 2012).

Not only viral proteins are possible anti-cancer agents from microbial origin. Some researchers showed the anti-cancer activity of surfactin, a cyclic lipopeptide produced by some *Bacillus* species. Additionally iturin is a lipopeptide produced by *Bacillus subtilis* and other related species, with a molecular mass of 1.036 Da and a high resistance for thermal treatments. Its amphiphilic structure is characteristic and contains a hydrophobic β-hydroxy fatty acid of variable chain length connected to a hydrophilic α amino acid ring of variable contents. Production of the lipopeptide isomers depends on several external factors, stress factors and strain qualities (Kluge et al., 1988).
In biomedical research, biosurfactants became of increasing interest.
Wang and his coworkers showed inhibitory activity of the surfactin lipopeptide on the growth of human leukemia K562 cells. The apoptotic activity of the cyclolipopeptide was associated with caspase-3 and poly(ADP-ribose) polymerase proteins which are important parts in the apoptotic pathway (Wang et al., 2007). Similar results of dose dependent induced apoptosis by *Bacillus subtilis* excreted surfactin were obtained for MCF-7 human breast cancer cells (Lee et al., 2011).
These results lead to the hypothesis to investigate surfactin as a possible natural anti-cancer agent. Structurally similar peptides like iturin and fengycin are also produced by *Bacillus* spp., but these lipopeptides have not been investigated as potential anticancer agents.

Based on this cited data for the AGV II VP3, *B. amyloliquefaciens* fengycin and *B. amazonenis* fengycin, their anti-cancer activity should be investigated in this present study.

General objectives

The general aim of the study is the investigation of potential anti-cancer activity of AGV II VP3, iturin from *B. amyloliquefaciens* LBM 5006 and fengycin from *Bacillus* sp. P34.

Specific objectives

Production of the Biosurfactants iturin and fengycin:

- Enhanced biocin production with the help of composed ferrous ion medium
- Ammonium precipitation of the iturin and fengycin cyclolipopeptides
- Size Exclusion Chromatography of the precipitated Biocin samples

Production of two different AGV II VP3 protein variants with n-terminal PTD4 transduction domain

- Construction of the PTD4-VP3 recombinant proteins, cloned into the pET-SUMO vector to obtain the final 6xHIS-SUMO-PTD4-VP3 (Thais / Santa Maria) fusion proteins
- IPTG induced overexpression of the obtained fusion protein constructs
- Immobilized metal ion affinity chromatography for purification of the 6xHIS tagged viral fusion proteins
- Cleavage of the 6xHIS-SUMO protein with the help of the SUMO-protease to obtain the final PTD4-VP3 (Thais / Santa Maria) proteins

Analysis of the anti-proliferative effect in human cancer cells and fibroblasts

- Inoculation of the obtained biosurfactants iturin and fengycin as well as the viral fusion protein PTD4-VP3 (Thais / Santa Maria) with human tumor cell (SiHa, A549) and human fibroblats (AS405) for different incubation times
- Cell viability tests with the help of the MTT assay and analysis of the death / survival rates

2. Bibliographic revision

2.1 What is Cancer, and how does it work?

Behind the word cancer stands a group of diseases characterized by unregulated cell growth resulting in a tumor or high quantity of abnormal cells. Thereby, cancer can derive of every cell tissue which means, the initially affected cell is classifying the cancer type. The five main groups to categorize cancer are:

1) Carcinomas (derived from cells which cover internal or external parts, like lung, breast or colon)

2) Sarcomas (located in bone, muscle, cartilage, fat, blood vessels)

3) Leukemia (affects the bone marrow and causes large numbers of abnormal blood cells)

4) Lymphomas and myelomas (derived from cells of the immune system)

5) Central nervous system (CNS) cancer (unstopped cell growth begins in the brain or spinal cord)

When cells start to divide abnormally, resulting in a tumor, there are also two ways to distinguish this abnormal cell-growth in benign or malignant tumors. The former type does not spread by invasion or metastasis and grows only locally. Thereby, they are most likely easy to treat and considered as „not aggressive“. In almost every case of benign tumors, good chances are proposed for the patient.

Malignant tumors, by contrast, are able to spread by invasion through the blood vessels, neighboring tissue and/or the lymphatic system and cause metastasis in different sites all over the body. Because of this invasive capability, malignant tumors are considered aggressive (potentially life threatening) and are also classified in grades, depending on the status of the tumor growth and the presence of metastasis.

A low grade tumor, grade I or II, refers to a tumor with lower cell abnormalities, whereas grade III or IV refers to tumors which grow more quickly with large numbers of abnormal cells.

The statistics show patients chances for recovering and survival rate decrease continuously depending on the grade of the tumor (National Cancer Institute, 2013). These chances are also influenced by the treatments and how is the cancers reaction on it.

Cancer is one of the world's most common diseases nowadays which equally concerns men as well as women. On top of the list of cancer diseases in 2008 is lung cancer with 12.7% increase (1.6 million) of new cases and 18.2% (1.4 million) of cancer deaths, followed by breast cancer (10.9% new cases, 1.4 million; 6.1% deaths), colon- and rectum cancer and cancer of stomach, prostate and liver (Section of Cancer Information, International Association of Cancer Registries - IACR, Biennial Report 2010/2011). The number of deaths and new cases in the statistics of cancer will increase due to the world's population growth and increased life expectancy as well as due to unhealthy common habits like smoking and unhealthy eating habits. In 2030 cancer might be responsible for 13.1 million deaths (Jemal *et al*, 2011).

Whether or not a normal cell turns into an abnormal cancer cell is determined by the possible genetic alterations (mutations) that normal cells suffer during their lifespan. This transformation into cancer caused by somatic mutations and genetic instability is frequently linked to carcinogenic influences like intense UV radiation, chemicals and other agents, fungus or virus. Cancer does not follow conclusively on every incident; it is rather the increasing probability to evolve cancer by raising numbers of genetical mutations caused by the carcinogenic substances (Rang et al., 2012).

To understand cancer it is helpful to understand how a normal cell progresses to become „cancer" (Figure 1).
The human body is made of different types of tissues and cells which grow, proliferate to produce more cells in order to replace old ones and keep the body healthy. And finally, old cells die when their lifespan is over. Normally, when cells become old or their DNA get damaged, cells own key-mechanisms like the p53-pathway, activation of the Bcl2-family

and the caspase-cascade ,will be activated and induce apoptosis, the programmed cell-death.
If now a mutation within a gene responsible for DNA-damage repair occurs in a normal cell, causing a dysfunction or complete loss of function, the probability in which this cell suffers more and more genetic mutations increases rapidly, besides the resulting genetic instability.
While these mutations have not been recognized by the damaged DNA-repair mechanisms, the cell passes such mutations to their daughter cells via mitosis, creating genetically distinct cells from the surrounding tissue.

From this step on every new daughter cell has major probability to suffer more mutations which also may affect their proliferating behavior resulting in unregulated cell division, unstopped cell multiplication, unstopped tissue growth (tumors) and, following as consequence, this formally unique distinct cell dominates the tissue-population (Lewin et al., 2004).

Also, the abnormal cancer cells have, in account of the occurred mutations, a clear advantage over the normal cells by increased cell proliferation rates, upregulated metabolism and their increased probability to acquire mutations favorable for their survival (e.g. resistances against cancer-drugs).

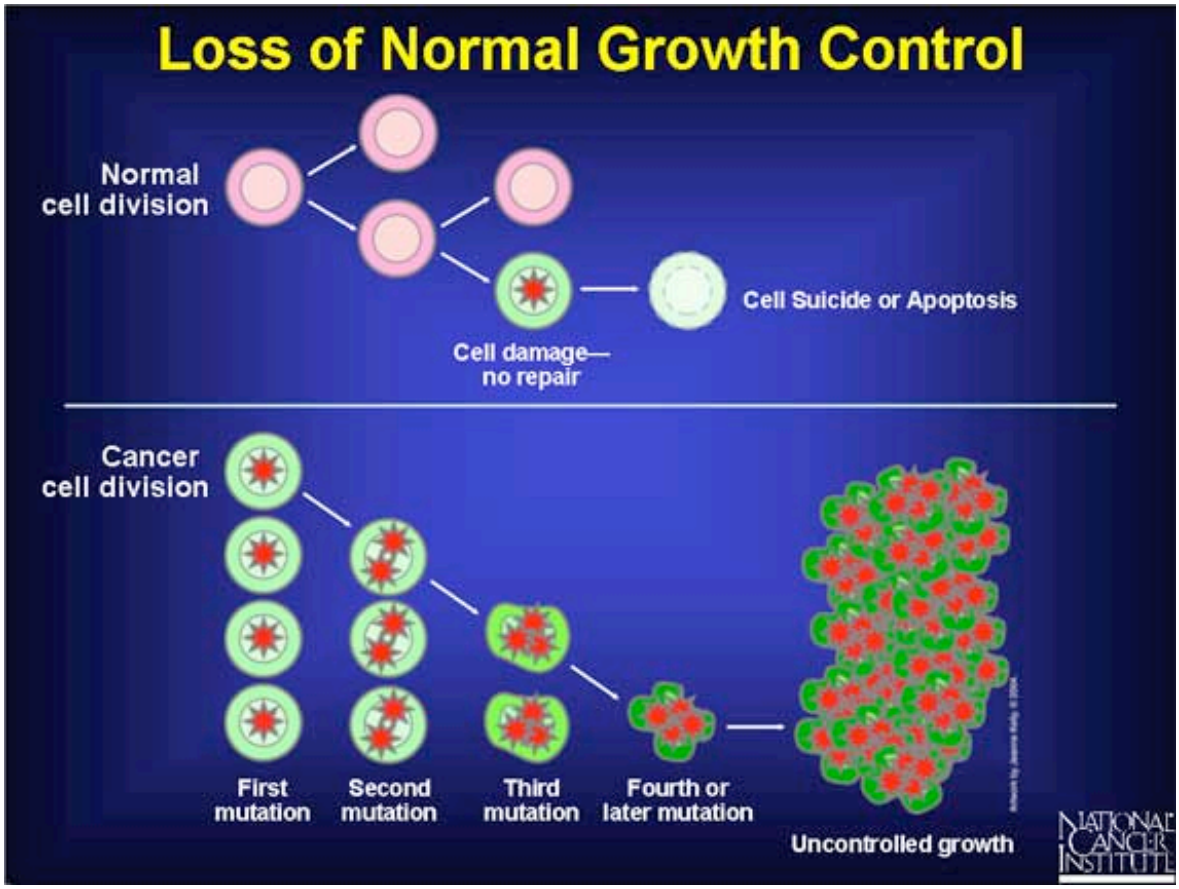

Figure 1: **Loss of normal Growth Control** - A: during cell cycle in normal cells, without damaged DNA-repair mechanisms, cells undergo apoptosis after a DNA-damage or detected mutation;
B: in abnormal cancer cells, which suffered mutations or DNA-damage, cells may proliferate unconditionally, while adquiring more mutations; increasing numbers of mutation, raised advantage of cell survival, resulting in unstopped tissue growth
(adapted from original Figure: National Cancer Institute)

Under normal conditions in normal cells, the cells‘ survival and proliferation is regulated by many proteins, some of them are known as Tumor-Suppressor-genes. The transcription of these tumor suppressor genes gives rise to so called key-proteins which have several functions during the cell cycle. For example, some proteins are involved in the continuation of the cell cycle. Other proteins are essentially coupled to recognition of

damaged DNA and DNA-repair. Further, some proteins are known as metastasis suppressors (Yoshida et al., 2000).
The most mentioned and studied tumor suppressor genes, also named anti-oncogenes, are neurofibromin 1, adenomatous polyposis coli, INK4, p19 - and with an outstanding literature report and a research history of more than 30 years - the tumor suppressor gene *TP53*, also called p53.
Since its discovery in 1979, the importance of the tumor suppressor gene 53 was interpreted in many research works which described its relevance on the essential connections with cell growth, cell cycle arrest and apoptosis. Until today there is no other gene involved in cancer development so highly explored as the p53 gene. This gene and the encoding protein are in main focus of cancer research to understand cancer development, inhibition of cancer and to find new effective, well-directed cancer treatments.

However, anti-oncogenes are only one side of „cancer-genes“. Like in nature, in order to keep equilibrium in all organisms and metabolisms, everything has its antagonist partner. Following this law of equilibrium, there certainly are existing and scientifically proven also the opposite numbers of tumor suppressor genes, the oncogenes, or more specialized, the proto-oncogenes.
Oncogenes are genes which may cause cancer when suffered a mutation, is highly expressed or when the antagonist is missing. An example is the NF-κB protein, also known as an anti-apoptotic gene by controlling the caspase-activating genes, responsible for DNA transcription and cell proliferation. Many cancers show a mutation in the NF-κB gene or its control activity. These defects may result in elevated ability to avoid apoptosis (Escárcega et al., 2007).
Unmutated proto-oncogenes are closely involved in regulation pathways. Further they drive cell differentiation, proliferation and general cell growth. When suffered a point mutation, the oncogenes gain higher probability to induce cancer by letting the cell evade/ escape apoptosis in combination with the induction of higher proliferation rates (Chial. H, 2008). Even so in some cases, their antagonist anti-oncogenes are not damaged or mutated.

How does cancer finally develop from a single cell?
In general, cancer develops after a series of mutations which accumulate in the DNA. Depending on where the mutation happened there is the possibility of activating a proto-oncogene, which turns into an oncogene and induces cancer by driving the uncontrolled proliferation of the tissue. The mutation(s) could happen in the anti-oncogene, too, for example p53, inactivating the tumor suppressor genes. Also a combination of both, anti-oncogene inactivation and proto-oncogene activation, is a cancer inducing event (Lewin et al, 2004).
Regarding mutations, mutant tumor suppressor alleles are usually recessive, whereas, mutant oncogene alleles are typically dominant. This means, when only one anti-oncogene allele of one chromosome is damaged the resting wild type gene may be sufficient for producing the correct „working“ protein. In contrary stands the oncogene-mutation. Only one allele needs to be mutated for causing major damage in the normal cell cycle (Chial, H., 2008).

2.2 The Tumor Suppressor Gene p53

The tumor suppressor gene *TP53* is maybe the most important gene concerning cancer development and protection. As already mentioned, the loss of the p53 encoding protein function causes an imbalance and results generally in cancer disease. As genetic observations show, more than one half of human cancer diseases are directly connected with the loss of the p53 protein or mutations of it (Lewin et al., 2004). Vogelstein showed clearly the importance of p53 signaling pathways in stress-response of cells (Vogelstein et al., 2000). This fact raises possibilities for clinical diagnosis and treatments.

When the p53 gene was discovered in 1979 (Lane and Crawford, 1979; Chang et al., 1979, Kress et al., 1979), it was taken as an oncogene at first. It was suggested that it is directly connected to the cell proliferation pathways and as it was detected in higher quantity in transformed (mouse) cells, the p53 protein was connected to the presence of cancer. Reich

and Levine showed in their experiments that the mRNA and protein levels of p53 increased remarkably until reaching their peak at the G1/S phase during the cell cycle, just before the beginning of DNA replication.
It was shown that the *TP53* expression was always associated to cell growth induction and cell growth could be inhibited by plasmids encoding anti-sense p53.
After the observations that p53 cooperates with the *Ha-ras* oncogene similar to interactions with *myc* or *E1A* (both strong oncogenes) and the immunohistochemical and immunohistocytochemical analysis, which showed the accumulation of p53 in the nucleus of tumor cells, the p53 gene and protein were classified as an nuclear dominant oncogene (Reich and Levine, 1984; Shohat et al., 1987, Eliyahu et al. 1984; Parada et al. 1984; Jenkins et al. 1985; Jenkins et al. 1984).

Later it was found that the *TP53* gene is closely connected to DNA-damage repair and the induction of apoptosis in case the detected DNA-damage is irreparable (Finlay CA et al., 1989).
In healthy human cells the tumor suppressor gene p53 is continuously expressed and degraded via ubiquitination almost at the same time, resulting that only low levels of p53 are found in normal cells. But as a response to stress, the protein levels are rising while the ubiquitination is blocked.
The protein is located in the nucleus and binds DNA after scanning for mutations, which may result from UV radiation, carcinogenic agents or toxic chemicals. Therefore, this protein plays a critical role in whether the DNA is repaired or the cell will be destructed.
If the DNA can be repaired the tumor suppressor p53 activates other genes to fix the damage. If the DNA cannot be repaired, this protein prevents the cell from dividing and signals it to undergo apoptosis. This process prevents cells with mutated or damaged DNA from dividing, which itself helps to prevent the development of tumors.
Because of the discoveries that (a) *TP53* is not a tumor inducing oncogene but a tumor suppressing anti-oncogene and (b) it is essential for regulating cell division and preventing tumor formation, it has been nicknamed the "guardian of the genome" (Lane, 1992).
During the years the tumor suppressor gene *TP53* gained several other names like „Death

Star“ (Vousden, 2000) and it was entitled with „molecule of the year“ attributed by Science in 1993 (Harris, 1993).

But what happened to the *TP53* gene in cancer cells or why did this guardian angel not fulfill his purpose?

The answer lies on the occurrence of mutations in the p53 gene. It was proven that the tumor suppressor gene was mutated in cancer cells. An interesting case and maybe also one of the first hints of p53 dysfunction in cancer cells (not wild type behavior) is the mutation or deletion of the p53 gene in tumors induced by the Friend virus (Mowat et al, 1985).

In general, in almost all studied cancer types, a point mutation of the p53 gene (Nigro et al., 1989; Baker et al., 1990) or a complete loss of the gene by loss of the short chromosome arm of the sister chromosomes nr. 17 was observed(Takahashi et al., 1989).

But nevertheless, not every mutation will result in cancer, which leads to a classification of even these mutations, according to the site and its associated phenotype (Michalovitz et al., 1991). There are some mutations which do not inhibit the normal function of the p53 gene, but these mutations will not further be discussed.

Generally, anti-oncogenes follow the „two-hits hypothesis“ by Knudson (Knudson, 1971). This theory states that as long as only one allele of an anti-tumor gene is mutated the resting wild type gene can still produce the working protein to maintain the normality. Only when both alleles are mutated it will have an effect on the cell cycle. This explains why certain mutations of tumor suppressor genes are recessive, whereas mutations of only one allele of oncogenes are typically dominant because of the gain-of-function mutation (Michalovitz et al., 1991).

But not all p53 mutations do always follow the „two-hits rule“ (Baker SJ, Markovitz S, Fearon ER, Willson JK, Vogelstein B., 1990). The term „dominant negative“ comes from mutations of the tumor suppressor gene *TP53*, encoded by only one mutated allele, preventing the function of the not-mutated allele.

Other classifications of altered p53 functions are

I) null mutations which inactivate *TP53* but do not actively intervene in transformation

II) positive dominant mutations of the anti-tumor gene where the mutant protein acquires an oncogenic activity and interferes directly in cell proliferation.

To sum it up, the anti-tumor gene p53 and its role in cancer development is of high importance to understand cancer and to find new treatments but it is also very complicated and full of exceptions. As already mentioned, in almost every studied cancer type, mutations of the p53 protein were detected which cause that the normal reaction of undergoing apoptosis is evaded. The possibility to induce apoptosis in even these cancer cells is a promising way for new treatment possibilities. This agrees with the aims of the present study which are associated to test new possibilities of treatment agents where also the impact on healthy cells of the patient may be reduced to a minimum.

2.3 Cancer treatments and differences of therapies

When cancer is diagnosed the next step is to determine the tumor grade and the patients´ condition. Based on this information, a suitable treatment plan, aiming always for the patients best outcome, is set up. As there is no single treatment and, depending on the type and the stage of the cancer (how much it has spread), age, health status and additional personal characteristics of the patient, patients often receive a combination of therapies and palliative care. A treatment plan usually contains a compilation of some of the following treatment-options: surgery, radiation, chemotherapy, immunotherapy, hormone therapy or gene therapy.
Hereby, chemo- and radiation therapies might be the *most-known* therapies which are most likely used before surgery to shrink the tumor and after surgery to compromise the remaining cancer cells (American Cancer Society, National Cancer Institute).
Nowadays, there exist various different anti-cancer treatments and almost every anti-cancer treatment tries to attack the tumors in its own special ways.
The hormone therapy for example is often used for tumors which derive from a hormone-influenced tissue, most notably known breast and prostate cancer, and their growth may be

hormone-dependent. Thereby, a possible positive effect could be achieved by administering antagonists or inhibitors of the thriving hormone. The idea is to alter the hormone production to reduce or to stop the tumor-growth or, as the best out-come, to kill it.

To trigger the body's own immune system to fight the cancer is achieved by immunotherapy. Hereby, it can be considered non-specific when the whole immune system is stimulated and improves cancer-fighting abilities but also specific when the immune system destroys well-directed only cancer cells (Alberts et al, 2007; American Cancer Society).
Using cancer-specific immune system cells scientists from the RIKEN research Centre for Allergy and Immunology, Japan, published in the journal *Cell Stem Cell* a research work with induced pluripotent stem cells (iPSCs). Mature T-lymphocytes, which targeted specifically skin cancer, were reprogrammed into iPSCs. Afterwards, the iPSCs turned into fully active cancer-specific T-lymphocytes which actively targeted and destroyed cancer cells (Vizcardo, Masuda et al, 2013).

Gene therapy tries to fight cancer on DNA-level. One idea is to replace damaged genes within cells with working genes and in this way to attack cancer on his „origin" - the DNA damage.
The other idea tries to fight cancer with a contrary method. The aim is based on introduced genes to damage the cancers DNA to such an extent that should trigger the cells to commit suicide.
However, this type of therapy has not yet resulted in successful treatments.

The most-known anti-cancer treatments are chemotherapy and radiation therapy and there is a wide spread diversity of chemo-drugs and also lots of possibilities in radiation therapy to treat cancer.

For both types of therapies the positive effect always depends on many factors, like the tumor size and location, how close the tumor is to radiation sensitive tissues or if the possibly used drug will be concentrated enough to cause positive effect.
For either both therapies, the general aim is to kill cancer cells by damaging the DNA to that extent, where the cells own signal pathways, like the p53 pathway, are triggered and the cells are forced to undergo apoptosis (Taylor A et al., 2004; Schulz-Ertner et al., 2006; National Cancer Institute; American Cancer Society).
The application of radiation therapy is carefully determined by a strict treatment plan to evade probable tumor counter effects and minimize common side effects. But nevertheless, in some cases the treated tumor shows resistances, due to former applications, or develops resistances during the actual treatment. It is shown also, that cancer cells derived from adult stem cells display a higher resistance level than cancer cells which are not of stem cell origin (Baumann et al., 2008; Ajani et al., 2009).
Chemotherapy, the application of „chemo-drugs“, often it is not only one, and how it works, is quite more extensive.
In general, the aim of chemotherapy-drugs is to damage cancer cells during their proliferating phase by using this typical behavior as an advantage for the treatment. The idea is to destroy cancer cells more effectively, while they are proliferating faster in comparison with healthy, normal cells.
And to obtain the maximum rate of possible cell death, chemo-drugs of different mechanisms of action and different dose-dependent toxicity-limits are given in individual combinations within the range of toxicity-tolerance of the patient.
Despite all this useful variety of chemo-drugs, a typical problem of this therapy is drug resistance.
„Repeated exposure of a tumor to a single anti-neoplastic agent will generally result in cross-resistance to the drug and agents of the same drug class as the original drug [...] whereas MDR1-gene (Multidrug resistance gene-1) expression presents one of the most important mechanisms of acquired drug resistance.“ (Malhotra & Perry, 2003).
As cancer are abnormal cells with a increased possibility for mutating the result of „repeated exposure“ to radiation or chemo-drugs leads to adaptations of the tumor and

further to resistant cancer cells. These cancers are nearly untreatable, only in high concentration of chemo and/or radiation, whereas the side effects and toxicity for the patient are raising.

Besides these common and specific side effects, there are still some other important aspects.
Neither both therapies are able to distinguish between normal cells and cancer cells. For this, every treatment will harm also healthy cells and force them to undergo apoptosis, too. Respectively this events occur in a lower frequency as for cancer cells. But still this presents a further burden for every patient.
Also radiation and chemo may induce other mutations within normal cells, which may lead to follow up cancer diseases. Examples for follow up cancers are acute myelogenous leukemia (AML) or acute lymphocytic leukemia (ALL) possibly induced either by radiation or chemotherapy (Krishnan et al., 2007; American Cancer Society).
And after all, chemo as well as radio therapy may fail in inducing apoptosis in some types of cancer when the p53 gene is already damaged and the control for DNA-damages caused by therapy applications will not induce the apoptotic signals.

The lack of differentiation between abnormal cancer and normal cells, the possible induction of follow up cancers and also the possible failure of therapy lead to the necessity to develop other cancer treatments with lower risks for the patients. To find alternative therapies, which may not harm the whole body in these dimensions, may be a hope for future cancer treatments. An aim of this present study is to test putative anti-cancer agents of a low-level to no detectible range of side effects.

2.4 Apoptosis

A wide range of cancer therapies try to fight cancer via the induction of apoptotic cell death.
Apoptosis is a natural end after every cells life, also known as programmed cell death. After splitting into two daughter cells and passing several checks of DNA control-screening/repair, cells undergo apoptosis. This very organized process is a control mechanism for the elimination of „old“, damaged or altered cells, without triggering the immune response of the organism (Earnshaw, 1995). This effect should help the organism to defend itself against bacterial or viral infections.
To maintain a balanced healthy organism, the control of cell death and the connected supervision of the cells, which should undergo apoptosis is as important as the regulation of cell proliferation (Thompson, 1995).

During an organism lifetime, cell death and building of new cells is part of all growth and development processes. For example during the embryonic state, the correct forming of tissues and organs is controlled, amongst other aspects, via apoptosis. In adults the cell death is a useful mechanism to supervise healthy and organized cell populations, where almost 10 trillion cells are produced every day, only to replace the ones, which died via apoptosis (Renehan et al., 2001)

The mechanism of apoptosis is complex and involves several cascading molecular events. The main components during cell death are the proteases called caspases. By the conversion of the pro-caspases into caspases, the apoptosis will be executed (Russo et al., 2006).
If and how a cell enters the apoptotic event, depends first on the signals, which trigger apoptosis, from the extracellular or the intracellular space. A distinction exists between the extrinsic and the intrinsic pathway, whereas both also activate different caspases to start the apoptotic process (Ashkenazi, 2002) (Figure 2).

The extrinsic pathway involves the cell surface located transmembrane death receptors (Locksley et al., 2001), members of the „tumor necrosis factor (TNF) receptor gene family“. Extrinsic ligands bind to these receptors and transduce signals into the cell which results ultimately in cell destruction (Elmore, 2007; Bazzoni & Beutler, 1996).
Until today, the most well characterized ligands of these receptors to date are FasL, TNF-alpha, Apo3L, and Apo2L and, among others,FasR, TNFR1, the corresponding receptors, respectively (Ashkenazi et al., 2008; Elmore, 2007). Once a ligand binds to its respective receptor, adaptor proteins inside the cell bind on the intracellular receptor domain, the „death domain“ (Wajant, 2002). This interaction of death receptor and intracellular proteins causes the conversion of pro-caspase 8, forming the death-inducing signaling complex (DISC) into active caspase 8 which leads to start the apoptotic process starts (Kischkel et al., 1995).

The intrinsic pathway, starting from the inside of the cell, different to the receptor mediated extrinsic pathway, involves intracellular signals which induce the release of pro-apoptotic proteins out of the intermembrane space of the mitochondria into the cytoplasm. The intrinsic pathway might be stimulated by cell damages caused by toxins, free radicals, radiation, damages to the DNA or also by viral infections (Elmore, 2007; Saelens et al., 2004). The p53 protein plays an important role in the intrinsic pathway and therefore, is in the focus of anti-cancer research and treatment development (Lane, 2004). The release of the pro-apoptotic proteins cytochrome c and Smac/DIABLO activates first the apoptotic protease activating factor Apaf-1, which oligomerizing forms the star-like apoptosome. Apaf-1, for its own, converts pro-caspase 9 into active caspase 9 and as a result starts the apoptotic process via intrinsic events (Du et al., 2000; Garrido et al., 2006; Elmore, 2007). The regulation of the pro-apoptotic events is part of the Bcl-2 protein family, while some of these may be anti- or pro-apoptotic. To the anti-apoptotic proteins, which are currently investigated as potential anticancer therapy targets, belong Bcl-2, Bcl-x, Bcl-x_L, Bcl-X_S, Bcl-w, BAG, the pro-apoptotic include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk. An upregulation or an activity increase might result in an option for cancer therapies via regulated induced apoptosis (Elmore, 2007).

The pro-apoptotic protein Bid demonstrates a connection between the extrinsic and intrinsic apoptosis pathway. Bid is cleaved by the caspase 8 during the extrinsic pathway and translocated into the mitochondria, where it induces the release of cytochrome c (starting point of the intrinsic pathway) (Esposti, 2002).

After the pro-caspase 8 of the extrinsic pathway as well as the pro-caspase 9 of the intrinsic pathway have been converted into their activated caspase forms, they both activate the caspase 3 (and also 6 and 7) which is thought to have the most important effect during apoptosis. Caspase 3 enters the nucleus and is responsible for DNA condensation, reorganization of the cytoskeleton, causing the apoptosis typical bulbed cell form, and the cells breaking apart into apoptotic bodies (Hengartner, 2000; Elmore, 2007).

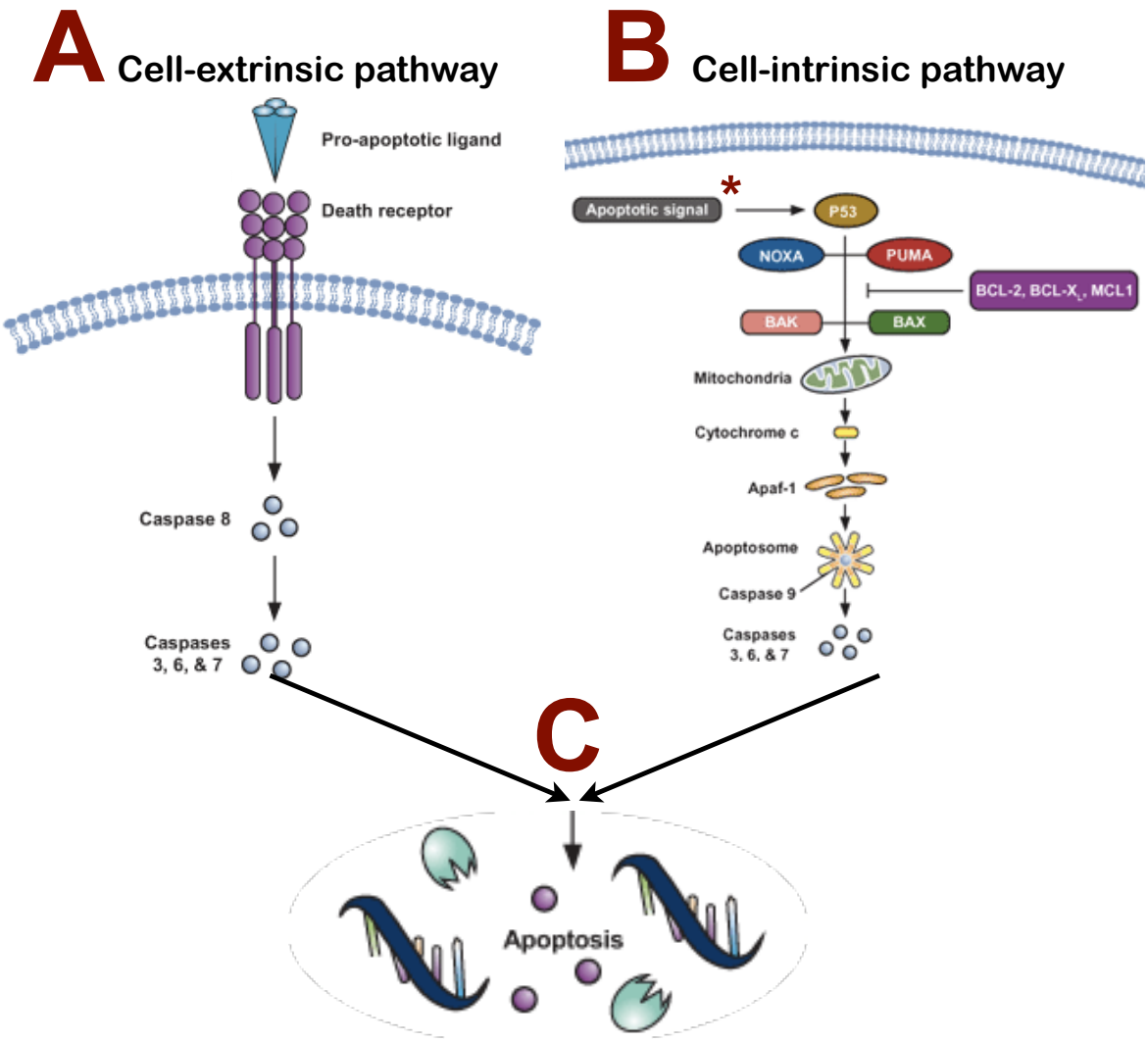

Figure 2: **Programmed Cell Death** - The main 2 apoptotic pathways with the involved elements, resulting in apoptosis.
A: The extrinsic pathway - A proapoptotic ligand binds to the death receptor TNF, resulting in the activation of caspase-8 and the initiation of the caspase cascade
B: The intrinsic pathway - Intracellular signals (*) activate the p53 tumor suppressor gene / members of the Bcl-2 family and induce the release of pro-apoptotic proteins (cytochrome c) into the intermembrane space of mitochondiras. Subsequently Apaf-1 is activated and stimulated caspase 9.
C: Apoptosis - DNA fragmentation due to the transduced caspase 3 into the nucleus and other apoptotic signals.
(based on: Ashkenazi et al., 2008; adapted from original figures: biooncology.com, Genentech USA, 2014)

As described, the programmed cell death is a well-organized, physiological process where, after all, signaling via extrinsic or intrinsic pathways the actual apoptotic process starts in the nucleus. During the first phase of apoptotic action alterations of the cell structure are visible via optic microscopy. The presented characteristics of the first state of cell degradation are cell shrinking which causes that the cytoplasm becomes dense and pyknosis. Pyknosis, from Greek, *pyknono* for „to condense“, describes the irreversible chromatin condensation. This process is known as a hallmark of apoptosis.
Afterwards the nuclear lamina becomes discontinuous, the DNA is fragmented and the nucleus breaks into nucleosomal units (Karyolysis). DNA fragmentation is one of the characteristics for apoptotic cell death and helps also to differentiate between ischemic or toxic cell death and apoptosis.
Subsequently to the nucleus separation in fragments, the cell shows, while continuously shrinking, irregular blebs, comparable to indentations around the cell center.
Finally, the cell breaks into several vesicles, also called apoptotic bodies, consisting of cytoplasm, organelles with or without nucleic fragments, surrounded by an completely intact cell membrane. Until the cell breaks apart, the intact membrane is another important characteristic for apoptosis. These apoptotic bodies are taken up via phagocytosis by macrophages and/or neighbor cells and finally, degraded without any further signal of immune response on the phagocytosis (Manjo & Joris, 1995; Kurosaka et al., 2003; Elmore, 2007;).

Cancer and other diseases, like neurodegenerative and autoimmune diseases are associated with an imbalanced relation between cell proliferation and cell death (Favarolo et al., 2012). Therefore, studying anti-cancer therapies based on inducing apoptosis agents and the application of these agents involves always to prove the apoptotic and necrotic function of the questioned agents.

2.5 Natural anti-cancer agents

Anti-tumor activity and inducing apoptosis are not only provided by designed drugs or specific anti-cancer treatments. There are many natural substances which show a scientifically proven anti-cancer activity. These substances can derive from plants, bacteria or fungi.
In this study two different types of natural potential anti-cancer agents were investigated for their anti-proliferative activity on cancer cells.

2.6 Surfactin

Surfactin is a biosurfactant lipopeptide, produced by *Bacillus subtilis*. Recently, the scientific interest in probiotics, defined as live microbial feed supplements that can benefit the host's health and immune system by improving its intestinal balance (Fuller, 1991; Lee et al., 2011), arise and attracts many scientists for several research reasons. In Asia many food preservation processes, like fermentation, involve the use of such probiotic cultures of *Bacillus subtilis*.
Lee and coworkers published a research work about the lipopeptide surfactin produced by the probiotic *Bacillus subtilis* strain CSY191, isolated from „doenjang" (traditional korean fermented soybean paste). During the fermentation process, CSY191 produces high amounts of surfactin, which are consumed with the soybean paste. Tests with this isolated surfactin show that growth of human breast-cancer cells MCF-7 is inhibited in a dose-dependent manner (Lee et al., 2011). Other experiments with surfactin show its inhibitory effect on to NF-κB activation (Byeon et al., 2008).
The search for anti-cancer agents also turned into analysis of biosurfactants, whereas the biosurfactant family produced by *Bacillus* species (Wang et al., 2007; Byeon et al., 2008; Cameotra et al., 2004; Singh et al., 2004; Bessler et al., 2003) received an increasing interest because of their proved anti-tumor activity (Cao et al., 2009; Cao et al., 2009; Cao et al., 2010).

Surfactin derived from *B. subtilis* and other *Bacilli,* is a cyclic lipopeptide with a molecular mass of 1,036 Da that presents a high resistance to heat, cold and steric influences. It contains a β-hydroxy fatty acid of variable chain length of 13-15 carbon atoms and seven α amino acids. The amino acid chain can vary in its sequence, whereas the surfactin can be classified into 3 isoforms, surfactin A, surfactin B and surfactin C (Figure 3).
It possesses an amphiphilic structure, a hydrophilic ring of amino acids and a hydrophobic chain of fatty acids. This helps the biocin to be soluble in aqueous solutions as well as in lipid bilayer membranes (Izadpanah & Gallo, 2005). Under natural conditions surfactin is produced within a mixture of different isomers. The composition of this cyclolipopetide mixture depends on external factors, like the bacteria growth medium or physical-chemical factors (Kluge et al., 1988) but also on certain stress factors and strain qualities. The production of surfactin and its isomers begins during the logarithmic growth stage and extends over the complete growth stage, whereas the expression is regulated by the culture density and not by the cell cycle (Breukink & De Kruijff, 1999).

Surfactin A	CH_3-$(CH_2)_{10\text{-}12}$-CH-CH_2-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu O
Surfactin B	L-Val
Surfactin C	L-Ile

Figure 3: **Cyclolipopeptide surfactin A** - surfactin A and isoforms B and C with alterations within the aminoacid circle (L-Val for surfactin B and L-Ile for surfactin C)
(adapted from original figure: „Peptide und Lipopeptide aus *Bacillus subtilis* und *Bacillus brevis*“, Andrea Sokoll, doctoral dissertation)

Surfactin shows activity like anti-viral (Vollenbroich, Ozel, Vater, Kamp & Pauli, 1997) and anti-microbial (growth inhibition of microorganisms) (Cho et al., 2003), whereby the bacteriocin excreting bacteria is not affected by the anti-microbial activity by possessing specific immune mechanisms (Klaenhamer, 1988; Caplice & Fitzgerald, 1999; Sablon et al., 2000; Cotter et al., 2005).

For clinical research questions bacteriocins have already been proposed as an alternative for disease control, because of the treatments effect and non-toxicity to humans and animals (Motta et al, 2007; Oliveira et al. 1998; Twomey et al. 2000).

Also, surfactin and its isomers receive increasing clinical interest due to their lack of toxicity for higher organisms and its antimicrobial activity against pathogens and multi-resistant pathogens (Niu & Neu, 1991).
The apoptotic function of surfactin can be put down to its incorporation into the phospholipid bilayer membranes where it forms ion channels and induces target cell permeabilization and perturbation due to its amphiphilic nature (McElhaney & Prenner, 1999;Wang et al., 2007).
surfactin was already characterized by an anti-cancer activity test against human breast cancer cells MCF-7 (Lee et al., 2011), LoVo (human colon cancer cells) (Kim et al., 2007) and also human leukemia K562 cells (Wang et al., 2007). The use of surfactin presented an increasing dose dependent apoptotic activity in reaction with cancer cells. However, the apoptotic activity of surfactin is still not sufficiently tested in normal cell lines, to investigate a selective activity for normal and mutated cells. Based on these results and the characteristics for surfactin, the two similar lipopeptides fengycin and iturin A were analyzed for their anti-tumor activity and their activity on normal human fibroblasts.

2.7 Iturin

As already mentioned, not all *Bacillus* strains produce equal amounts of every cyclolipopeptide. Some strains show an enhanced production of surfactin, while other strains can produce elevated amounts of similar lipopeptides like iturin A or fengycin (Figure 4).

A *B. amyloliquefaciens* strain isolated from the woodlands of southern Brazil was characterized in our laboratory and labeled LBM 5006 (Lisboa et al., 2006). This strain

demonstrates a very similar biochemical behavior to the surfactin-producing *B. subtilis*. Nevertheless the two species can be distinguished by the higher G+C content and the increased production of α-amylases by *B. amyloliquefaciens*.

Further characterization of LBM 5006 showed that the organism produces an antimicrobial substance that inhibits microbial growth of *B. cereus* and *L. monocytogenes*. *B. amyloliquefaciens* is known to produce iturins (Hidrate et al., 2002) and based on the properties of the antimicrobial substance it was assumed that LBM 5006 produces iturins (Lisboa et al., 2006).

Further studies confirmed that two antimicrobial peptides are produced by strain LBM5006: a major iturin-like peptide has a molecule mass around 1058 Da and a fengycin-like peptide of around 1464 Da (Benitez et al., 2010). These peptides presented heat stability for temperatures higher than 80°C (exposure time 30 min) but were inactivated by autoclaving (121°C for 15 min). They showed resistance to all proteolytic enzymes tested (papain, trypsin, proteinase K and pronase E). Also, PCR analysis showed the presence of the *ituD* gene, essentially for iturin production (Benitez et al., 2010; Tsuge et al., 2001).

The antifungal activity of the strain LBM 5006 was also investigated resulting in growth inhibition of filamentous fungi such as *Aspergillus niger* ATCC16404, *Aspergillus flavus*, *Aspergillus phoenicis*, *Diplodia sp.*, *Bipolaris sorokiniana* and *Apiosordaria sp* (Benitez et al., 2010).

The apoptotic activity of the iturins produced by *B. amyloliquefaciens* on cancer cells and normal human cells has not been investigated yet. Thus, the present study should give an insight on the activity of the LBM5006 iturin on cancer cells and normal human fibroblasts.

The basis for this study are the previous results for surfactins which was shown to induce apoptosis and cell cycle arrest in cancer cells (McElhaney & Prenner, 1999;Wang et al., 2007; Kim et al., 2007; Lee et al., 2011;) and the fact that iturin is one of the cyclolipopeptides produced by *Bacillus* most related with surfactin. The probability to

achieve positive results with a highly related cyclolipopeptide are high, but also the use of a chemically related molecule may show different activities, either negative or positive. The LBM 5006 iturin already showed positive results against important pathogens in antimicrobial and antifungal tests (Benitez et al. 2010). The investigation on its anti-cancer or in general on its anti-proliferating activity in cells might bring an additional useful function for this lipopeptide and also might represent new possibilities for anti-cancer treatments.

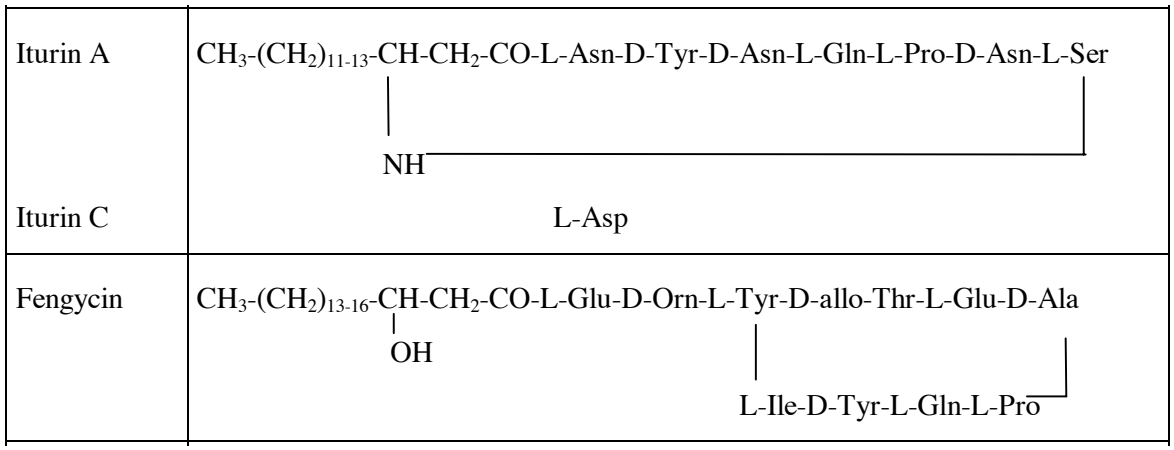

Figure 4: **Structures of cyclolipopeptides iturin A, iturin C and fengycin** -
A: Iturin A and its isomer iturin C (aleration in the aminoacid circle L-Asp)
B: Fengycin
(adapted from original figure: „Peptide und Lipopeptide aus *Bacillus subtilis* und *Bacillus brevis*“, Andrea Sokoll, doctoral dissertation)

2.8 Fengycin

The other investigated lipopeptide is also structurally related to surfactin, called fengycin. The fengycin producing bacterium in this present study was a newly discovered microorganism, isolated from the intestinal content of a teleost fish „Piau-com-pinta“ (*Leporinus sp*.) of the Amazonas basin. The isolate was identified as a *Bacillus spp.* (Motta et al., 2007) and was designated as strain P34. Phylogenetic analysis revealed that P34 shared low similarity with most *Bacillus* species but high similarity (91%) with *B. infernus.* Further, analysis of the physiological properties showed essential differences between *Bacillus sp*. P34 and the *B. infernus*, which has the capability to proliferate under heat conditions and anaerobic culture (Boone et al. 1995). This suggests that P34 is a new *Bacillus species* (Stackebrandt and Goebel 1994; Palys et al. 1997; Goto et al. 2000; Motta et al. 2007).

Also, like surfactins and iturins, the P34 antimicrobal substance inhibited microbial growth of gram-positive bacteria like *Corynebacterium fimi, Bacillus cereus, Listeria monocytogenes, and Lactobacillus acidophilus* (Motta et al., 2007).

Testing the resistance against proteolytic enzymes suggested that the antimicrobial substance of P34 might be a cyclic peptide. Further, experiments showed heat stability up to 100°C for 15min (Motta et al., 2007). Purification and characterization revealed that the substance is a lipopeptide of 1456 Da which match with the molecular mass for fengycin isomers and their different fatty acid chain length (Benitez et al., 2010; Hu et al., 2007).

Fengycin and its isoforms are like iturins, related to surfactin, which was proven to induce apoptosis and cell cycle arrest in cancer cells (McElhaney & Prenner, 1999;Wang et al., 2007; Kim et al., 2007; Lee et al., 2011). Based on the same assumptions, which were taken for the LBM5006 iturin, the P34 fengycin is investigated in this present study for its apoptosis inducing activity in cancer cells. This outcome might represent one more possible anti-cancer treatment.

2.9 The VP3 protein of the *avian girovirus 2*

In 2011 Dr. Rijsewijk and his coworkers detected by analyzing samples of ill poultry production chicken, taken for clinical studies to examine the apathy and loss of weight, a new type of gyrovirus to which the name *avian gyrovirus* II (AGV II) was proposed (Rijsewijk et al., 2011). In chicken this virus may be associated to symptoms like anemia and is, as it was proven, seem to be widespread, like the chicken anemia virus CAV (Dos Santos et al., 2012).

CAV was discovered by Yuasa and his coworkers in 1979 and is known as a widespread infectious virus (Yuasa et al., 1979). Chickens, infected with the CAV virus, present symptoms among others of anorexia, anemia, immunosuppression and may also die (Zanella & Brenato, 2012). During the investigation of the CAV proteins it was shown, that the CAV VP3 protein, expressed *in vitro* in absence of the other CAV proteins, is responsible for the induction of apoptosis. This led to the proteins nomination „apoptin“ (Noteborn et al., 1994). Because of the high identity between CAV apoptin and AGVII VP3 amino acid sequences it is assumed that the VP3 may also induce apoptosis, similar as apoptin.

Since 1997 it was also proven that the apoptin protein was capable to induce cell death in various human cancer cell lines like osteosarcomas, melanomas, lymphomas, breast cancer, lung-cancer, and gliomas. It could also be demonstrated that normal human cells, like firboblasts, endothelian cells and blood stem cells are not affected by the apoptotic effect of apoptin (Danen van Oorschot et al., 1997; Noteborn et al., 1998; Backendorf et al., 2008; Li et al., 2012; An et al., 2013). But if the apoptin was inoculated with human fibroblasts transformed with the SV40 virus, which is known as cell-DNA transforming and cancer inducing, the fibroblasts entered in apoptosis (Danen van Oorschot et al., 1997; Zhang et al., 2004). Further, according to Backendorf and his coworkers, this indicated the capability of apoptin to identify the initial oncogenic transformation and also that the apoptin‘s activity depends on the oncogenic transformation (Backendorf et al., 2008).

In vivo experiments, where mice received implants of human tumor-cells and were further treated with apoptin protein, intra-tumoral injected or were expressed via vectors. This treatment resulted in a significant tumor shrinking and in some cases in a complete tumor regression. While no healthy tissues around the tumors area were affected negatively, it was assumed that the CAV apoptin reacts as an efficient anti-tumor agent (Pietersen et al., 1999; Sun et al., 2009; Van der Eb et al., 2002; Pan et al., 2010; Li et al., 2012).
Also, as already proven for other treatments, when cancers are treated with a combination of anti-neoplastics with apoptin a significant higher tumor regression is observed, in comparison to the separate use of these agents (Olijslagers et al., 2007; Liu et al., 2008; Jin et al., 2011; Fang et al., 2012; Yuan et al., 2013).
But one of the most important characteristics of the apoptin was described by Zhuang and his coworkers as well as by Li and his coworkers (Li et al., 2010; Zhuang et al., 1995). The CAV VP3 is capable of inducing cell death in cancer cells, without the p53 protein. This opened a wide range in the field of anti-cancer treatments for possible applications. There are already recognized tumor cell lines which present mutations in the p53 gene and therefore are not capable of entering the apoptosis pathway on their own (Berglind et al., 2008). Further, it was demonstrated that inhibitory proteins of the p53 induced apoptosis do not act negatively on the apoptin activity (Danen van Oorschot et al., 1997; Schoop et al., 2004). Other results showed a positive effect of the Bcl-2 gene over-expression on the apoptin (Danen van Oorschot et al., 1999). Under normal conditions the Bcl-2 gene inhibits the apoptotic activity of the tumor-suppressor p53 protein. In some cancers the Bcl-2 proto-oncogene suffered mutations which lead to an abnormal over-expression of this gene and further this may inhibit and stop the via the p53 protein induced apoptosis. The loss of function of the p53 gene via mutations and the gain of function of the over-expressed Bcl-2 gene are frequently occurring alterations in tumor cell lines which also might result in an increased resistance against common used anti-cancer agents (Liu, 2009).
These characteristics of not affecting normal healthy cells, inducing apoptosis also in the presence of inactive or non functional *TP53* gene and the Bcl-2-enhanced apoptotic activity of the apoptin, bring up the possibility of a new efficient, non-toxic anti-cancer

agent which may also be used for treatments of cancer diseases which do not react on nowadays common cancer treatments.

Until today the differentiating mechanism of apoptin for normal or abnormal cells (tumor, transformed cells) is not completely elucidated. But nevertheless, a different subcellular localization has been described for apoptin in normal and abnormal cells. Danen van Oorschot, Los and Sun already demonstrated that the CAV apoptin is localized in the nucleus of cancer cells whereas in normal cells, it is in the cytoplasm (Danen van Oorschot et al., 1997; Danen van Oorschot et al., 2003; Sun et al., 2009; Los et al., 2009). The functional domains of the apoptin like Leucine rich sequence (LRS), Nuclear export signal (NES) and Nuclear localization signal (NLS) sequences, described later in this chapter, help to explain certain reactions within the cells. Apoptin is described as a macromolecule by his protein size of 121 amino acids and therefore it needs to be actively transported into or out of the nucleus by special transporting proteins. Poon and his coworkers could obtain results for the Importin IMPβ1 mediated apoptin transport into the nucleus via the nuclear pore complex (NPC), whereas the apoptins NLS sequence interacted with the importin. Further, it was proven that during this transport, the importin/apoptin complex enters the nuclear pore and apoptin is sent into the nucleus, while the importin returns back to the cytoplasm side of the pore (Poon et al., 2005; Ding et al., 2010). But not only the nuclear localization of the apoptin induces the cell death in cells. When the viral protein was forced into the nucleus of normal cells, neither apoptotic signals nor apoptosis could be detected. This indicates that the apoptins activity is not directly connected with its localization, and it necessitates other abnormal cell signals to stimulate its pro-apoptotic activity (Danen van Oorschot et al., 2003; Guelen et al., 2004).
Where importin IMPβ1 is responsible for the transport of apoptin into the nucleus via the NPC, the exportin CRM1 is responsible for the export into the cytoplasm (Poon et al., 2005; Maddika et al., 2006). This led to the assumption that the apoptin, both in normal and abnormal cells is first transported into the nucleus via importin and, only in the normal cells, exported via CRM1 to the cytoplasm again, where the protein suffers proteosomic degradation (Lanz et al., 2012). It is thought, that the proteins accumulation in the nucleus

is based on the disguise of the NES sequence by CDK2 thrived phosphorylation of threonine-108 (Poon et al., 2005; Maddika et al., 2006).

The cell death is caused via the intrinsic pathway (also see intrinsic pathway), beginning with the release of cytochrome c of the intermembrane space. As already mentioned, the cytochrome c release further activates Apaf-1, which activates caspase 9 and with this starts the caspase cascade (Danen van Oorschot, van der Eb, Noteborn, 2000; Elmore, 2007). As many cancer types are directly connected with a loss of function of the DNA-damage control gene *TP53* (Nigro et al., 1989), which is responsible for the initiation of the intrinsic pathway, questions remain about the apoptins interaction within the nucleus of the tumor cells and further which signaling pathways are activated past the apoptosis initiation.

Through the cellular localization of the apoptin in the nucleus of cancer cells, it is thought, that the protein´s N- and C-termini interact with the DNA and also with various pro-apoptotic proteins (Bcl-10, FADD, DEDAF) which might clarify the induction of apoptosis (Leliveld et al.,2004, Zhou et al., 2012). Based on these investigations, it could be demonstrated that the initiation does not exclusively depend on one type of signaling but involves a combination of various reactions, like the influx or eflux of proteins in /out of the nucleus or molecular interactions (Zhou et al., 2012).
The effect of imbalanced protein concentrations within cancerous cells was also associated with apoptin. For example, it was shown that in tumor cells the apoptin favored the stability of TAp73, a member of the p53 related proteins and known as a downstream target of the p53 protein within the apoptotic signaling. At the same time apoptin induces degradation of ΔNp73, the dominant-negative inhibitor of p53 and TAp73. These alterations lead to activation of the pro-apoptotic modulator PUMA which further results in the induction of apoptosis independent of the *TP53* gene (Taebunpakul et al., 2012).

2.10 Amino acid sequence and structure of CAV apoptin

As already mentioned, the apoptin has several functional domains. Within the 121 amino acids, between amino acid nr. 33 and 46 there is a hydrophobic leucine rich sequence (LRS), which is thought to be responsible for the interaction with proteins. Further, on the C-terminus are found first a NLS sequence divided in two parts (1st part between 82-88 and 2nd part 111-121). In between the two NLS sequence-parts, at amino acid nr. 97-105, a NES sequence is located. In addition, high numbers of serine and threonine as well as Proline-rich or basic regions are found at the C-terminus end of the apoptin (Noteborn, 2005). At spot 108 of the amino acid sequence, a threonine is located which presented to be an accessible spot for phosphorylation (Maddika et al, 2006). This phosphorylation spot permits interaction with other nucleus located proteins and modifications via quinases. Until the discovery of the AGV II VP3 and its sequencing of the complete amino acid sequence, the CAV apoptin did not demonstrate any homology to other proteins. So far it can be said, that the VP3 of the AGV II is the first described homologous protein to the CAV apoptin.

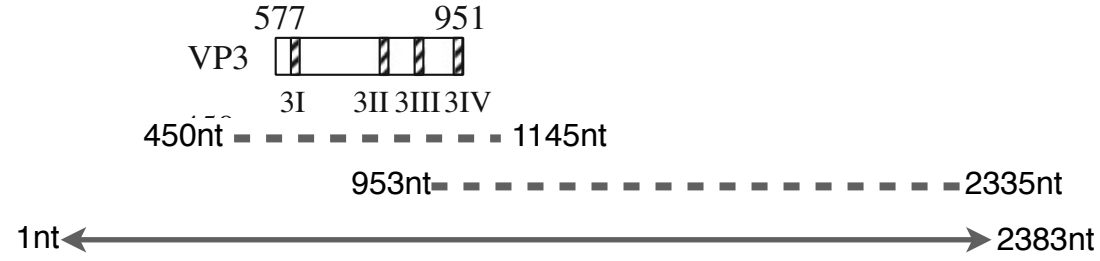

Figure 5: **Schematic representation of the genomic organization of the AGV II** - The horizontal line in the lower part represents the 2383nt long AGV II genome. The arrows at the end indicate the closed circular genome structure. The numbers besides the bars indicate the nucleotides. The broken line from 953nt to 2335nt displays the VP1 coding gene, the broken line from 450nt to 1145nt the VP2 coding gene.
The horizontal bar represents the VP3 gene from 577nt to 951nt. The hatched regions within the bars indicate the positions of conserved motifs. Within VP3: 3I = isoleucine stretch, 3II = NLS1, 3III = NES and 3IV = NLS2.
(adapted from the original figure of Rijsewijk et al., 2011)

2.11 The *Avian Gyrovirus* VP3 protein

In 2011 the AGV II was described and analyzed by Dr. Rijsewijk and his collegues (Rijsewijk et al., 2011).
The AGV II virus has a circular genome of a single strand DNA of 2383 nucleotides. By analyzing the sequence, a 40% identity of the whole genome to the CAV genome appeared. Also the virus´ organization is similar to the CAV.
The analysis of the nucleotide sequence identified three open reading frames (ORFs), partially overlapping, which are coding homologous proteins to the CAV VP1, VP2 e VP3. Comparing the amino acid sequences of the AGV II proteins with the encoded CAV peptides, the ORF1 codified VP2 protein of AVG II shows 40.3% of amino acid identity. VP2 is thought to be a dual-specificity protein phosphatase, playing a role in virulence, viral replication and cytopathology.
38.8% similarity to the CAV counterpart is shown by the ORF3 expressed VP1 protein. Its arginine and lysine rich region at the very start of the protein is typical for viral capsid proteins, and three conserved replication motifs could be identified (Figure 5).

And finally, ORF2 is coding the protein VP3 of 124 nucleotides, 3 nt longer than the CAV VP3, with 32.2% identity to the CAV apoptin. Within the VP3 protein, some in CAV VP3 conserved amino acid sequences were identified (Rijsewijk et al., 2011).
First there is a short hydrophobic isoleucine stretch (also LRS) between the residues 38-51 in the N-terminal region. This stretch is important for the binding with cellular proteins (Heilman et al., 2006). Also a in two parts divided NLS (nuclear localization signal) sequence (NLS1 and NLS2), like in CAV, was detected between the residues 84-88 and 120-121, respectively. Between these two NLS parts a NES (nuclear export signal) sequence was clearly conserved between residues 97-105.

To this day, the VP3 has not been examined as much as the CAV apoptin, but as the VP3 protein shows up to 32.2% identity of the functional domains of apoptin and also the VP3

is considered to be the first homologous protein for the apoptin, all expectations for AGV II VP3 may be based on the CAV apoptin and the previously obtained results.

2.12 Anti-proliferative activity tests

The two antimicrobial lipopeptides from different *Bacillus* strains, iturin A and fengycin, and two isoforms of the viral peptide VP3 from the *Avian Gyrovirus* II were tested for their anti-proliferative activity on human cancer cells and normal fibroblasts to investigate their potential anti-cancer effect.

Heretofore, for the lipopeptides and viral protein isoforms tested in this study no detailed studies for their anti-cancer activities have been found in literature yet. As references for the investigated bacterial and viral proteins serve the homologous peptides, surfactin for iturin A and fengycin, and apoptin of the chicken anemia virus (CAV) for the AGV II VP3. This connection helps to understand and predict a possible anti-tumor function (Wang et al., 2007; Cao et al., 2009; Cao et al., 2010; Kim et al., 2007; Noteborn et al., 1994, Danen van Oorschot et al., 1997; Noteborn et al., 1998).

3. Materials and Methods

3.1 Cultivation and storage of *Bacillus* strains

Bacillus cereus, used as the indicator bacteria for the anti-microbiotic tests, was cultivated on BHI-agar plates ON at 37°C and stored at 4°C for further experiments.
The strains *B. amyloliquefaciens* LBM 5006 (Lisboa et al., 2006), for iturin A production, and *Bacillus sp*. P34 (Motta et al., 2007), for fengycin production, were directly inoculated in 25 mL liquid BHI medium for 24h at 37°C from general glycerol stocks. Following, the cultures were spinned down in 1 mL aliquots for 5 min at 6000 x *g* in an EPPENDORF centrifuge 5415 R, half of the supernatant was aspirated and the remaining culture was mixed with 60% sterile glycerol 1:1. These glycerol stocks were stored at -20°C and used directly for further pre-cultures. The strains were stored under these conditions as it turned out to keep a necessary raised stress level for lipopeptide production.

3.2 Cultivation conditions for enhanced iturin A and fengycin production

For the enhanced biosurfactant production by *B. amyloliquefaciens* LBM 5006 and *Bacillus sp.* P34, the strains were cultivated in two different media.
First, for the inoculation for pre-cultures of each strain, 100 µL of glycerol stock culture (-20°C) were transferred directly to 25 mL BHI medium and incubated at 37°C and 190-200 rpm for 10-18 h.
To test the production media, 1% of the precultures were inoculated either in BHI (reference) or in composed ferrous ion medium containing 1% peptone, 0.1% yeast extract, 0.01% $CaCO_3$, 0.01% NaCl, 2% soft sugar, 0.2 mM $FeSO_4$ at pH 6.64. The ferrous sulfate was added after autoclaving (Lin et al., 2007). The cultures were inoculated at 30°C for 144 h (6 d) at 190-200 rpm.

All culture supernatants were obtained by centrifuging at 9.000 x *g,* for 15 min at 4°C in a Thermo Scientific Heraeus Megafuge 16R centrifuge. Volumes above 150 mL were centrifuged in a HITACHI High-Speed Refrigerated Centrifuge CR 21GIII.

3.3 Culture supernatant precipitation for iturin A or fengycin

To concentrate the iturin A and fengycin and to eliminate contaminating proteins, the previously obtained culture supernatants were precipitated with ammonium sulfate ($(NH_4)_2SO_4$) at 4°C to a 20% $(NH_4)_2SO_4$ final concentration (Motta et al., 2007). Protein precipitation was done by using ammonium sulfate. Previous tests (Motta et al., 2007; Lisboa et al., 2006) showed that fengycin as well as iturin A precipitate at a final concentration of 20% ammonium sulfate.

As the calculation of the correct $(NH_4)_2SO_4$ amount depends on the sample volume, the temperature, and also on the increasing sample volume by adding solid ammonium sulfate, the EnCor Biotechnology Inc. ammonium sulfate calculator, available online (*http://encorbio.com/protocols/AM-SO4.htm*), was used to calculate the specific $(NH_4)_2SO_4$ amounts for every sample. First the ammonium sulfate was added continuously in small amounts under permanent stirring and further mixed for one additional hour on ice. The complete salting out reaction of iturin A and fengycin was obtained by incubating the samples for 24 h at 4°C. Afterwards the samples were centrifuged in max. 40 mL fractions at 12.000-12.400 x *g* to pellet the precipitated iturin A or fengycin, respectively. Each pellet was immediately dissolved in 1 mL fresh 10 mM phosphate buffer (Na_2HPO_4) at pH 7.0 and stored at -20°C for further purification steps via Size Exclusion Chromatography (SEC).

3.4 iturin A and fengycin purification via size exclusion chromatography

For further purification of the iturin A or fengycin, the 1 mL buffer-resuspended lipopeptides were applied to a size exclusion chromatography (also known as gel filtration) column.

To achieve protein separation for each lipopeptide sample, two glass columns (35 cm height, 1 cm diameter) were packed with 10 mL of Sephadex G-100 resin (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and equilibrated each with 3x column volume (CV) of 10 mM phosphate buffer, pH 7.0.

Each iturin A or fengycin sample was applied to one of the columns, and eluted with phosphate buffer; eluting proteins were collected in 1 mL fractions. Fractions were monitored by measuring the protein content via spectrophotometry at 280nm.

The elution was stopped, when the absorbance returned to +/- 0.0, followed by column washing with 3x CV 10 mM phosphate buffer. After every third purification, the resin was cleaned by washing with 0.2 M NaOH (3 CV), subsequent washing with sterile ultrapure H_2O (Milli Q) and equilibrated in 10 mM phosphate buffer pH 7.0 for further purifications. The collected fractions with the highest absorbance values were further analyzed for their antimicrobial activity against *B. cereus*.

3.5 Lyophilization and final protein quantification for iturin A and fengycin

SEC fractions, positive for growth inhibition of *B. cereus,* were pooled and lyophilized (Freeze Dryer, Labconco®).

After the lipopeptide samples were completely freeze-dried, they were resuspended in 1 mL 10 mM phosphate buffer pH 7.0 and analyzed for their protein contents at 280 nm. The measured absorbance values were converted, based on the BSA equation curve (Figure 6) and the resulting linear regression equation y = 0,5558*x, and the protein samples quantified (mg/mL).

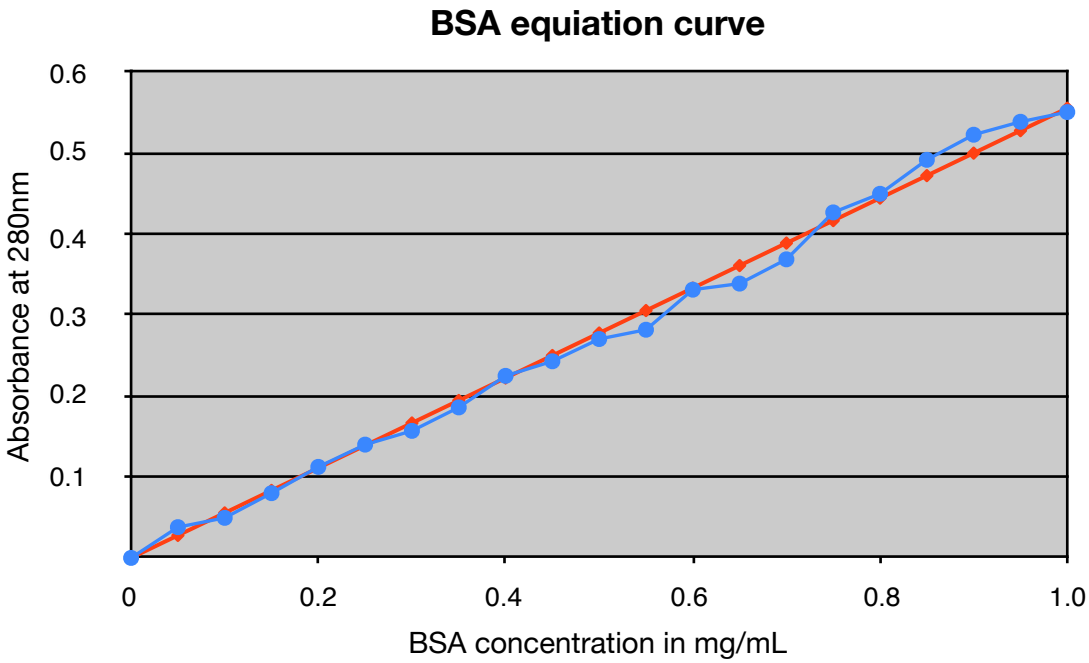

Figure 6: **BSA equiation curve for protein quantity determination** -
blue graph: Avarage values of 3 samples, drawn against the aborbance value at 280nm
red graph: linear regression line of the BSA graph

3.6 Antimicrobial activity test of iturin A and fengycin

To test the antimicrobial activity of the purified and pooled samples of iturin A and fengycin, a modified "critical dilution assay“ was performed, adapted from the standard protocol of A. Mayr-Harting (Mayr-Harting et al., 1972).

On BHI-agar plates a very thin uniform layer of the indicator bacteria *B. cereus* resuspended in saline solution (0.85% NaCl), with an optical density of OD_{600} = 0.6 was seeded and let dry under a hood. For further use, the plates were divided in equal areas.

To define the arbitraic units of the lyophilized lipopeptides, iturin A or fengycin, 10 µL of every sample were dropped into an area on the indicator plate.

Serial dilutions (*up to* $1:2^{10}$) of the iturin A and fengycin samples were prepared in saline solution.

Following single uniform drops of 10 µL of each lipopeptide sample and every dilution were put onto the indicator plates, in which each drop was placed within one area.

All closed plates were dried under a hood and were incubated at 37°C overnight.

The activity units (AU) were calculated based on the growth inhibition of the lowest concentration (Mayr-Harting et al., 1972).

3.7 Cultivation and preparation of *E. coli* strains

The *E. coli* strains used in this study, DH5α for plasmid transformation and BL21 (DE3) pLysS for induced protein expression of toxic proteins, were cultivated in Luria Bertani (LB) medium with antibiotics for general cultivation purposes and stored on LB-agar plates with antibiotics, after incubating for 24 h at 37°C, at 4°C.
For selection of positive transformed colonies the bacterias DH5α and BL21 (DE3) pLysS, transformed with the pET SUMO-PTD4-VP3 constructs, were grown on LB-Agar plates with 50 µg/mL of kanamycin, added to all cultivation mediums. Respectively, for the BL21 (DE3) pLysS strain the final selective antibiotic concentration of 34 µg/mL, on account of the pLyS plasmid, is added additionally to all mediums after autoclaving.

3.8 Transformation of the BL21(DE3) pLysS with pET SUMO-PTD4-VP3

Via a PCR with the appropriate primers, the VP3 gene of the AGV II virus of two different isolates, isolate **Thais** (T) and isolate **Santa Maria** (SM), were amplified. For the amplification of AGV2 VP3 (T) and (SM) the following primers were used:
forward primer: 5'-TATGCCCGCGCGGCAGCACGACAAGCTCGAGCCCAGACCCCCCGCTCTCGCCGAC-3' and reverse primer: 5'-TCACAGTCTTAGTTTTTTATGGGG-3'; designed based on the nucleotide sequence of AGV2 VP3 gene. The protein transduction domain 4 (PTD4) is fused N-terminally to both forward primers (sequence:TATGCCCGCGCGGCAGCACGACAAGCTCGAGCC). The amplicon has a size of 405bp. The fusion between PTD4 and VP3 is expected to facilitate the transport into the cells and the nuclear localization of VP3, as it was proven for the PTD4-apoptin

fusion protein by Sun (Sun et al., 2009). Afterwards the PTD4-VP3 PCR constructs were cloned into the pET SUMO *empty* vector, which obtains a strong T7*lac* promoter. The PCRs and final pET SUMO PTD4-VP3 (T) and (SM) (also designed as 6xHIS SUMO PTD4 VP3 (T) and (SM)) constructs were performed by Marcus Braga Knack (Knack et al., 2011).

The plasmids were transformed into chemo-competent *E. coli* DH5α cells for plasmid reproduction and storage. The cultures were cultivated as described elsewhere (see **2.5 Cultivation and preparation of *E. coli* strains**).

After sequencing and analyzing the DNA sequences of positive selected colonies, two different constructs, due to DNA sequence differences of the two isolated AGV II T and SM samples, were obtained. Therefore chemo-competent *E. coli* BL21 (DE3) pLysS cells were transformed with one pET SUMO PTD4-VP3 construct and the obtained cultures cultivated as described elsewhere (see **2.5 Cultivation and preparation of *E. coli* strains**). The transformation was performed after standard protocols for chemo-competent cells transformation .

The obtained colonies, grown on selective LB medium agar plates, were further analyzed via colony PCR (in 20µL reaction: 14.0 µL H2O, 2 µL 10x PCR standard buffer, 1.0 µL 50mM $MgCl_2$, 2.0 µL 10mM dNTPs, 0.5 µL 10pM of each primer and 0.25 µL Taq Polymerase) with the appropriate primers for the VP3 gene and the vector backbone. The used forward primer anneals on the VP3 gene: 5´-GATGCTCGTATGCCGTTAATAG-3´, the reverse primer anneals on the vector backbone: 5´-TAGTTATTGCTCAGCGGTGG-3´. The amplicon has a size of 476bp.

3.9 IPTG induced protein overexpression of PTD4-VP3 (T) and PTD4-VP3 (SM) in *E. coli* BL21 (DE3) pLysS cells

BL21 (DE3) pLysS colonies, tested positive for the pET SUMO PTD4-VP3 (T) or pET SUMO PTD4-VP3 (SM) construct, were used for induced protein overexpression with isopropyl β-D-1-thiogalactopyranoside (IPTG).

For this, precultures of each PTD4-VP3 construct were inoculated in 25 mL LB medium with the appropriate antibiotics (50 µg/mL kanamycin; 34 µg/mL chloramphenicol) for 10-18 h at 37°C and 180-200 rpm.
Subsequently 1% preculture was inoculated in fresh liquid LB medium without antibiotics for induced overexpression. The cultures were cultivated at 37°C and 180-200 rpm until an OD_{600} of 0.4-0.6 was reached. The protein overexpression was induced with IPTG (final concentration 1 mM). After induction with IPTG the overexpression cultures were further cultivated under different conditions: 12°C, 180 rpm for 10-12 h.
The cultures were harvested by centrifugation at 12,000 x *g* for 10min at 4°C and immediately stored at -20°C for further purifications. Samples for SDS-PAGE analyzes of the non-induced and induced cultures were stored separately.

3.10 Purification of the SUMO-PTD4-VP3 (T) and (SM) constructs via immobilized metal ion affinity chromatography (IMAC) with nickel ions

The purifications of the pET SUMO-PTD4-VP3 (T) and (SM) constructs were performed with the Pro Bond purification Kit (Invitrogen). The columns were prepared as described in the kit manual and equilibrated in native binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0)
The following described sample preparation and also the protein purification were carried out on ice.
The stored overexpression culture pellets were gently resuspended in 4°C cold native binding buffer in a weight to volume ratio of 1:2, in a final volume of 6 mL. Further, cell lysis was carried out on ice by ultrasound, treating the solution for 20 seconds at high intensity (20 kHz, 250 W). Subsequently the lysate was centrifuged at 3000 x *g,* at 4°C for 15 min and the supernatant, containing the soluble 6xHIS-tagged SUMO-VP3 (T) or (SM) protein, separated from the remaining cellular debris.
The purification was carried out based on the manuals instructions with adaptations. The pre-packed Ni^{2+}-columns were loaded with the HIS-tagged VP3 fusion protein, and via

gentle agitation for 60 min the protein was bound to the resin. After the resin settled by gravity, the flowthrough was collected in 1 mL fractions. Subsequently the columns were washed once with 8 mL native washing buffer 1 (50 mM imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0) and afterwards with 8 mL native washing buffer 2 (100 mM imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The washing step fractions were collected in volumes of 1 mL. Following, the elution of the bound proteins was carried out with 8 mL native elution buffer (250 mM imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0) and the elution fractions were collected in 1 mL volumes. All fractions were analyzed for their protein content on a 12% SDS-PAGE (29:1 acrylamide/bisacrylamide, BioRad). The elution fractions, positively tested for the 30kDa SUMO-PTD4-VP3 proteins, once for isolate (T) and isolate (SM), were stored at -20°C.

3.11 Cleavage of the 6xHIS-SUMO-PTD4-VP3 constructs

To obtain only the PTD4-VP3 proteins, without the N-terminally fused 6xHIS anchor and SUMO protein, the fractions, positive for the presence 30 kDa fusion protein were pooled and filtered with Amicon Ultra-15 centrifugal filter units, with cutoff of 10 kDa, to separate the remaining imidazole from the samples. Filtration was carried out by 5.000 x *g* and 2 h at 4°C. Afterwards, the filtered samples were cleaved with the SUMO protease (Invitrogen) and the reaction was carried out as described by the products‘ manual with adaptations. The reaction volume was calculated for 1 mL total volume, with 100 µL reaction buffer and 20-50 µL SUMO protease enzyme and a NaCl salt concentration of 150 mM. The enzymatic reaction was incubated overnight at 4°C.
Subsequently, the final PTD4-VP3 (T) and PTD4-VP3 (SM) proteins were separated from the 6xHIS-SUMO fusion protein and the SUMO protease by IMAC purification, because the fusion protein and the protease both bound to the Ni^{2+} resin due to their 6xHIS anchors. The PTD4-VP3 isoforms were eluted in the flowthrough. The protein contents of the final VP3 samples were determined via spectrophotometry analysis at 280 nm and visualized by a 15% SDS-PAGE (29:1 Acrylamid/Bisacrylamid, Biorad).

3.12 Cell culture

The human cancer cell lines A549 (Lung cancer) and SiHa (Cervix cancer) used in this study were used. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/mL streptomycin in 25 cm^2 culture flasks. Human skin fibroblasts AS 405 (gently provided by Larissa Milano de Souza student of Dr. Jenifer Saffi) were grown in DMEM or RPMI-1640 medium, with 15-20% fetal bovine serum, 100 U/mL penicilin and 100 µg/ mL streptomycin in 25 cm^2 culture flasks. All cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. The growth medium was changed once to three times a week.

3.13 Cell viability tests with MTT

Viable cells were detected using MTT dye, which forms blue formazan crystals that are reduced by mitochondrial dehydrogenase present in living cells. All cell lines were suspended and each line was seed in 96-microwell plates (tissue culture test plate 96F, TPP, Switzerland) and incubated for 24 h at 37°C. Before the cells were treated separately with 20 µL of LBM 5006 iturin, *P34* fengycin, AGV II VP3 (T) or AGV II VP3 (SM) at varying concentrations, the medium was removed and 180 µL fresh medium was added to each well.

After 1, 3 and 5 days of incubation with viral peptides or biosurfactants, 20 µL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (2.5 mg/mL in PBS), purchased from Sigma-Aldrich. Co, were added and the 96-well plate was subsequently incubated for 4 h. After the incubation the supernatant was carefully removed and 200 µL 96% ethanol were added to dissolve the formazan crystals, followed by gentle shaking for 10 min. The optical densities were measured on an ELISA plate reader at 540 nm. The treated groups were compared with the control groups in the absence of lipopeptides or

viral peptides. The growth inhibition rates were calculated according to the following equation:

$$\text{growth inhibition rate (\%)} = [\, A\text{-}B / A \,] * 100$$

where A is the average of OD of control group, and B is the average of treated group.

3.14 Statistical analysis

The obtained data were expressed as the means (+/- standard deviations) of three replicates of every experiment. The results were subjected to analysis of variance, using the MAC Numbers or Microsoft Office Exel 2007 software to analyze differences and further analyzations with SASS were performed with the help of MSc. Elsa C. Mundstock and Gilberto P. Mesquita at the "Núcleo de Assessória Estatística- NAE, UFRGS“ .

4. Results

4.1 Iturin A and fengycin

4.1.1 Medium dependent cell growth and biosurfactant production

The strains *B. amyloliquefaciens* LBM 5006 and *B. spp.* P34 were inoculated for lipopeptide production in liquid BHI, as reference, and in a composed ferrous ion medium FeSS (1% peptone, 0.1% yeast extract, 0.01% $CaCO_3$, 0.01% NaCl, 2% soft sugar, 0.2mM FeSO4 at pH 6.64) and grown for 144h (6 days) at 30°C, 180-200rpm.
First pre-cultures of each strain were grown in 25 mL liquid BHI for 10 to 18h at 37°C, 180-200rpm.
Every 24h a sample was taken of each condition and analyzed for cell growth and lipopeptide quantity comparing the reference medium BHI with the composed ferrous ion medium.
The data were statistically analyzed for significant differences in culture growth via measuring the OD_{600} and for the lipopeptide contents by measuring the spectrophotometric absorbance at 280nm of the final protein solution. The received absorbance values were used to calculate the lipopeptide contents with the help of the BSA equilibration curve and the resulting equation

$$y = 0.5558x$$

The charts of the medium dependent cell growth data of both *Bacillus* strains, as well as the charts for the medium dependent lipopeptide quantity are displayed in figure 7. In every demonstrated chart, the blue symbols represent the cell growth and peptide quantity of cultures grown in BHI medium, the green symbols refers to the composed ferrous ion medium conditions for cell growth and lipopeptide quantity. For every graph the standard error of mean is added to every measured point. Further results were statistically analyzed with the SAS software and with the support of the NAE, UFRGS. The hours marked with asterisks showed significant differences between the BHI and FeSS condition.

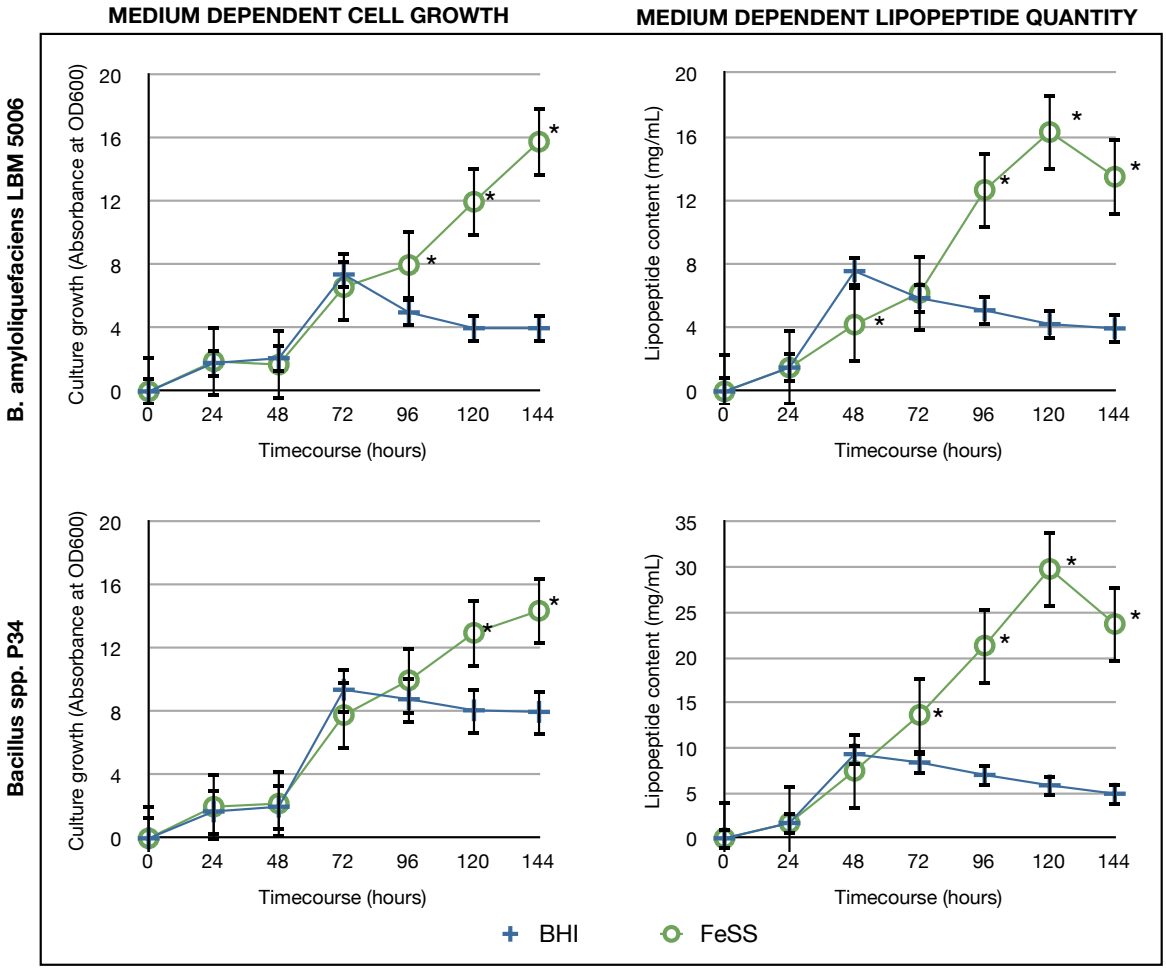

Figure 7: **Charts for medium dependent cell growth and the medium dependent lipopeptide quantity for *B. amyloliquefaciens* LBM5006 and *Bacillus spp.* P34**.
Graphs for the BHI reference medium are displayed in blue, the composed ferrous ion medium is displayed in green.
The charts for the medium dependent cell growth are plotted for the timecourse against the mean values of the measured optic densities for every culture.
The data for the lipopeptide quantities are plotted for the timecourse against the mean values of the determined lipopeptide contents.
All demonstrated results are obtained via the calculated mean values of three replicates.
* indicates the measuring points, where a significant difference between the used composed culture medium and the reference medium was detectable.

For the analysis of the medium dependent cell growth of both *Bacillus* strains it was not possible to perform ANOVA tests, due to the lack of homogeneity of the standard deviations (SD) in both cases. To perform statistical analysis, the data needed to be transformed. For the LBM5006 strain, the calculated SD were modulated to inverted variances, for the *B. spp*. P34 the SD datas were transformed to $\log_{10}([OD]+0,5)$. Following the medium dependent cell growth of both *Bacillus* strains were analyzed with transformed SD datas in SAS for significance of different cell growth dependent on the used culture medium.

As it is illustrated in fig. 7, for both cases, the cultures increase in cell numbers until 72 h post inoculation, whereas already at 48 h there was a significant difference between the BHI-culture and the FeSS culture detectable. At 72 h, the cell numbers for the cultures, grown in BHI, reached their maximum (OD_{600} 7.4 for LBM5006; OD_{600} 9.4 for *Bacillus* sp. p34) and decreased from 72 h onwards. In contrary, for the FeSS grown cultures, the culture density measured with OD_{600} increased continuously until 144 h (OD_{600} 15.8 for LBM5006; OD_{600} 14.4 for *B. spp.* p34). Concomitant with the detected culture growth, significant differences of medium dependent cell growth are detected from 72 h onwards and indicated with asterisks for every measuring.

Further the results for the detected lipopeptide quantities of medium dependent cultivation conditions (after precipitation and following SEC purification) are displayed in table 1. As it is illustrated, for the lipopeptides production of both *Bacillus* strains, cultivation in BHI reached its maximum production (7.56 mg/mL for LBM5006 in BHI; 9.32 mg/mL for *Bacillus* sp. p34 in BHI) at 48 h. At this measuring point a significant difference between the peptide production in BHI-cultures and FeSS-cultures was detected, displaying a higher production in BHI for the same cultivation time. From 48 h onwards, the lipopeptide content decreased continuously for the BHI grown cultures, whereas in contrast, the lipopeptide production for cultures cultivated in FeSS continued rising and reached a maximum at 120 h (16.30 mg/mL for LBM5006 in FeSS; 29.83 mg/mL for *Bacillus* sp. p34 in FeSS). For the in FeSS grown cultures, the lipopeptide quantity decreased after 120 h.

For the LBM5006 produced iturin, as well as for the P34 produced fengycin, from 72 h onwards significant differences for the lipopeptide production were detected and marked with an asterisk in the chart.
For further experiments, the activity units were determined for the lipopeptide samples of the BHI cultivation at 48 h and for the FeSS grown biosurfactant samples at 96 h, 120 h, and 144 h.

4.1.2 Antimicrobial Activity Tests - Activity Units (AU) per mL

The chosen iturin and fengycin samples of different cultivation conditions were further examined for their antimicrobiotic activity. Therefore, a modified „critical dilution assay“ was performed, adapted from the standard protocol of A. Mayr-Harting (see **2.5 Antimicrobial activity test of iturin A and fengycin**).
In table 1 are demonstrated the determined AU/mL for the purified lipopeptides iturin and fengycin in the "critical dilution assay“ (Mayr-Harting et al., 1972) against the indicator bacterium *B. cereus*.
The lipopeptides, iturin (FeSS / 120 h) with 16.3 mg/mL and arbitrary units of 3.200 AU/mL as well as fengycin (FeSS / 120 h) with 29.83 mg/mL and arbitrary units 6.400 AU/mL were used for testing the anti-proliferative activity in cancer cells and normal human fibroblasts via the cell viability tests with MTT.

Table 1 - selected lipopeptides produced under different cultivation conditions with the calculated peptide quantity (total sample protein content) and determinated arbitrary units

Lipopeptide / culture medium / time point	calculated Lipopeptide quantity	determined AU ./. *B.cereus*
Iturin / BHI / 48h	7.56 mg/mL	1.600 AU/mL
Iturin / FeSS / 96h	12.66 mg/mL	1.600 AU/mL
Iturin / FeSS / 120h	16.3 mg/mL	3.200 AU/mL
Iturin / FeSS / 144h	13.49 mg/mL	3.200 AU/mL
Fengycin / BHI / 48h	9.32 mg/mL	800 AU/mL
Fengycin / FeSS / 96h	21.34 mg/mL	3.200 AU/mL
Fengycin / FeSS / 120h	29.83 mg/mL	6.400 AU/mL
Fengycin / FeSS / 144h	23.74mg/mL	3.200 AU/mL

4.2 *Avian Gyrovirus* II VP3 isomers Thais and Santa Maria

4.2.1 PTD4-VP3 constructs /pET SUMO plasmids

The PTD4-VP3 constructs for the Thais and Santa Maria isolates were obtained via Polymerase Chain Reaction (PCR). During this reaction the PTD4 sequence was added N-terminally to the VP3 forward primers and the obtained 405 bp PCR products (Figure 8, PTD4-VP3 (T): lane 1; PTD4-VP3 (SM): lane 2) were cloned into the pET SUMO expression vector and sequenced. The created construct expresses the PTD4-VP3 protein with a N-terminally fused 6xHIS tag and SUMO Protein of ca. 30 kDa. The final PTD4-VP3 (T) and PTD4-VP3 (SM) constructs were obtained by Marcus Knack during his IC work at the virology lab at ICBS/ UFRGS. The results are demonstrated to show consistency of the further results.

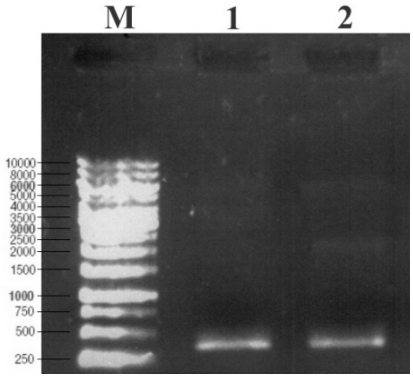

Figure 8: **PCR amplicons of AGV II VP3 (T) and AGV II VP3 (SM)** - PCR amplification of the VP3 coding regions of AGV2-T (lane 1) and AGV2-SM (lane 2) producing a fragment of 405bp (Obtained from Knack et al., 2011).

After sequencing, (sequences are displayed in figure 9) the coding recombinant gene size was determined for 762 bp and the expressed protein calculated for 6xHIS-SUMO-PTD4-VP3 (T) of 28.12 kDa and, due to the different amino acid sequence of the Santa Maria isomer, 28.13 kDa for the 6xHIS-SUMO-PTD4-VP3 (SM), respectively.

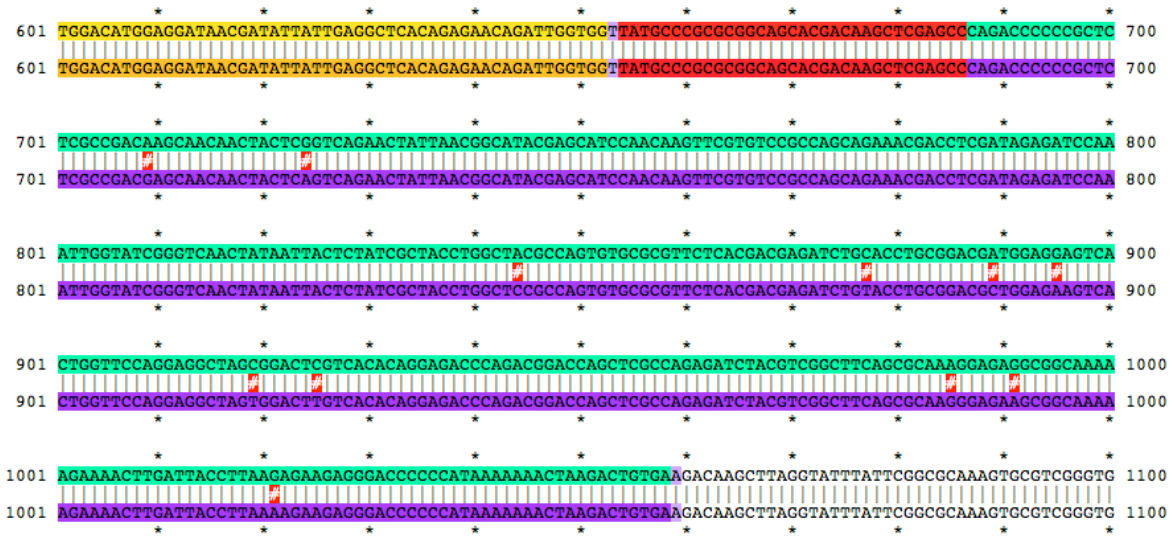

Figure 9: **Alingment of AGV II VP3 (T) and AGV II VP3 (SM)** - Alingment of the coding regions of the nucleotide sequences for the AGV II VP3 isomers VP3 Thais (green line) and VP3 Santa Maria (violet line).
The end of the fused SUMO protein is marked in yellow (nt 601 - 652). Marked in red: the N-terminally to the VP3 peptide fused PTD4 sequence (nt 654 - 686). The sequence differences of the Thais isomer and Santa Maria isomer are marked with rhombos #.

The resultant pET SUMO PTD4-VP3 (T) and (SM) plasmids were separately transformed first into *E. coli* DH5α (plasmid reproduction) and further into BL21(DE3) pLysS cells for IPTG induced protein overexpression of toxic proteins.

After every transformation on selective agar plates (see **2.5 Cultivation and preparation of *E. coli* strains**) grown colonies were analyzed via Colony PCR (see **2.6 2.6 Transformation of the BL21(DE3) pLysS with pET SUMO-PTD4-VP3**).

As illustrated in figure 10, the (Colony) PCRs products of 476 bp for the pET SUMO PTD4 VP3 (T) construct were analyzed by agarose gel electrophoresis. Similar results for the (Colony) PCRs of pET SUMO PTD4 VP3 (SM) were obtained (data not shown).

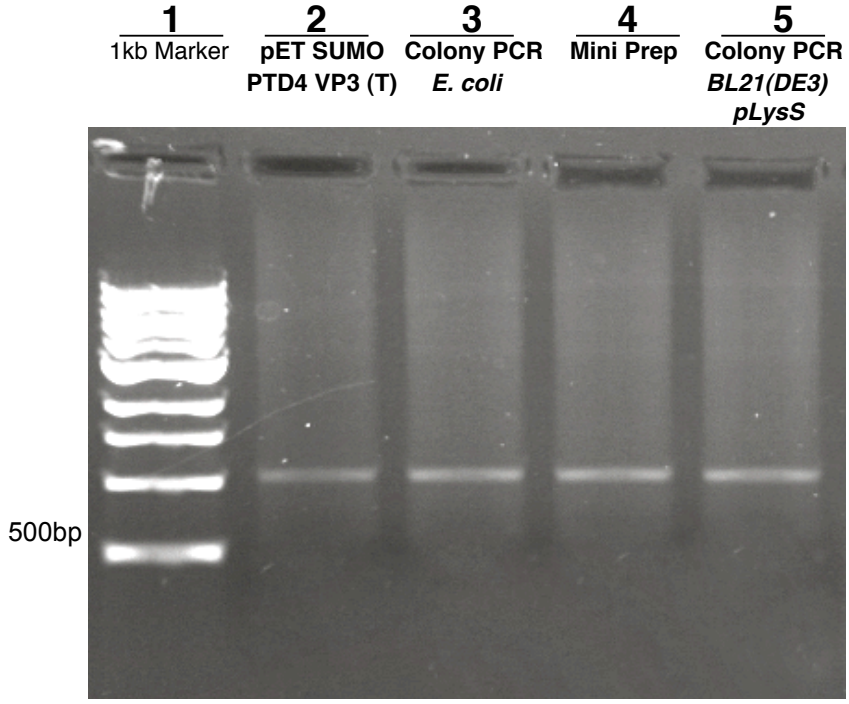

Figure 10: **PCR products for pET SUMO PTD4 VP3 (T) constructs** - the pET SUMO PTD4 VP3 (T) construct and MiniPrep, Colony PCR products of transformed *E. coli* DH5α and BL21(DE3) pLysS. Amplicon has a calculated size of 476bp.
1: 1kb Marker (NEB)
2: PCR product of the pET SUMO PTD4 VP3 (T) construct
3: Amplicon of *E. coli* DH5α transformed with pET SUMO PTD4 VP3 (T)
4: Amplicon of the plasmid extraction of *E. coli* DH5α pET SUMO PTD4 VP3 (T)
5: *E. coli* BL21(DE3) pLysS transformed with pET SUMO PTD4 VP3 (T)

All displayed PCR products in figure 10 lane 2 to 5 show an amplicon of ca. 500bp PCR product in lanes 2 to 5.

In lane 2 the amplified DNA fragment for the pET SUMO PTD4-VP3 (T) construct, produced by Marcus B. Knack (Knack et al., 2011), is shown. The same construct was subsequently used for transformation of *E. coli* DH5α to obtain the initial *E. coli* DH5α pET SUMO PTD4 VP3 (T) strain.

A Colony PCR of the transformed *E. coli* DH5α was performed (lane 3) and afterwards the pET SUMO construct via plasmid extraction isolated (PCR product is shown in lane 4). This sample was used for further transformation of BL21(DE3) pLysS, the obtained PCR product after transformation is shown in lane 5.

The positive tested colony of BL21 (DE3) pLysS pET SUMO PTD4 VP3 (T), as well as 3 sister colonies (data not shown) were stored on LB-agar plates for further induced overexpression with IPTG.

Similar results for (Colony) PCRs were obtained for the pET SUMO PTD4 VP3 (SM) construct. The initial pET SUMO PTD4 VP3 (SM) construct, obtained by Marcus Knack (Knack et al., 2011), showed a amplicon of ca. 500bp and was used for further transformation of *E. coli* DH5α. Further the PCR product of the *E. coli* DH5α pET SUMO PTD4 VP3 (SM) strain and the extracted pET SUMO PTD4-VP3 (SM) plasmid, both displayed amplified DNA bands at a size of ca. 500bp. Finally the Colony PCR product of the final BL21(DE3) pLysS pET SUMO PTD4 VP3 (SM) strain demonstrated a DNA band at ca. 500bp (data not shown).

One positive tested colony of the BL21(DE3) pLysS pET SUMO PTD4 VP3 (T) as well as BL21(DE3) pLysS pET SUMO PTD4 VP3 (SM) were used for the induced protein overexpression with IPTG.

4.2.2 IPTG induced overexpression for the 6xHIS-SUMO-PTD4 VP3 isomers

Induced protein overexpression with IPTG was performed like elsewhere described (**2.7 IPTG induced protein overexpression of PTD4-VP3 (T) and PTD4-VP3 (SM) in *E. coli* BL21 (DE3) pLysS cells**).

Analysis of the pre and post-induction samples of both 6xHIS SUMO PTD4-VP3 isomers showed detectable protein expression only after the induction with 1mM IPTG with continuously increasing protein amount (data not shown).

Cell lysis and lysate preparation was carried out as described in **2.8**.

Further the protein purification was performed with Ni^{2+}-columns under native conditions. The collected fractions were analyzed for their protein content on a 12% SDS PAGE (see **2.8**).

In figure 11 the SDS PAGE analysis for the pET SUMO PTD4 VP3 (T) sample is demonstrated.

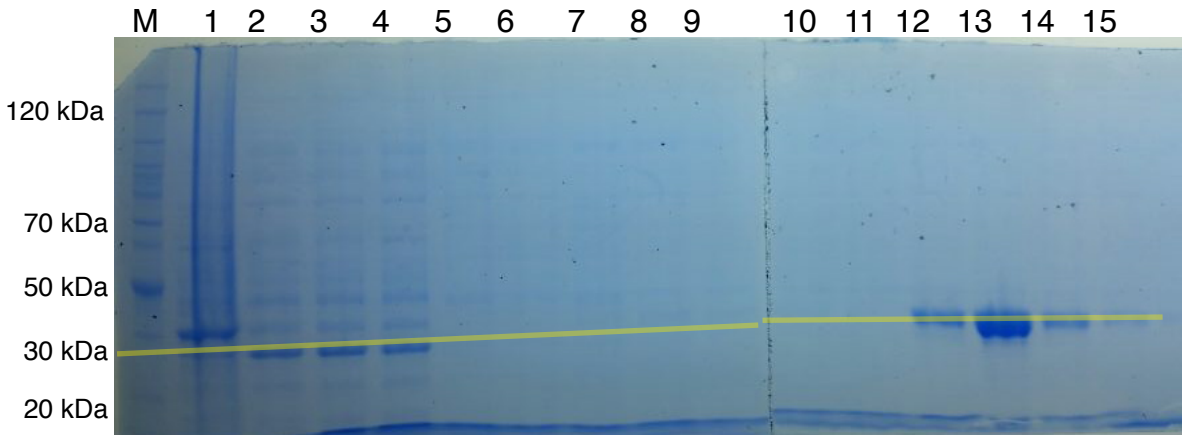

Figure 11**: SDS PAGE of the 6xHIS SUMO PTD4 VP3 IMAC purification** - SDS PAGE analysis of the 6xHIS SUMO PTD4 VP3 (T) protein expressed in recombinant *E. coli*, purified with Ni^{2+}-columns under native conditions
Analysis of the pellet after cell lysis (1) and the lysate (2). Lane 3 represents the sixth (last) flowthrough fraction. Lane 4 and 5 show the first and last (6th) fractions of the 1st washing step with 50mM Imidazole. Lanes 6 tp 11 display the fractions 1 to 6 of the 2nd washing step with 100 mM Imidazole. The protein elution fractions 1 to 4 with 250 mM Imidazole are shown in lane 12 to 15.
M: BenchMark™ Protein Ladder (Invitrogen)
12% SDS PAGE; yellow line indicates 30 kDa size

As illustrated in Fig. 11 after cell lysis the expressed 6xHIS SUMO PTD4 VP3 (T) protein (28.12 kDa) was almost undetectable by SDS PAGE gel analysis (fig. 11, lane 1). The lysate (fig. 11, lane 2) shows various detected native proteins of *E. coli* as well as enhanced expressed protein at the calculated protein size of ca. 30 kDa. After the His-tagged protein bound to the Ni^{2+}-resin, in the sixth flowthrough fraction (fig. 11, lane 3) native proteins, which could not bind to the column, and excess recombinant VP3 (T) protein was detected. During the first washing step with 50mM Imidazole only for the first fraction native *E. coli* proteins and 6xHIS SUMO PTD4 VP3 (T) could be detected, whereas in the 6th fraction of washing step 1 the protein amount either for recombinant VP3 (T) or other peptides was undetectable (fig. 11, lane 4 and 5). All fractions of the second washing step with 100mM Imidazole (fig. 11, lane 6 to 11) showed only very faint bands of different peptides (at ca. 45 kDa, ca. 75 kDa and ca. 110 kDa) but no detectable 6xHIS SUMO PTD4 VP3 (T) protein.

Finally, in lane 12 to 15, the elution fractions with 250mM Imidazole are demonstrated. As illustrated, the first elution fraction (fig. 11, lane 12) shows already detectable amount of the recombinant 6xHIS SUMO PTD4 VP3 (T) at the expected size of 28.12 kDa. The eluted protein increases in fraction 2 (fig. 11, lane 13), where it seems to reach a peak. In lane 14 and 15, the protein amount diminishes gradually (fig. 11, lane 14 and 15). The following fractions did not show any detectable amounts of protein (Data not shown).

The elution fractions which showed detectable 6xHIS SUMO PTD4 VP3 (T) (fig. 11, lane 12 to 15) were pooled and used for further processes.

For the 6x His-tagged PTD4 VP3 (SM) isomer similar results were obtained during the protein purification. Whereas the pellet showed almost no detectable protein of 28.13 kDa, the lysate presented soluble 6xHIS SUMO PTD4 VP3 (SM). Comparable to the protein amount of the PTD4 VP3 (SM) in lane 2, the last flowthrough fraction and the first fraction of washing step 1 (50 mM Imidazole) showed also a protein band at almost 30 kDa. In the following fractions, the sixth fraction of the first washing step, as well as all fractions of washing step 2 (100 mM Imidazole) no protein amounts at 30 kDa were detected.

The elution fractions displayed the eluted protein with 250 mM Imidazole at ca. 30 kDa (data not shown).
Also for 6xHIS SUMO PTD4 VP3 (SM) the elution fractions with detected VP3 peptide were pooled and stored for further processing at -20°C.

4.2.3 Filtering and Cleavage of the recombinant fusion protein isomers

To obtain the final PTD4-VP3 products for the Thais and Santa Maria isomers, the in 3.2.2 described pooled elution fractions were filtered with Amicon Ultra-15 centrifugal filter units, with a pore size of 10 kDa, to separate the remaining imidazole from the samples (see **2.8 Cleavage of the 6xHIS-SUMO-PTD4-VP3 constructs**). Subsequently the enzymatic reaction to separate the 6xHIS-SUMO fusion protein was carried out overnight. On the next day, the 6xHIS-SUMO protein was together with the HIS-tagged SUMO protease separated from the PTD4-VP3 peptide with an additional IMAC purification step. The final protein was kept in 10mM phosphate buffer pH 7.0.
To determine the protein contents, the final PTD4-VP3 samples were analyzed at 280nm and the protein quantity calculated via equation obtained with BSA as standard:

$$\mathbf{y = 0.5558x}$$

The protein content for the PTD4-VP3 (T) protein was determined of 1.9 mg/mL and for the PTD4-VP3 (SM) of 1.8mg/mL.
Additionally, to verify complete cleavage of the 6xHIS-SUMO protein, a SDS PAGE was performed. The 15% SDS PAGE for the purified 6xHIS-SUMO-PTD4-VP3 (T) and (SM) isoforms, as well as the pre and post cleavage samples (in listed order: lanes 1, 2 and 3) are shown in figure 12.
As it is illustrated (figure 12), after the fractions were pooled (lane 1) and filtered (lane 2) the detected protein content for the ca. 30 kDa sized fusion proteins decreased for the same applied sample volume of 10 µL. After cleavage of the 6xHIS-SUMO protein with SUMO protease, the protein size for the detected protein was determined as 14.73 kDa, both isomers. In lane 3 a protein band of ca. 15 kDa was expected, but for the Thais isomer as

well as for the Santa Maria isomer, no protein of 15 kDa could be detected. For both isoforms a protein band of ca. 30 kDa (VP3 (T), lane 3: very faint band; VP3 (SM), lane 3: clear protein band) could be detected.

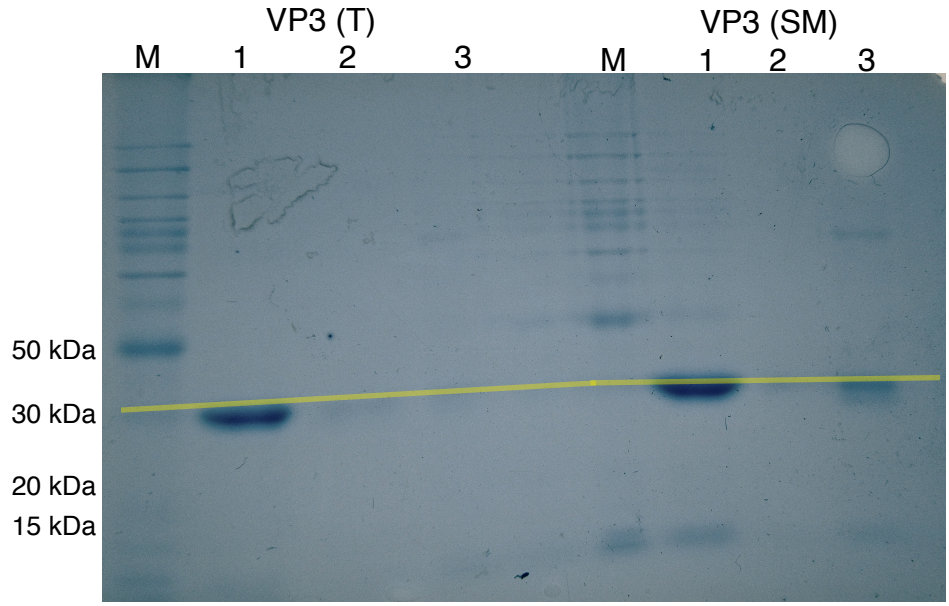

Figure 12**: SDS PAGE of the final 6xHIS-SUMO-PTD4-VP3 isoforms** - SDS PAGE of the enzymatic reaction with SUMO Protease (cleavage) to obtain the final 6xHIS-SUMO-PTD4-VP3 isoforms.
Left side: samples for the VP3 (T) isomer;
right side: samples for the (SM) isomer;
1:purified and pooled HIS-tagged fusion protein
2: sample after filtration
3: final PTD4-VP3 fusionprotein after 6xHIS-SUMO cleavage
M: BenchMark™ Protein Ladder (Invitrogen)
12% SDS PAGE; yellow line indicates 30 kDa size

Although the results of the SDS PAGE could not show detectable amounts of the final 15 kDa sized PTD4-VP3 isomers, the obtained final protein samples were used to test the anti-proliferative activity of the fusion protein on cancer cells and normal human fibroblasts via the cell viability test with MTT.

4.3 Cell viability studies (MTT)

To evaluate if the purified lipopeptides and viral peptides have the capacity to induce apoptosis and to distinguish between cancer and normal cells, the 3 different cell lines, 2

cancerous lines A549 and SiHa and the human fibroblast AS405, were inoculated with different peptide concentrations. Additionally the lipopeptides were also inoculated for 3 different periods of time (24 h and 120 h).

4.3.1 Anticancer properties of purified iturin and fengycin

The well-characterized MTT assay was used to determine whether iturin or fengycin could inhibit the growth of the human cancer cells A549 and SiHa, but may not affect human fibroblasts AS405. All cells were incubated with iturin or fengycin at concentrations ranging from 50 to 500 µg/mL, for 24 h and 72 h (data not shown), respectively at a concentration ranging from 100 to 1000 µg/mL for 120 h of incubation.
As it is illustrated in the following figures, iturin as well as fengycin inhibited the growth of cells in a concentration dependent manner (Figure 13: 24 h treatment - iturin, figure 14: 24 h treatment - fengycin).
Analyzing the data for the cells incubated 24h with the lipopeptides iturin or fengycin, a general increased growth inhibition could be observed for the SiHa cells onwards from a protein concentration of 100 µg/mL for fengycin and of 150 µg/mL for iturin respectively, whereas the A549 cells do not show similar growth inhibition during concentration dependent treatment. For iturin as well as for fengycin cell viability and growth inhibition, varying dose independent means between 0 and 20% for iturin and between 5 and 40% for fengycin are shown. For the normal human fibroblast cells AS405 no direct connection between protein concentration and growth inhibition could be detected, though the cell viability ratios vary between 0 and 20% for iturin and 0 and 15% for fengycin.
The figures 15 and 16 show the treatments of A549, SiHa and AS405 cells with iturin and fengycin for 5 days. As it is illustrated for iturin as well as for fengycin, the lipopeptides inhibited cell proliferation of the cancerous A549 and SiHa cells in a dose dependent manner.
Both lipopeptides showed a growth inhibition of ca. 90% for A549 and ca. 80% for SiHa. The human fibroblasts AS405 showed growth inhibition of max. ca. 20% for both lipopeptide treatments.

The results for the time dependent analysis of iturin for instance are shown in figure 17. For all used cell lines during the treatment, a time dependent and also dose dependent effect of the lipopeptide could be observed.

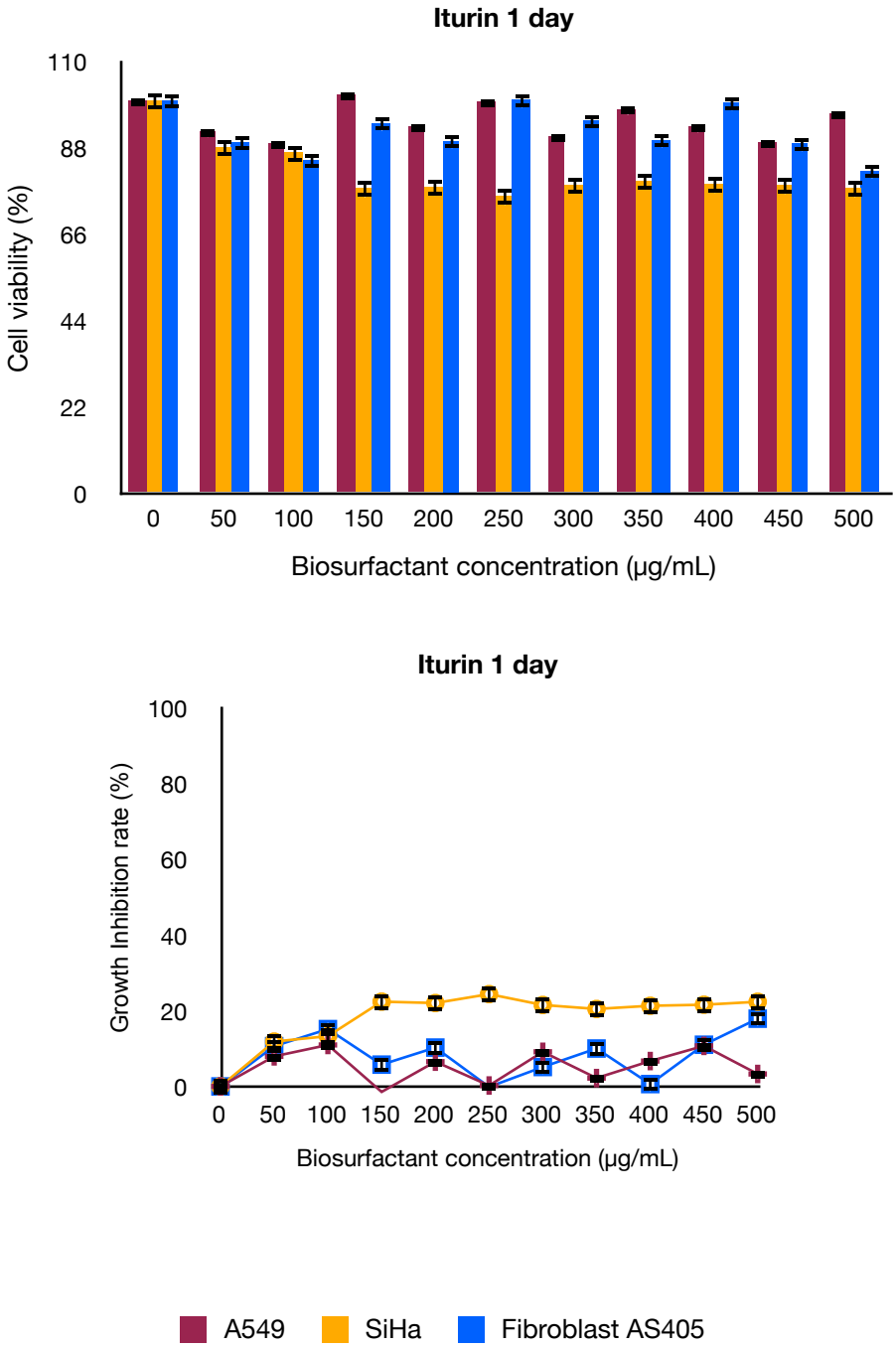

Figure 13: **Anti-proliverative effect of iturin for 24 h (1 day)** - the anti-proliferative activity of iturin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (50 - 500 µg/mL) of iturin for 24 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

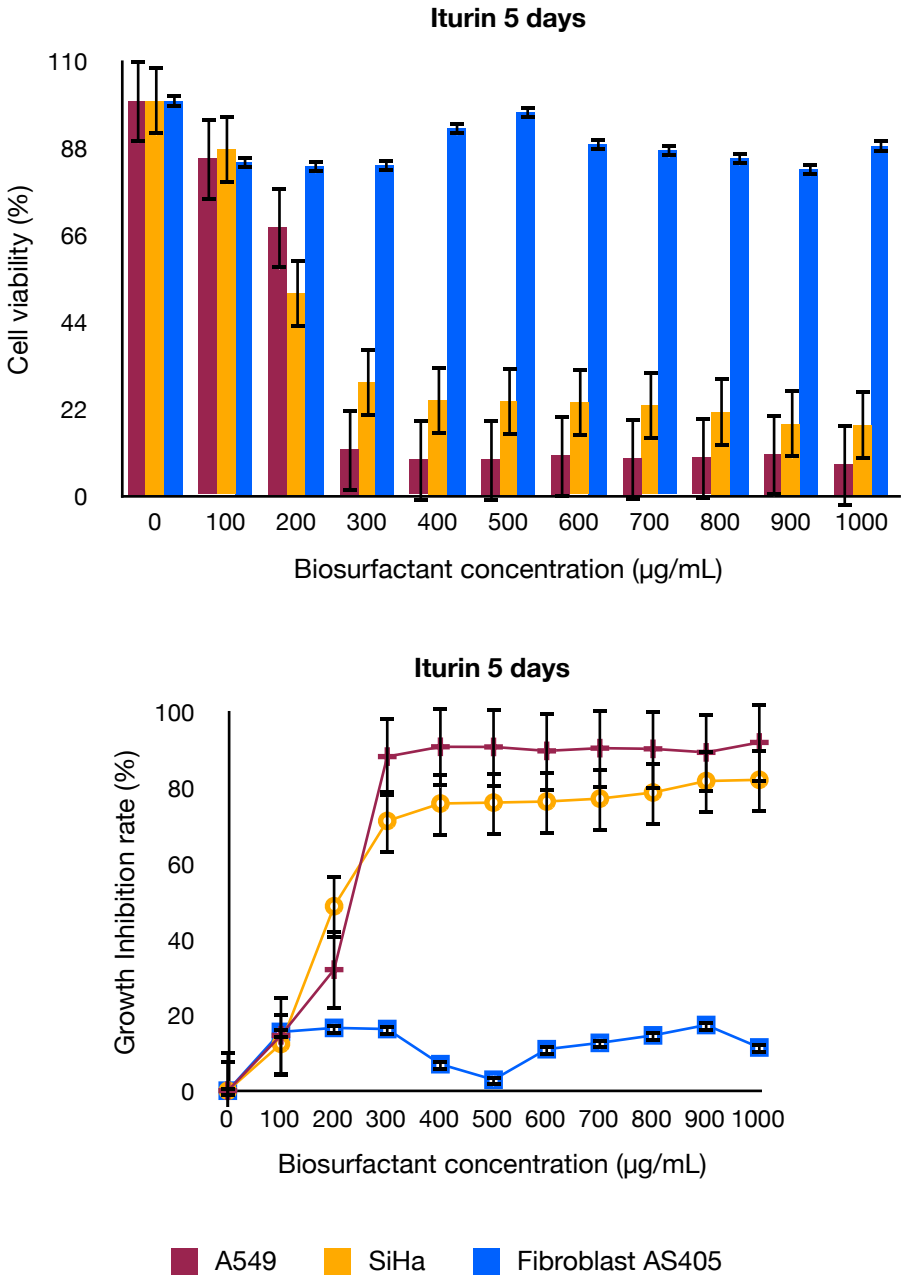

Figure 14: **Anti-proliverative effect of iturin for 120 h (5 day)** - the anti-proliferative activity of iturin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (100 - 1000 µg/mL) of iturin for 120 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

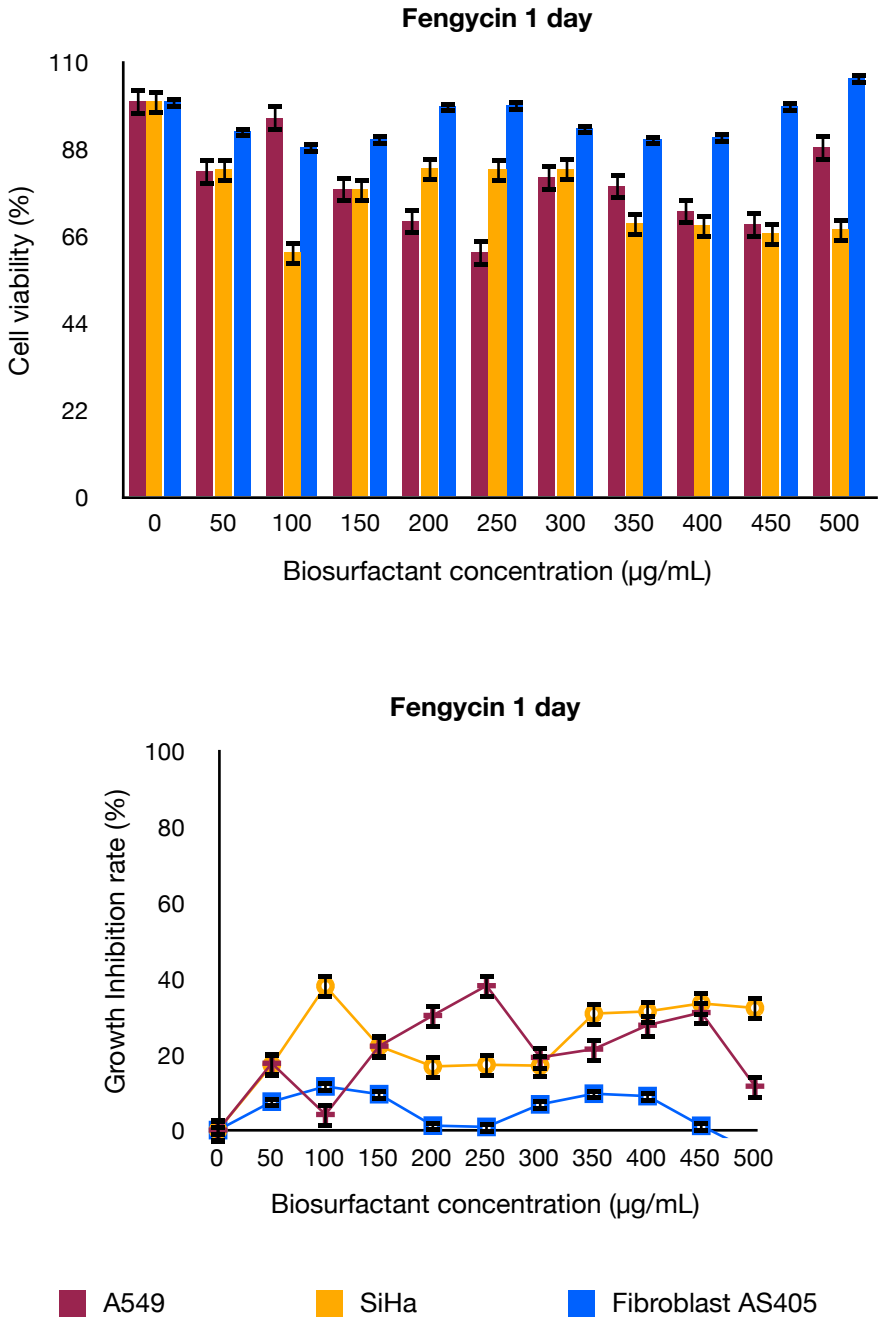

Figure 15: **Anti-proliverative effect of fengycin for 24 h (1 day)** - the anti-proliferative activity of fengycin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (50 - 500 µg/mL) of iturin for 24 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

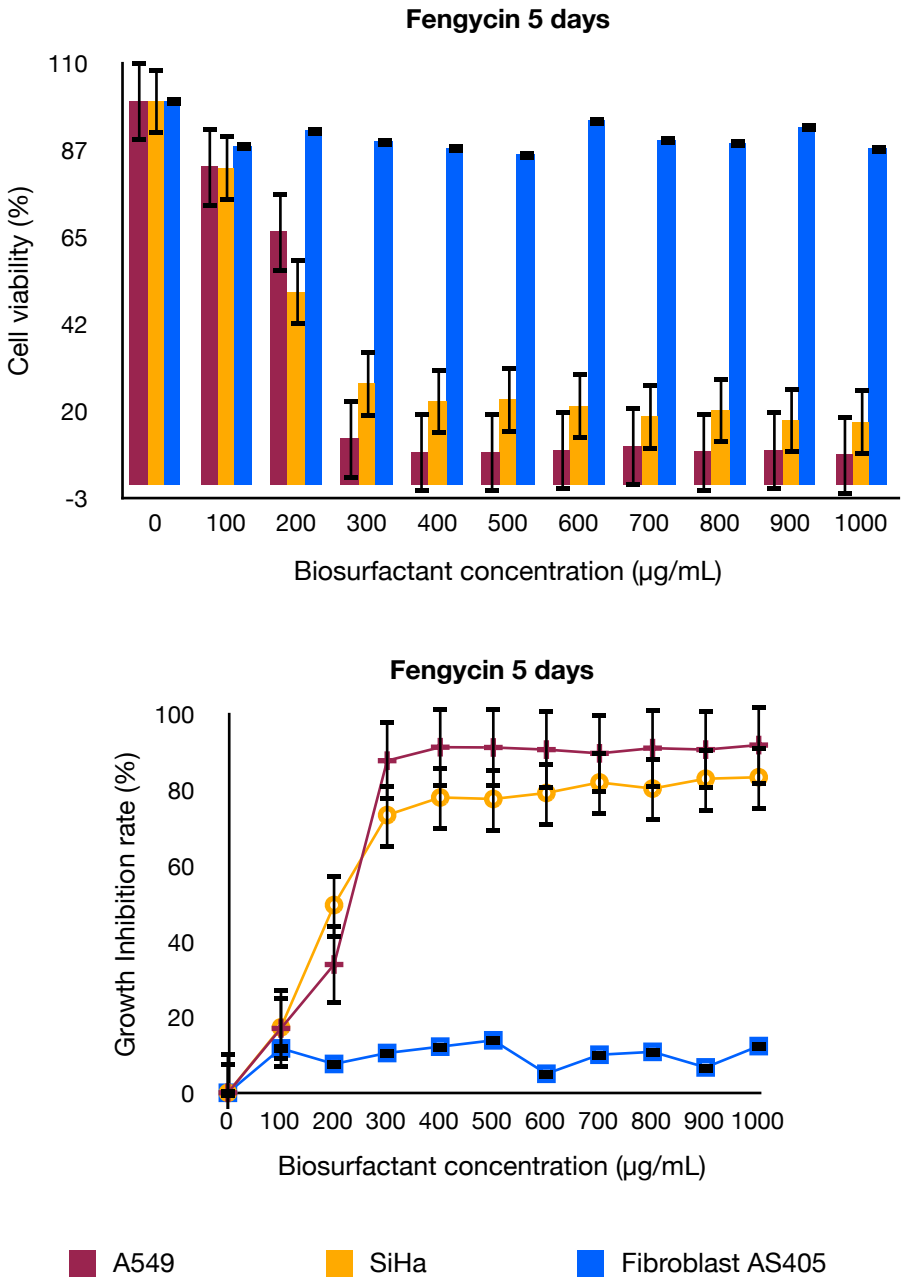

Figure 16: **Anti-proliverative effect of fengycin for 120 h (5 day)** - the anti-proliferative activity of fengycin on A549, SiHa and human fibroblast AS405. Cells were treated with various concentrations (100 - 1000 µg/mL) of iturin for 120 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

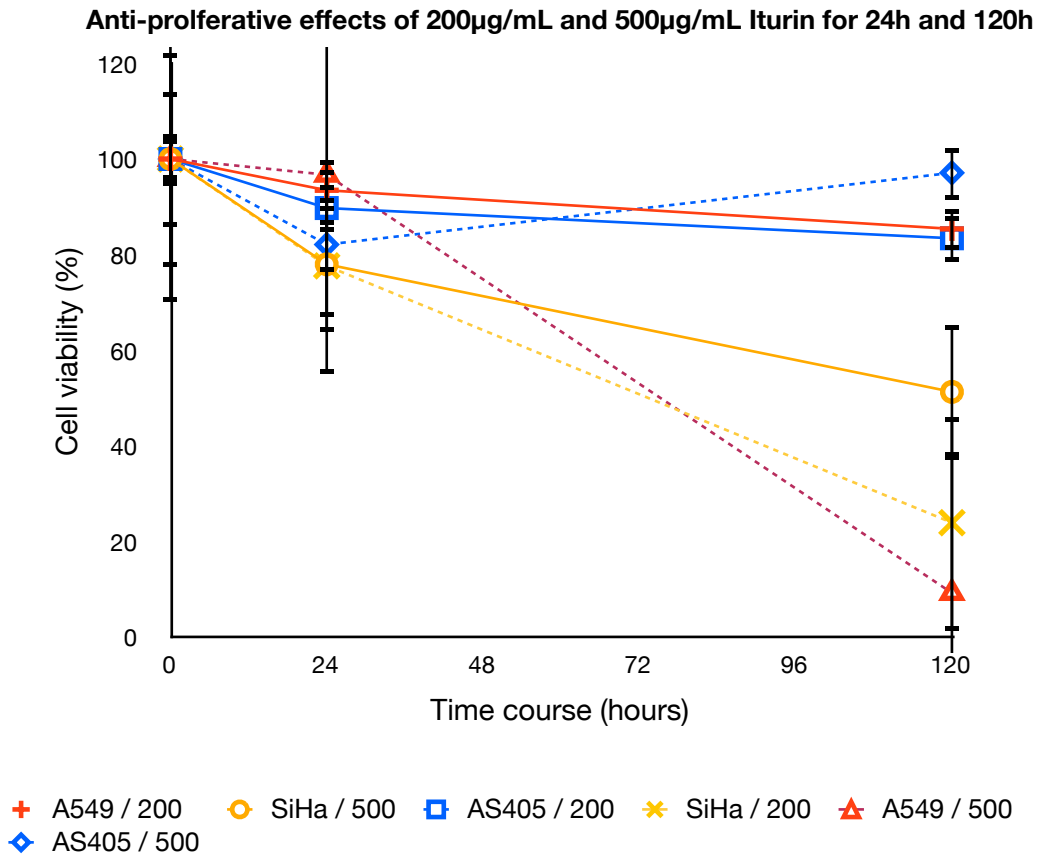

Figure 17: **Anti-proliferative effect of 200 µg/mL (lines) and 500 µg/mL (broken lines) iturin for max. 120 h (5 days)** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (200 and 500 µg/mL) of iturin for max. 120 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

4.3.2 Growth Inhibition induced by PTD4-VP3 isomers

As well as for the lipopeptides, the VP3 isomers were incubated with the mentioned cell lines for 24h at different concentrations. Afterwards the cell viability was determined via the MTT assay.

As it is illustrated in figure 18 and figure 19, both PTD4-VP3 (Thais) and PTD4-VP3 (SM) showed dose dependent growth inhibition, whereas the inhibition effect on A549 and SiHa is very remarkable (for PTD4-VP3(Thais) 90% for both cell lines; for PTD4-VP3 (SM) ca. 80% for SiHa; ca. 90% for A549). The inhibition of cell growth on AS405 for both PTD4-VP3 isomers appear less important than on the cancerous cells (40% for both VP3 isomers).

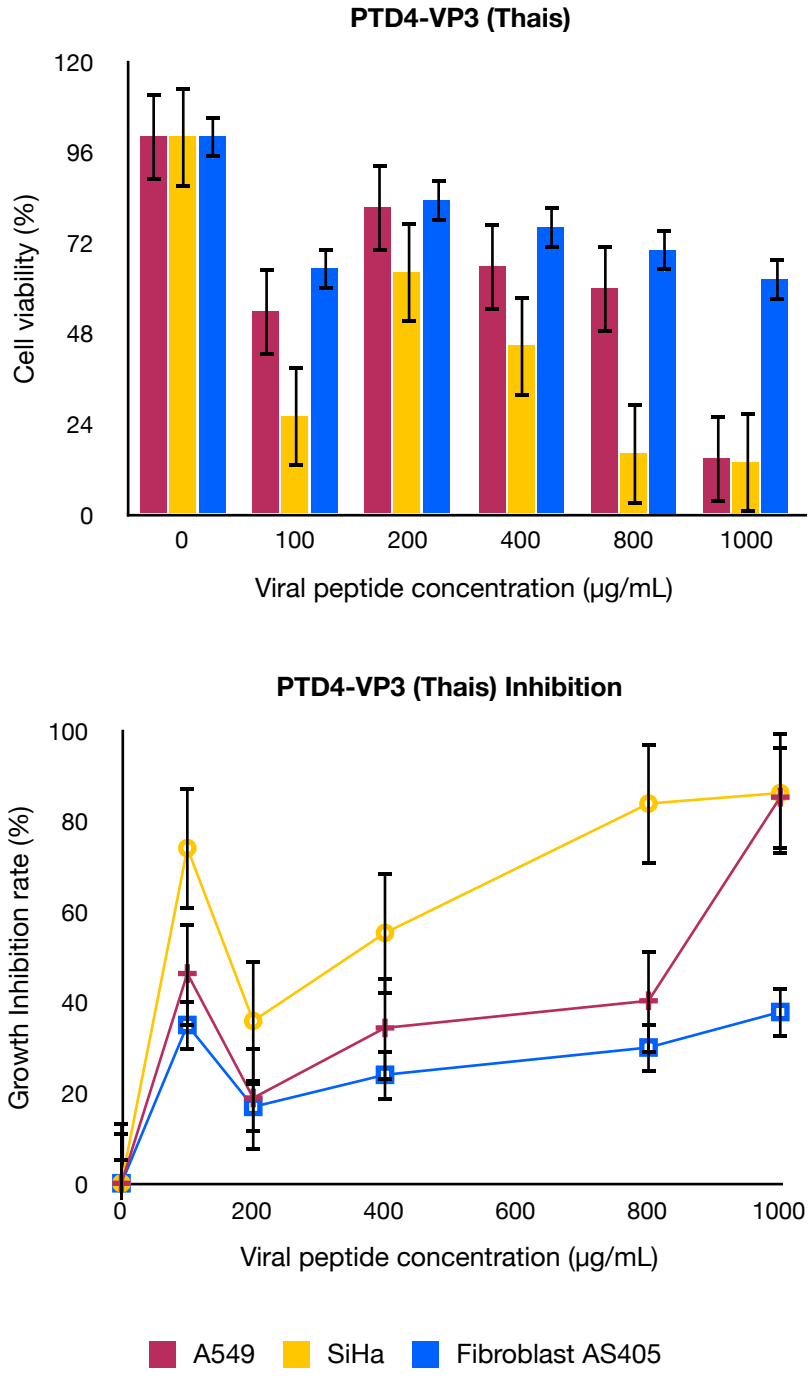

Figure 18: **Anti-proliverative effect of PTD4-VP3 (T) for 24 h** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (100 - 1000 μg/mL) of PTD4-VP3 (T) for 24 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

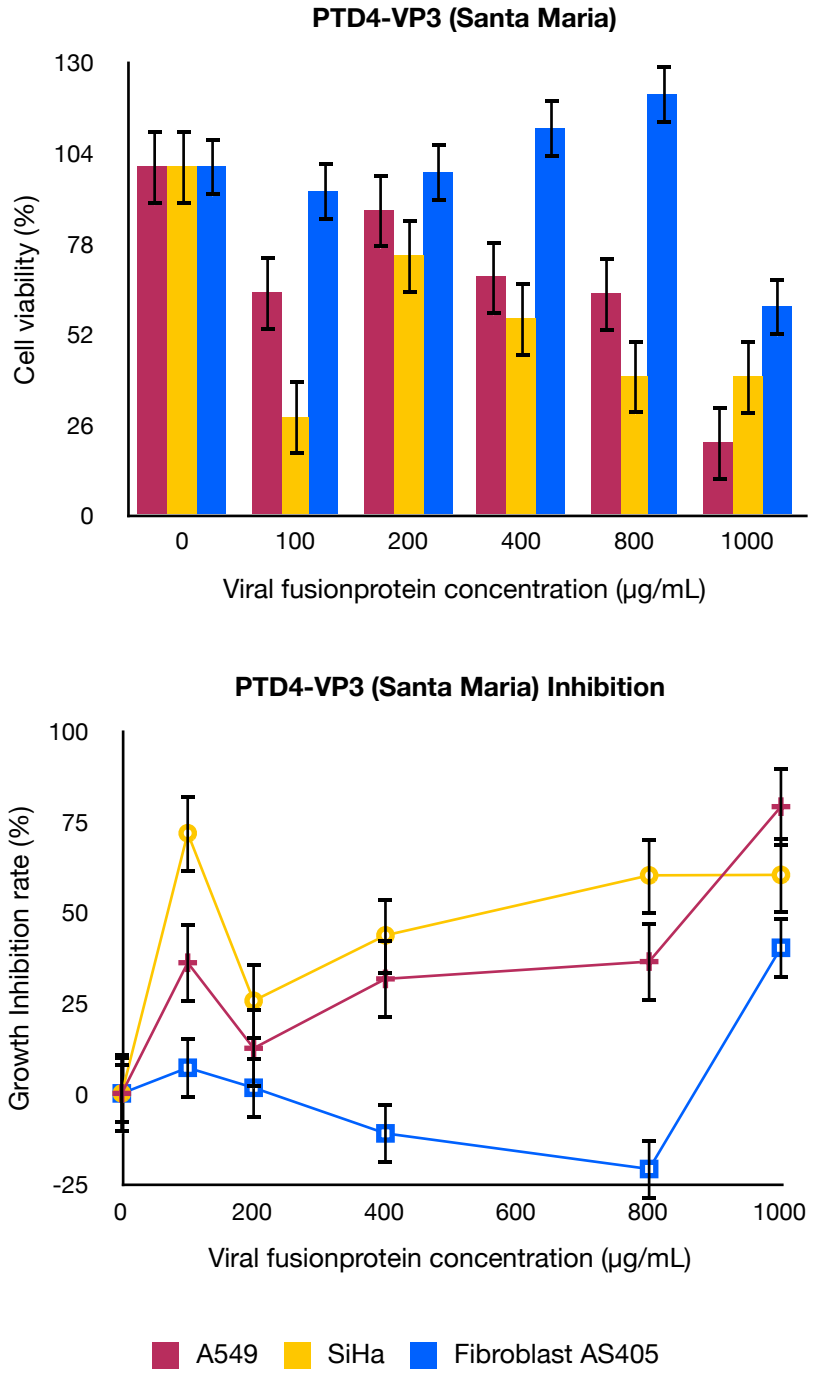

Figure 19: **Anti-proliferative effect of PTD4-VP3 (SM) for 24 h** - A549, SiHa and human fibroblast AS405 were treated with various concentrations (100 - 1000 µg/mL) of PTD4-VP3 (SM) for 24 h. Cell viability was determined by MTT and expressed as the mean +/- S.E. of three replicates.
Bars and graph in red: A549
Bars and graphs in yellow: SiHa
Bars and graphs in blue AS405

5. Discussion

The search for new anti-cancer agents among natural substances is a result of arising side effect problems of common anti-cancer therapy with designed chemo drugs or radiation. Nowadays cancer therapies are directed against the cell proliferation process, aim to induce apoptosis, and with this approach there is no distinction between normal and abnormal cancer cells. More likely the applied drugs attack the cancer, but in the end, also the normal healthy cells. Every treatment brings various negative side effects, like nausea and vomiting, fatigue, hair loss, pain and several others. Further cancer cells may develop resistances against the applied drugs during treatment.
(American Cancer Society; National Cancer Institute; Malhotra & Perry, 2003; Krishnan et al., 2007)
Based on these known problems, search for anti-cancer agents which may not cause further resistances or the well-known side effects arose since the last decades.
In this study the two viral peptide isomers, AGV II VP3 (T) and AGV II VP3 (SM), and the biocins iturin and fengycin were investigated for their potential anti-proliferative effect, which further may indicate an apoptosis inducing anti-cancer activity, on the cancerous cell lines A549 and SiHa. Their effect on the normal human fibroblasts AS405 was also tested. The aim of this study was to investigate possible new anti-cancer agents on natural basis, with low toxicity on normal cells.
Since the 1970s lipopeptides, like surfactin, from the genus *Bacillus* have been studied for their potential anti-cancer activities, whereas the research on viral peptides began during the 1990s with the discovery of apoptin of the chicken anemia virus (CAV) (Wang et al., 2007; Cao et al., 2009; Cao et al., 2010; Kim et al., 2007; Noteborn et al., 1994, Danen van Oorschot et al., 1997; Noteborn et al., 1998).

Studies by Kameda´s group have provided the first evidence that lipopeptides have anti-cancer activities by demonstrating that extracted lipopeptides from *B. natto* KMD 1126 can induce apoptosis in cancer cells (Kameda et al., 1974). From this time on, many researchers already studied the interactions of surfactin-like lipopeptides with various cell

lines (Kameda et al., 1974; Wang et al, 2007; Cao et al., 2010). Among others, Wakamatsu discovered that lipoproteins induce neuronal differentiation in PC12 cells (Wakamatsu et al., 2001). This discovery provided the groundwork for the use of microbial extracellular lipopeptides as novel reagents for anti-cancer treatments.

It was reported that a cyclic lipoprotein purified from *B. subtilis subsp. natto* T-2 inhibited the growth of human leukemia K562 cells in a dose-dependent manner (Wang et al., 2007). In the same year, the negative effects of surfactin on the proliferation of human colon carcinoma cells (LoVo) were reported by Kim and coworkers, suggesting, that surfactin might have anti-cancer activities as a result of its ability to down-regulate the cell cycle and suppress cell survival (Kim et al., 2007). Later the time-dependent apoptotic effect of surfactin on MCF-7 breast cancer cells was demonstrated (Cao et al., 2009). These results lead to the assumption to investigate the potential value as a novel anti-tumor agent. Together with the apoptotic activity of the lipopeptides, the parameters (medium composition, temperature, cultivation time) for enhanced peptide production were also studied.

Because of the close relation of the lipopeptides to each other, in this study, for behalf of the possible anti-cancer substances produced by microorganisms, the lipopeptides iturin and fengycin were the focus of attention.

A possible anti-cancer agent from virus, the apoptin protein from the CAV virus was tested positive for its apoptosis inducing activity in cancer cells, while not affecting any normal cells (Danen-van Oorschot et al. 1997). The anti-cancer effect of the CAV apoptin was already tested in 70 different tumor cell lines and various normal human cell lines (Backendorf et al., 2008). As the apoptin already was expressed with the help of different expression systems like *E. coli* (Sun et al. 2009; Jin et al. 2011; Li et al., 2012) also in a model fused to the PTD4 protein transduction domain protein (Sun et al., 2009).

In 2011 the *Avian Gyrovirus* II (AGV II) was discovered by Rijsewijk and his group (Rijsewijk et al., 2011). After sequence analyzing, the AGV II virus showed a genetic structure highly similar to the CAV virus, where the encoded VP3 protein of AGV II presents 32.2% homology to the CAV apoptin in the functional protein domains. Based on the homology, the AGV II VP3 peptide was thought to possibly have an anti-cancer

activity on cancer cells, too.
The AGV II VP3 peptide was tested in this present study for its negative effect on cell proliferation on cancer cells. To deliver the viral peptides across the cellular membrane, the PTD4 transduction domain was fused N-terminally to the VP3 proteins.

To examine the potential apoptosis induction of the named lipopeptides fengycin and iturin and the isomers „Thais“ and „Santa Maria“ of the viral fusion-proteins PTD4-VP3, their anti-proliferative activity was tested in tumor cells and normal cells *in vitro*.

In general, the cell viability tests displayed that all used peptides, lipopeptides as well as recombinant viral peptides, inhibited cell proliferation with clear higher effect on tumor cell lines A549 and SiHa than on the normal human fibroblasts AS405. Also a dose-dependent inhibition of cell proliferation could be demonstrated for all peptides. For iturin and fengycin a time-dependent increase on inhibition could also be demonstrated.

Comparing the anti-proliferative effect of the iturin lipopeptide on the cancerous cells, growth inhibition of SiHa cells was stronger than A549 cells after 24 h. An increased inhibition of cell growth was detectable from 50 µg/mL to 100 µg/mL, which indicated dose-dependent cytotoxic effect of iturin on cancer cells. Reaching a peptide concentration of 150 µg/mL, apparently the growth inhibition rate evens out at a certain inhibition level around 20% also with a simultaneous increasing lipopeptide concentration. This might point out to a maximum effect of iturin within 24 h of treatment, as also with rising lipopeptide concentration stronger effects of growth inhibition could not be reached.
The effect of iturin on A549 after 24 h was not a regular increasing inhibition of cell proliferation. Cell viability varied notwithstanding with increasing lipopeptide concentrations. A possible reason for these results with A549 might be a necessarily longer incubation time to achieve regular dose-dependent cytotoxic effect.
The results of the 5 days treatment support the assumption of the time-dependent anti-proliferative activity of iturin on cancerous cells. After 120 h the SiHa cells as well as the A549 cells suffered growth inhibition of almost 80% for SiHa and ca. 90% for A549 from

300 µg/mL peptide concentration upwards. These results confirm on one hand the dose-dependent cytotoxic activity on both cancerous cell lines, since from 0µg/mL to 300 µg/mL peptide concentration the numbers of viable cells display a strong negative correlation to the peptide concentration. From 300 µg/mL the inhibition rate continuously increases but with almost no incline.

On the other hand the obtained results indicate a time-dependent inhibition of cell proliferation. Whereas after 24 h for the SiHa cells only 20% maximum inhibitory effect could be detected and for A549 no clear inhibitory effect could be demonstrated, after 120h the cell inhibitory rates raised remarkably for both cell lines (80% for SiHa and ca. 90% for A549).

Analyzing the anti-proliferation effect of the lipopeptide fengycin on the SiHa and A549 cells, in general the data display similar results regarding dose- and time-dependent cytotoxic activity. Remarkable difference is shown for the 24 h treatment, where neither the A549 nor the SiHa cells show regular increase of anti-proliferation effect. Also here a longer incubation time might be necessary, to obtain regular dose-dependent growth inhibition.

After 5 days of treatment with fengycin the obtained results, as well as for iturin, point out to a dose- and time dependent growth inhibition of cancer cells. Similar to the iturin-results, a negative correlation between the increasing lipopeptide concentration and the decreasing numbers of viable cells for both cancerous cell lines are detectable between 0 µg/mL and 300 µg/mL, followed by a nearly stable inhibition rate of 80% for SiHa and 90% for A549 until a tested maximum lipopeptide concentration (1000 µg/mL).

Concerning the anti-proliferation effect of iturin and fengycin on the normal fibroblasts AS405, no clear evidence, neither positive nor negative, can be made. Throughout all cell viability tests a certain irregularity referring the viable cell numbers along increasing lipopeptide concentrations was detected.

Analyzing the datas of the 5 days incubation with iturin or fengycin, respectively, observations about the effect on AS405 could be made.

On one side, it can be seen for the tests with iturin as well as with fengycin that the cell viability rates for the AS405 cells do not decrease with rising peptide concentrations, like it

is demonstrated for the cancerous cell lines. In both treatments, the growth inhibition rate does not pass the 20% value, unlike 80% and 90% for SiHa or A549, respectively.
On the other side, the irregularity of the AS405 cell viability rates shows a lack of consistency regarding the possible correlation between an anti-proliferating effect and increasing peptide concentrations.
This in turn argues that the lower measured viability rates of the normal fibroblast cells, compared with the negative control of 0 µg/mL peptide content, are not causally related to the applied lipopeptide and their induced anti-proliferation activity.
As possible explanations for the observed irregularities might be on the one hand a unfortunate unstable distribution of fibroblast in the wells during the seeding process. On the other hand, as AS405 is a normal not cancerous cell line, the cells own characteristics for higher sensitivity and lower survival rate after isolating might be also a reason for diminished viability rates.
With regard to these points, it might be assumed that the lipopeptides do not show a anti-proliferating effect on normal AS405 fibroblasts.
The obtained results are supported by similar results for the anti-proliferative and apoptosis inducing activity of the related lipopeptide surfactin (Wang et al., 2007; Jeong et al., 2007; Liu et al.,2010; Lee et al., 2012). Wang showed a cytotoxic effect of surfactin on cancer cells K652, where the cytotoxicity involves mainly the induction of apoptosis, associated with cell arrest in G1 phase and caspapse-3 activation. In this study it was demonstrated that surfactin could inhibited cell growth in a time- and dose dependent manner (Wang et al., 2007). When K652 cells were incubated for 48 h with 64 µg/mL of surfactin, the growth inhibition reached more than 70%.
Comparable results were displayed by Jeong and coworkers (Jeong et al., 2007). The cell proliferation of different human colon carcinoma cells could be inhibited up to 90% after 72 h incubation time and 10 µg/mL surfactin concentration.
Nevertheless, it was also demonstrated by Wang and coworkers, that a surfactin concentration higher than 32 µg/mL would affected significantly the proliferation of normal cells, too (Wang et al., 2007).

As the used lipopeptides were applied in concentrations higher than 32 µg/mL, iturin and fengycin might show a basal anti-proliferation activity on normal cells because of higher lipopeptide content.
It might be shown, that also with higher concentrations than 32 µg/mL the negative effect on normal cells is still low and varies little with rising peptide concentrations.
Also it is necessary to test the anti-proliferating activities of iturin and fengycin in lower concentrations (< 100 µg/mL), to evaluate their effectiveness on cancer cells in low-dose ranges and their general effect on normal cells.

In conclusion for evaluating the anti-proliferating activity of the tested lipopeptides iturin and fengycin on the cell lines SiHa, A549 and AS405, it could be shown that iturin as well as fengycin present a clear inhibitory effect on the proliferation of cancerous cell lines A549 and SiHa, whereas, with reservations, an inhibitory effect on AS405 is not clearly demonstrated.
With regard to the presented evaluation and based on these first results on the anti-proliferating test for iturin and fengycin, both lipopeptides present a high inhibitory rate on cancer cells and though might be promising candidates for alternative anti-cancer treatments with low side effects.

Analyzing the anti-proliferative effect of the PTD4-VP3 isomers after 24 h of treatment with viral peptides, a higher increase of growth inhibition rates could be observed in cancer cells than in normal fibroblasts. Further, a dose-dependent anti-proliferating activity could be observed for both viral peptides.
Comparing the inhibitory effects on cancer cells of the Thais isolate with the Santa Maria isolate a higher effectiveness could be detected for Thais. In detail, the cancer line SiHa suffered in 24 h higher proliferation inhibition during the treatment with increasing contents of PTD4-VP3 (T) compared with the same conditions during the treatment with PTD4-VP3 (SM). Nevertheless, a stable inhibition rate for both tested viral peptides (90% for treatment with Thais; 60% for SM) could be observed for 800 µg/mL onwards.

In contrary, A549 does not present a difference for applied PTD4-VP3 isomers, the inhibitory rates for the Thais as well as for the Santa Maria isomer show very similar values.
Analyzing the influence of the PTD4-VP3 isomers on the normal fibroblasts the already mentioned complexities of unfortunate seeding and the characteristics of normal cell lines need to be taken into consideration and the observed growth inhibition rates of Thais and the increased cell viability rates of Santa Maria analyzed critically.
As the treatment was carried out for 24 h, a normal cell-cycle dependent mitosis might probably have taken place.
Analyzing the peptides influence on the AS405 cells, already mentioned complexities for this cell line need to be taken into consideration.
For the Thais isomer a clear decrease of the cell viability rates for AS405 could be observed till the final concentration (1000 µg/mL). This observation might indicate a anti-proliferating effect of the Thais VP3 isomer on normal fibroblasts.
In contrary, for the Santa Maria isomer an increase of viable cells was detectable until 800 µg/mL. Notwithstanding, for 1000 µg/mL a clear break (drop to ca.55%) on the viability rate is observed.
With regard to the already mentioned varying initial cell numbers, these increased cell numbers indicate that VP3 Santa Maria did not inhibit cell proliferation. The observed break could also be explained with a probable saturated dose of viral peptide, which similar to the saturated effect of lipopeptides results in a negative effect on healthy normal cells, too.

In 2011, Jin and coworkers showed similar results for dose-dependent increasing anti-proliferating activity of the PTD4-apoptin protein of the CAV virus (Jin et al., 2011). Three different melanoma cell lines and two normal cell lines were incubated with PTD4-apoptin. As Jin displayed in his study, the cancer cell lines were affected in a dose-dependent manner, whereas the normal cell lines did not suffer cell growth inhibition with rising PTD4-apoptin concentrations. After an incubation time of 48 h the cell survival rate for the used cell lines B16-F1, A875 and SK-MEL-5 (melanoma cells) dropped to 20% at a

concentraiton of 25 µg/mL, the hepatocytes L-02 and lung fibroblasts WI-38 did not show significant signs of growth inhibition (Jin et al., 2011).
Comparable results were obtained by Sun and coworkers (Sun et al., 2009). It could be shown that PTD4-apoptin induced selectively apoptosis in human cancer cells HepG2 (almost 60% apoptosis after 48 h), where no significant effect on L-02 cells could be detected under the same conditions. (Sun et al., 2009).
Regarding these results, the CAV apoptin showed only anti-proliferating activity in cancer cells, which compared to the obtained results in this present study shows more likely the Santa Maria isomer.

It could be demonstrated in this work, that the PTD4-VP3 Thais isomer has dose-dependent, partially a stronger effect on different cancer cells. But also the applied viral peptide concentrations showed a notable negative effect on normal fibroblasts as well. The Santa Maria isomer, in contrast, showed dose-dependent, but (for SiHa cells) weaker anti-proliferating activity, but up to a 800 µg/mL concentration of PTD4-VP3 (SM) no negative effect on cell proliferation of AS405.
Based on the obtained results for the recombinant PTD4-VP3 isomers, The PTD4-VP3 Santa Maria isomer seems to be the more promising PTD4-VP3 recombinant protein for further investigations for its anti-cancer activity with lower peptide concentrations (<100 µg/mL).

6. Conclusion

The present study is the first research work about the anti-proliferating effect of the lipopeptides iturin from *B. amyloliquefaciens* LBM5006, fengycin from *Bacillus* sp. P34 and the recombinant viral peptide isomers PTD4-VP3 Thais and Santa Maria of the *Avian Gyrovirus* II.

The aim of the study was to investigate and evaluate the anti-proliferative activity of the lipopeptides and the recombinant viral peptides, in order to provide possible new anti-cancer agents with low cytotoxic effects on healthy cells.

Whereas the lipopeptides were produced via medium-dependent enhanced native protein expression methods, followed by precipitation and SEC purification, the PTD4-VP3 proteins were obtained via IPTG induced overexpression for recombinant fusion proteins in BL21(DE3)pLysS *E. coli* cells, followed by IMAC purification and final cleavage of the SUMO-protein.

To test their cytotoxic activities, the peptides were separately incubated with two cancerous cell lines, A549 and SiHa, and with human fibroblasts AS405, to investigate their effect on healthy cells during simulated treatment conditions.

For the lipopeptides iturin and fengycin, a clear dose- and time-dependent cytotoxic activity on cancer cells could be determined. Under the same treatment simulation conditions for healthy cells a basal anti-proliferation activity could be detected, whereas the continuous low percentage of growth inhibition might be a result of high dosage of lipopeptides and should be investigated more detailed with peptide concentrations below 100 µg/mL.

The two PTD4-VP3 isomers Thais and Santa Maria showed both dose-dependent growth-inhibition on the used cancer cell lines A549 and SiHa. Analyzing their effect on AS405, the Thais isomer displayed inhibitory effect on AS405. Only the fusion protein PTD4-VP3 (SM) showed partially positive results as for un-stopped proliferation activity until a peptide concentration of 800 µg/mL.

Further treatment simulations need to be performed for iturin, fengycin and PTD4-VP3 (SM) with adjusted peptide concentrations to determine exact protein amounts for the optimized effect on cancer cells and further use as possible anti-cancer agent.

7. References:

AJANI, J.A., Chemotherapy and Radiation Resistance: Version 2.0, American Society of Clinical Oncology, 2009.

ALBERTS, B. et al., Cancer, Molecular Biology of the Cell. 5. ed. New York: Garland Science. Cap. 20, p. 1205-1268, 2007.

AMERICAN CANCER SOCIETY, www. cancer.org. latest access on 13th of August 2014.

AN, S. et al., Nonviral gene therapy in vivo with PAM-RG4/apoptin as a potential brain tumor therapeutic. International Journal of Nanomedicine, v. 8, p. 821- 834, 2013.

ASHKENAZI A., Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer. v. 2, p. 420-430, 2002.

ASHKENAZI A, HOLLAND P, ECKHARDT SG., Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/tumor necrosis factor–related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol. v. 26, p. 3621-3630, 2008.

BACKENDORF, C. et al., apoptin: therapeutic potential of and early sensor of carcinogenic transformation. Annual Review of Pharmacology and Toxicology, v. 48, p. 143-169, 2008.

BAKER SJ, MARKOWITZ S, FEARON ER, WILLSON JK, VOGELSTEIN B., Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249 (4971): 912–5, 1990.

BAUMANN, M., KRAUSE, M., HILL, R., Exploring the role of cancer stem cells in radioresistance, Nature Reviews Cancer v. 8, p. 545-554, 2008.

BAZZONI F, BEUTLER B., The tumor necrosis factor ligand and receptor families. N Engl J Med., v. 334, p. 1717-1725, 1996.

BENITEZ, L.B.; VELHO, R.V.; LISBOA, M.P.; MEDINA, L.F.; BRANDELLI, A., Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006. Journal of Microbiology (Seoul. Print), v. 48, p. 791-797, 2010.

BERGLIND, H., PAWITAN, Y., KATO, S., ISHIOKA, C., SOUSSI, T., Analysis of p53 mutation status in human cancer cell lines, A paradigm for cell line cross-contamination, Cancer Biology & Therapy v. 7, p. 699-708, 2008.

BESSLER WG, MITTENBUHLER K, ESCHER V, HUBER M., Lipopeptide adjuvants in combination treatment. Int Immunopharmacol, v. 3, p. 1217–1224, 2003.

BOONE DR, LIU Y, ZHAO ZJ, BALKWILL DL, DRAKE GR, STEVENS TO, ALDRICH HC, *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. Int J Syst Bacteriol v. 45, p. 441-448, 1995.

BREUKINK, E. & DE KRUIJFF, B., The lantibiotic nisin, a special case or not? Biochimica and Biophysica Acta, Amsterdam, v. 1462, p. 223-234, 1999.

BYEON et al., surfactin blocks NO production in Lipopolysaccheride-activated Macrophages by inhibiting NF-κB Activation, J. Microbiol. Biotechnol., v. 18, p. 1984-1989, 2008.

CAMEOTRA SS, MAKKAR RS., Recent applications of biosurfactants as biological and immunological molecules. Ecol Ind Microbiol, v. 7, p. 262–266, 2004.

CAPLICE, E.; FITZGERALD, G.F., Food fermetations: role of microrganims in food production and preservation. International Journal of Food Microbiology, Amsterdam, v. 50, n. 1-2, p. 131-149, 1999.

CAO, X., WANG, A.H., JIAO, R.Z., WANG, C.L., MAO, D.Z., YAN, L., ZENG, B., surfactin induces apoptosis and G(2)/M arrest in human breast cancer MCF-7 cells through cell cycle factor regulation, Cell Biochem Biophys v. 55, p. 163-171, 2009.

CAO, X.H., LIAO, Z.Y., WANG, C.L., CAI, P., YANG, W.Y., LU, M.F., HUANG, G.W., Purification and antitumor activity of a lipopeptide biosurfactant produced by *Bacillus natto* TK-1, Biotechnol Appl Biochem v. 52, p. 97-106, 2009.

CAO, X.H, WANG, A.H., WANG, C.L., MAO, D.Z., LU, M.F., CUI, Y.Q., JIAO, R.Z., surfactin induces apoptosis in human breast cancer MCF-7 cells through ROS/JNK mediated mitochondrial/caspase pathway, Chem Biol Interact v. 183, p. 357-362, 2010.

CHANG, C., SIMMONS, D.T., MARTIN, M.A., MORA, P.T., Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. J Virol., v. 31, p. 463–471, 1979.

CHIAL, H., Proto-oncogenes to Oncogenes to Cancer. Nature Education, v. 1, p. 33, 2008.

CHIAL, H.,Tumor suppressor (TS) genes and the two-hit hypothesis. Nature Education, v. 1, p. 177, 2008.

CHO, S.J., HONG, S.Y., KIM, J.Y., PARK, S.R. KIM, M.K., LIM, W.J. et al., Endophytic *Bacillus sp.* CY22 from balloon flower (Platycodon grandiflorum) produces surfactin isoforms, Journal of Microbiology and Biotechnology, v. 13, p. 859-865, 2003.

COTTER, P.D.; HILL, C.; ROSS, P.R., Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology, London, v.3, p. 777-788, 2005.

DANEN-VAN OORSCHOT, A. A. A. M. et al., BAG-1 inhibits p53-induced but not apoptin-induced apoptosis. Apoptosis, v. 2, p. 395-402, 1997.

DANEN-VAN OORSCHOT, A. A. A. M. et al., Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. Journal of Biological Chemistry, v. 278, p. 27729-27736, 2003.

DANEN-VAN OORSCHOT, A. A. A. M.; VAN DER EB, A. J.; NOTEBORN, M. H. M., Bcl-2 stimulates apoptin®-induced apoptosis. Drug Resistance in Leukemia and Lymphoma, v. 457, p. 245-249, 1999.

DANEN-VAN OORSCHOT, A. A. A. M.; VAN DER EB, A. J.; NOTEBORN, M. H. M., The chicken anemia virus-derived protein apoptin requires activation of caspases for

induction of apoptosis in human tumor cells. Journal of Virology, v. 74, p. 7072-7078, 2000.

DE MARTEL C, FERLAY J, FRANCESCHI S, Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. The Lancet Oncology, v. 13, p. 607-615, 2012.

DING, Q. ET AL., The nucleocytoplasmic transport of viral proteins. Virologica Sinica, v. 25, p. 79-85, 2010

DOS SANTOS, H. F. et al., Variants of the recently discovered *avian gyrovirus* 2 are detected in Southern Brazil and The Netherlands. Veterinary Microbiology, v. 155, p. 230-236, 2012.

DU C., FANG M., LI Y., LI L., WANG X., Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell , v. 102, p. 33–42, 2000.

EARNSHAW W. C., Nuclear changes in apoptosis. Current Opinion in Cell Biology, v. 7, p. 337-343, 1995.

ELIYAHU D, RAZ A, GRUSS P, GIVOL D, OREN M., Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature, v. 312, p. 646–649, 1984.

ELMORE S., Apoptosis: a review of programmed cell death. Toxicol Pathol., v. 35, p. 495-516, 2007.

ESPOSTI, M. D., The roles of Bid. Apoptosis, v. 7, p. 433-440, 2002

ESCÁRCEGA RO, FUENTES-ALEXANDRO S, GARCÍA-CARRASCO M, GATICA A, ZAMORA A, The transcription factor nuclear factor-κB and cancer. Clinical Oncology (Royal College of Radiologists (Great Britain)), v. 19, p. 154–61, 2007.

FANG, X. et al., Combination of apoptin with photodynamic therapy induces nasopharyngeal carcinoma cell death *in vitro* and *in vivo*. Oncology Reports, v. 28, p. 2077-2082, 2012.

FAVAROLO, B. et al., Role of apoptosis in disease. Aging, v. 4, p. 330-349, 2012.

FERLAY, J. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available on : <http://globocan.iarc.fr>, accessed, 30th of May, 2014.

FINLAY CA, HINDS PW AND LEVINE AJ, The p53 proto-oncogene can act as a suppressor of transformation. Cell, v. 57, p. 1083-1093, 1989.

FULLER, R., Probiotics in human medicine, Gut, v. 32, p. 439-442, 1991.

GARRIDO, C. ET AL., Mechanisms of cytochrome c release from mitochondria. Cell Death and Differentiation, v. 13, p. 1423-33, 2006.

GOTO K, OMURA T, HARA Y, SADAIE Y, Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J Gen Appl Microbiol, v. 46, p. 1-8, 2000.

GOTTESMANN, M. M., Mechanisms of cancer drug resistance. Annual Review of Medicine, v. 53, p. 615-627, 2002.

GUELEN, L. et al., TAT-apoptin if efficiently delivered and induces apoptosis in cancer cells. Oncogene, v. 23, p. 1153-1165, 2004.

HARRIS C., p53 - at the crossroads of molecular carcinogenesis and risk assessment. Science, v. 262, p. 1980–1981, 1993.

HEILMAN DW, TEODORO JG, GREEN MR, Apoptin nucleo- cytoplasmic shuttling is required for cell type-specific localization, apoptosis, and recruitment of the anaphase-promoting complex/cyclosome to PML bodies. J Virol, v. 80, p. 7535–7545, 2006.

HENGARTNER, M. O., The biochemistry of apoptosis. Nature, v. 407, p. 770- 776, 2000.

HIRADATE S, YOSHIDA S, SUGIE H, YADA H, FUJII Y, Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry v. 61, p. 693-698, 2002.

HU, L.B., Z.Q. SHI, T. ZHANG, AND Z.M. YANG, fengycin antibiotics isolated from B-FS01 culture inhibiting the growth of *Fusarium moniliforme* Sheldon ATCC 38932. FEMS Microbiol. Lett., v. 272, p. 91-98, 2007.

INTERNATIONAL ASSOCIATION OF CANCER REGISTRIES - IACR. Section of Cancer Information (CIN), Biennial Report 2010/2011, p. 27 - 39, Lyon France; Available on <http://www.iarc.fr/en/publications/>, accessed, 2nd of June, 2014.

IZADPANAH, A.; GALLO, R.L.,Antimicrobial peptides. Journal of the American Academy of Dermatology, St. Louis, v. 52, p. 381-390, 2005.

JEMAL, A. et al., Global cancer statistics. CA: A Cancer Journal for Clinicians, v. 61, p. 69-90, 2011.

JENKINS JR, RUDGE K, CHUMAKOV P, CURRIE GA., The cellular oncogene p53 can be activated by mutagenesis. Nature, v. 317, p. 816–818, 1985.

JENKINS JR, RUDGE K, CURRIE GA., Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature, v. 312, p. 651–654, 1984.

JEONG, S.-Y., PARK, S.-Y., KIM, Y.-H., KIM, M., LEE, S.-J., Cytotoxicity and apoptosis induction of *Bacillus vallismortis* BIT-33metabolites on colon cancer carcinoma cells, Journal of Applied Microbiology, v. 104, p. 796-807, 2007.

JIN, J. et al., PTD4-apoptin protein and dacarbazine show a synergistic antitumor effect on B16-F1 melanoma *in vitro* and *in vivo*. European Journal of Pharmacology, v. 654, p. 17-25, 2011.

KAMEDA Y, OIRA S, MATSUI K, KANATOMO S, HASE T., Antitumor activity of *Bacillus natto*. V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. Chem Pharm Bull (Tokyo), v. 22, p. 938–44, 1974.

KIM, S.Y., KIM, J.Y., KIM, S.H., BAE, H.J., et al., surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression, FEBS Letters 581, p. 865-871, 2007.

KISCHKEL, F. C. et al., Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO Journal, v. 14, p. 5579-88, 1995.

KLAENHAMER, T.R., Bacteriocins of lactic acid bacteria. Biochimie, Paris, v. 70, p. 337-349, 1988.

KLUGE, B., VATER, J., SALNIKOV, J., ECKART, K., Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. FEBS Lett. 231, p. 107-110, 1988.

KNAK, M.B., LUZ DE CASTRO, F., SLONGO, J., DOS SANTOS, H.F., MENGUE SCHEFFER, C., ROEHE, P.M., FRANCO, A.C., Clonagem e expressão da proteina VP3 codificada pelo girovírus aviário tipo 2 (AGV2), Salão de Iniciação Científica (23. : 2011 out. 3-7 : UFRGS, Porto Alegre, RS).

KNUDSON A, Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A, v. 68, p. 820–823, 1971.

KRESS M, MAY E, CASSINGENA R, MAY P, Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. Virol J, v. 31, p. 472–483, 1979.

KRISHNAN, B. & MORGAN CANCER G.J., Non-Hodgkin Lymphoma Secondary to Cancer Chemotherapy, Epidemiol Biomarkers Prev., v. 16, p. 377-380, 2007.

KUROSAKA, K. et al.,Silent cleanup of very early apoptotic cells by macrophages. Journal of Immunology, v. 171, p. 4672-4679, 2003.

LANE, D.P., Cancer. p53, guardian of the genome. Nature, v. 358, p. 15-6, 1992.

LANE D., p53 from pathway to therapy. Carcinogenesis, v. 25, p. 1077-1081, 2004.

LANE DP, CRAWFORD LV, T antigen is bound to a host protein in SV40-transformed cells. Nature, v. 278, p. 261–263, 1979.

LANZ, H. L. ET AL., Proteasomal insensitivity of apoptin in tumor cells. Biochemical and Biophysical Research Communications, v. 422, p. 169- 173, 2012.

LIN, H.Y., RAO, Y.K., WU, W.S., TZENG, Y.M., Ferrous ion Enhanced Lipopeptide Antibiotic iturin A Production from *Bacillus amyloliquefaciens* B128, International Journal of Applied Science and Engineering, v. 2, p. 123-132, 2007.

LISBOA, M.P., BONATTO. D., BIZANI, D., HENRIQUES, J.A.P., BRANDELLI, A., Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic Forest. Int Microbiol, v. 9, p. 111-118, 2006.

LEE, J.H., NAM, S.H., SEO, W.T., YUN, H.D., HONG, S.Y., KIM, M.K., CHO, K.M., The production of surfactin during the fermentation og cheonggukjang by potential probiotic *Bacillus subtilis* CSY191 and the resultant growth suppression of MCF-7 human breast cancer cells, Food Chemistry, v. 131, p. 1347-1354, 2011.

LELIVELD, S. R. et al. apoptins functional N- and C-termini independently bind DNA. FEBS Letters, v. 557(1-3), p. 155-158, 2004.

LEWIN, B. et al., Oncogenes and cancer. Genes VIII. 8. ed. Upper Saddle River: Pearson Prentice Hall, cap. 30, p. 889-937, 2004.

LI, J. et al., TAT-apoptin induces apoptosis in the human bladder cancer EJ cell line and regulates Bax, Bcl-2, caspase-3 and surviving expression. Experimental and Therapeutic Medicine, v. 3, p. 1033-1038, 2012.

LI, X. et al., Potent anti-tumor effects of a dual specific oncolytic adenovirus expressing apoptin in vitro and in vivo. Molecular Cancer, v. 9, p. 1-12, 2010.

LIU, F. S., Resistance in cancer therapy - a quick review. Taiwanese Journal of Obstetrics and Gynecology, v. 48, p. 239-244, 2009.

LIU, Q. et al., Survivin knockdown combined with apoptin overexpression inhibits cell growth significantly, Cancer Biology and Therapy, v. 7, p. 1053-1060, 2008.

LIU, X., TAO, X., ZOU, A., YANG, S., ZHANG, L., MU, B., Effect of the microbial lipopeptide on tumor cell lines: apoptosis induced by disturbing the fatty acid composition of cell membrane, Protein Cell, v. 1, p. 584-594, 2010

LOCKSLEY, R. M. et al., The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell, v. 104, p. 487-501, 2001.

LOS M, PANIGRAHI S, RASHEDI I, MANDAL S, STETEFELD J, ESSMANN F, SCHULZE-OSTHOFF K, apoptin, a tumor-selective killer. Biochim Biophys Acta, v. 1793, p. 1335–1342, 2009.

MADDIKA, S. et al., Cancer-selective therapy of the future. Cancer Biology and Therapy, v. 5, p. 10-19, 2006.

MALHOTRA, V., PERRY, M.C., Models of anti-cancer therapy, Classical Chemotherapy, Mechanisms, Toxicities and the therapeutic window, Cancer Biology & Therapy, v. 2, 4 Suppl. 1, S2-S4, p. 1-3, Landes Bioscience, 2003

Manjo, G. & Joris, I., Apoptosis, oncosis and necrosis. An overview of cell death. Am J Pathol., v. 146, p. 3-15, 1995.

MAYR-HARTING, A., HEDGES, A.J., BERKLEY, R.C.W., Methods for studying bacteriocins, In Methods in Microbiology, Vol 7. ed. Norris JB, Ribbons D. New York: Academic Press, p. 315-412, 1972.

MCELHANEY, R. N.; PRENNER, E. J., The interaction of antimicrobial peptides with model lipid bilayer and biological membranes. Biochimica et Biophysica Acta, Netherlands, v. 1462, p.1-234, 1999.

MICHALOVITZ D, HALEVY O AND OREN M., p53 mutations - gains or losses. J. Cell. Biochem., v. 45, p. 22-29, 1991.

MOWAT M, CHENG A, KIMURA N, BERNSTEIN A, BENCHIMOL S., Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. Nature, v. 314, p. 633–636, 1985.

MOTTA, A. S., CANNAVAN, F.S., · SIU-MUI TSAI, S.M., BRANDELLI, A., Characterization of a broad range antibacterial substance from a new *Bacillus* species isolated from Amazon basin, Arch Microbiol, v. 188, p.367–375, 2007.

NATIONAL CANCER INSTITUTE, www.cancer.gov, latest access on 21st of August 2014.

NIGRO, J.M.; BAKER, S.J.; PREISINGER A.C.; JESSUP, J.M.; HOSTETTER, R.; CLEARY, K.; BIGNER S.H.; DAVIDSON, N.; BAYLIN, S.; DEVILEE, P. et al, Mutations in the p53 gene occur in diverse human tumour types. Nature., v. 342, p. 705-708, 1989.

NIU, W.W.; NEU, H.C., Activity of mersacidin, a novel peptide, compared with that of vancomycin, teicoplanin, and daptomycin. Antimicrobial Agents and Chemotherapy, Washington, v. 35, n. 5, p. 998-1000, 1991.

NOTEBORN, M. H. M. et al., A single chicken anemia virus protein induces apoptosis. Journal of Virology, v. 68, p. 346-351, 1994.

NOTEBORN, M. H. M.; DANEN-VAN OORSCHOT, A. A. A. M.; VAN DER EB, A. J., The apoptin® gene of chicken anemia virus in the induction of apoptosis in human tumorigenic cells and in gene therapy of cancer. Gene Therapy and Molecular Biology, v. 1, p. 399-406, 1998.

NOTEBORN, M. H. M., Apoptin acts as a tumor-specific killer: potentials for a anti-tumor therapy. Cell Mol Biol (Noisy-le-grand), v. 51, p. 49-60, 2005.

NOTEBORN MH., Proteins selectively killing tumor cells. Eur J Pharmacol v. 625, p. 165–173, 2009.

OLIJSLAGERS, S. et al., Additive effect of apoptin and chemotherapeutic agents paclitaxel and etoposide on human tumour cells. Basic and Clinical Pharmacology and Toxicology, v. 100, p. 127-131, 2007.

OLIVEIRA SS, ABRANTES J, CARDOSO M, SORDELLI D, BASTOS MCF, Staphylococcal strains involved in mastitis are inhibited by *Staphylococcus aureus* antimicrobial peptides. Lett Appl Microbiol v. 27, p. 287-291, 1998.

PALYS T, NAKAMURA LK, COHEN FM, Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int J Syst Bacteriol v. 47, p. 1145-1156, 1997.

PAN, Y. et al., Antitumor effects of a recombinant pseudotype baculovirus expressing apoptin *in vitro* and *in vivo*. International Journal of Cancer, v. 126, p. 2741-2751, 2010.

PARADA LF, LAND H, WEINBERG RA, WOLF D, ROTTER V., Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature v. 312, p. 649–651, 1984.

PIETERSEN, A. M. et al., Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. Gene Therapy, v. 6, p. 882-892, 1999.

POON, I. K. et al., Apoptin nuclear accumulation is modulated by a *CRM1*- recognized nuclear export signal that is active in normal but not in tumor cells. Cancer Research, v. 65, p. 7059-7064, 2005.

RANG, H. P. et al., Fármacos anticâncer. Farmacologia. 7. ed. Rio de Janeiro: Elsevier. Cap. 55, p. 673-688, 2012.

REICH, N. & LEVINE, A.J., Growth regulation of a cellular tumor antigen, p53, in nontransformed cells. Nature v. 308, p. 199-201, 1894.

RENEHAN, A. G., What is apoptosis, and why is it important?. British Medical Journal, v. 322, p. 1536-1538, 2001.

RIJSEWIJK, F. A. M., DOS SANTOS, H. F., TEXEIRA, T. F., CIBULSKI, S. P., VARELA, A. P. M., DEZEN, D., FRANCO, A. C., ROEHE, P. M., Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus, Springer-Verlag 2011 Arch Virol, v. 156, p. 1097–1100, 2011.

RUSSO, A. et al., Apoptosis: a relevant tool for anticancer therapy. Annals of Oncology, v. 17, p. 115-123, 2006.

SABLON, E.; CONTRERAS, B.; VANDAMME, E., Antimicrobial peptides of Lactic Acid Bacteria: Mode of Action, Genetic and Biosynthesis. Advances in Biochemical Enginnering/Biotechnology, Berlin, v. 68, p. 21-60, 2000.

SAELENS, X. et al., Toxic proteins released from mitochondria in cell death. Oncogene, v. 23, p. 2861-2874, 2004.

SCHOOP, R. A. A. L. et al., Bcl-xL inhibits p53- but not apoptin-induced apoptosis in head and neck squamous cell carcinoma cell line. Carcinogenesis, v. 109, p. 38-42, 2004.

SCHULZ-ERTNER D, JÄKEL O, SCHLEGEL W., Radiation therapy with charged particles. Seminars in Radiation Oncology, v. 16, p. 249–259, 2006.

SINGH P, CAMEOTRA SS., Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol v. 22, p. 142–6, 2004.

SHOHAT O, GREENBERG M, REISMAN D, OREN M AND ROTTER V., Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. Oncogene v. 1, p. 277-283, 1987.

STACKEBRANDT E, GOEBEL BM, Taxonomic note: a place of DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol v. 44, p. 846-849, 1994.

SUN, J. et al., PTD4-apoptin protein therapy inhibits tumor growth *in vivo*. International Journal of Cancer, v. 124, p. 2973-2981, 2009.

TAEBUNPAKUL, P. et al., Apoptin induces apoptosis by changing the equilibrium between the stability of TAp73 and ΔNp73 isoforms throughubiquitin ligase PIR2. Apoptosis, v. 17, p. 762-776, 2012.

TAKAHASHI T, NAU MM, CHIBA I, BIRRER MJ, ROSENBERG RK, VINOCOUR M, LEVITT M, PASS H, GAZDAR AF, MINNA JD., p53 - a frequent target for genetic abnormalities in lung cancer. Science v. 246, p. 491–494, 1989.

TAYLOR A, POWELL ME., Intensity-modulated radiotherapy—what is it? Cancer Imaging, v. 4, p. 68–73, 2004.

THOMPSON, C. B., Apoptosis in the pathogenesis and treatment of disease. Science, v. 267, p. 1456-1462, 1995.

TSUGE, K., T. AKIYAMA, AND M. SHODA, Cloning, sequencing, and characterization of the iturin A operon. J. Bacteriol. v. 183, p. 6265-6273, 2001.

TWOMEY DP, WHEELOCK AI, FLYNNJ, MEANEY WJ, HILL C, ROSS RP, Protection against Staphylococcus aureus mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin lacticin 3147. J Dairy Sci v. 83, p. 1981–1988, 2000.

VAN DER EB, M. M. et al., Gene therapy with apoptin induces regression of xenografted human hepatomas. Cancer Gene Therapy, v. 9, p. 53-61, 2002.

VIZCARDO, et al., Cell Stem Cell. Brief Report: Regeneration of Human Tumor Antigen-specific T-Cells form iPSCs derived from mature CD8+ T-Cells, Cell Stem Cell, v. 12, p. 31-36, 2013.

VOGELSTEIN B., LANE D., LEVINE A.J., Surfing th p53 netowrk. Nature, v. 408, p. 307–310, 2000.

VOLLENBROICH, D., ÖZEL, M., VATER, J., KAMP, R.M., PAULI, G., Mechanismen of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals v. 25, p. 289-297, 1997.

VOUSDEN, K.H., p53: death star. Cell, v. 103, p. 691-694, 2000.

WAJANT, H., The Fas signaling pathway: more than a paradigm. Science, v. 296, p. 1635-1636, 2002.

WAKAMATSU Y, ZHAO X, JIN C, DAY N, SHIBAHARA M, NOMURA N, et al., Mannosylerythritol lipid induces characteristics of neuronal differentiation in PC12 cells through an ERK- related signal cascade. Eur J Biochem, v. 268, p. 374–83, 2001.

WANG C.L., NG T.B., YUAN F., LIU Z.K., LIU F., Induction of apoptosis in human leukemia K562 cells by cyclic lipopeptide from *Bacillus subtilis* natto T-2, Peptides 28, published by Elsevier Inc., p. 1344–1350, 2007.

WHO: The Global Burden of Disease: 2004 update. Geneva: World Health Organization; 2008.

YOSHIDA BA, SOKOLOFF MM, WELCH DR, RINKER-SCHAEFFER CW., Metastasis-suppressor genes: a review and perspective on an emerging field. J. Natl. Cancer Inst. V. 92, p. 1717–30, 2000.

YUAN, L. et al., Apoptin selectively induces the apoptosis of tumor cells by suppressing the transcription of HSP70. Tumor Biology, v. 34, p. 577-585, 2013.

YUASA, N.; TANIGUCHI, T.; YOSHIDA, I., Isolation and some properties of an agent inducing anaemia in chicks. Avian Diseases, v. 23, p. 366-385, 1979.

ZANELLA, J. R. C.; BRENTANO, L., *Circoviridae*. In: Flores, E.F., org. Virologia veterinária. 2. ed. Santa Maria: UFSM. Cap. 14, p. 419-438, 2012.

ZHANG, Y. H. et al., Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen**.** Journal of Virology, v. 78, p. 9965-9976, 2004.

ZHUANG, S. et al. (1995). Differential sensitivity to Ad5 E1B-21kD and Bcl-2 proteins of apoptin-induced versus p53-induced apoptosis. Carcinogenesis, v. 16, p. 2939-2944, 1995.

ZHOU, S. et al., Mechanisms of apoptin-induced cell death. Medical Oncology, v. 29, p. 2985-2991, 2012.