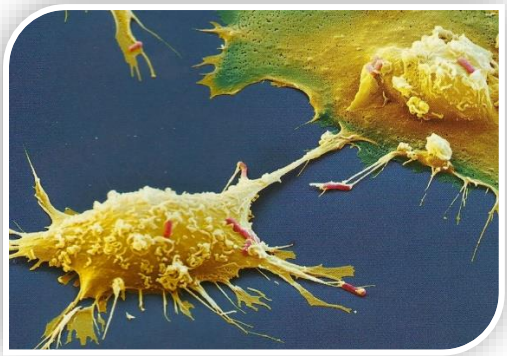
GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI



PHD THESIS SUMMARY

The modeling effect of some cytotoxic peptides on tumor cells metabolism

PhD Leader:
Prof. Dr. Magda BĂDESCU

Phd Candidate:
Bogdan Mihail DIACONESCU
Asst. Prof. Pathophysiology

2018

Contents

PERSONAL PART
MOTIVATION OF THE STUDY

Despite recent advances in treatment, cancer remains a major source of morbidity and mortality worldwide. Although some cases of cancer can often be treated successfully by surgery and / or radiotherapy, chemotherapy remains the usual treatment. It is known that most of the available cytostatics allow for control of tumor growth only at concentrations that affect healthy cells, resulting in undesirable side effects. Thus, it is imperative to find new products with new mechanisms of action, and one of the current research directions is the use of cytotoxic antimicrobial peptides. These could constitute new tumoricidal molecules that could be used in adjuvant cancer therapy, with the potential to reduce the doses of the cytostatic and their toxic side effects.

In the experimental studies conducted within this thesis we want to verify the hypothesis that cytotoxic peptides such as dermaseptin, lycotoxin I and II have a tumoricidal potential and whether the magnitude of the effect depends on the nature of the peptide used and its concentration in the cell's living environment , but also the cell line type used experimentally *in vitro*.

We proposed to analyze the biological effect of these peptides (dermaseptin, lycotoxin 1 and 2) on tumor cell lines: MDA-MB231 (mammary adenocarcinoma), M14K (human mesothelioma), HT-29 (colorectal adenocarcinoma) 549 (human alveolar carcinoma). In order to determine the modulating mechanism of the cytotoxic peptides studied, it is necessary to evaluate the viability and gene expression of the chosen molecular targets (Akt, HIF-1alpha XBP, NRF2, PERK, CHOP, BCL2, IRE1 alpha, PI3K) in the molecular pathways of survival, growth, proliferation and apoptosis of tumor cells in the presence or absence of the studied peptides, aspects not yet described in the literature.

PURPOSE AND OBJECTIVES FOLLOWED

PURPOSE: chemotherapy is a necessary method of treating cancer, but it also involves many side effects, sometimes hard to bear by the patient. The purpose of this doctoral thesis was to find new molecules that have tumoricidal potential and could be used in adjunctive cancer therapy in the long run without side effects but which would potentiate the effects of a chemotherapy with the possibility of reduces the dose of the cytostatic, in the sense that the toxic side effects of these drugs could be controlled more easily and even reduced.

General objectives

1. Establishing the experimental model by using tumor cell lines.
2. Choosing cytotoxic peptides with tumoricidal potential.
3. Establishment and optimization of cell viability assessment methods under the action of cytotoxic peptides.
4. Selection of molecular targets and optimization of molecular biology methods for the determination of gene expressions, which highlight the influence of these peptides on tumor cells in the ways of cell survival, growth and proliferation, with evaluation and pro and anti-apoptotic pathways.

Specific objectives

1. Establishment of tumor cell lines and optimization of the cellular experimental model. Adherent tumoral lines: HT-29 (colorectal adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), A-549 (pulmonary alveolar carcinoma) and suspension cell line: M14K (mesothelioma), all of these tumor lines being of human origin.
2. Selection of peptides with cytotoxic potential in correlation with data from the literature: dermaseptin and lycotoxin 1 and 2.
3. Choosing methods and optimizing them for cell viability determination: A colorimetric vital method (MTT) so that the lowest common concentrations of the tested cell lines can be selected, where the peptide has a significant cytotoxic effect, to make statistical comparisons with higher cytotoxicity tested and common to the cell lines tested.

4. Cellular viability assessment for selected peptide concentrations by flow cytometry technique.
5. Selection of molecular target-specific primers (Akt, HIF-1alpha XBP, NRF2, PERK, CHOP, BCL2, IRE1 alpha, PI3K) selected for gene expression assay.
6. Determination of the modulating effect of cytotoxic peptides on the molecular pathways of survival, growth and proliferation of tumor cells in the presence or absence of the studied peptides, by determining the gene expression for the chosen molecular targets.

MATERIALS, METHODS AND TECHNIQUES

Four adherent cell lines were used:

HT-29 is a colorectal adenocarcinoma adherent cell line. HT-29 cells produce embryonic carcinoma antigen, are human intestinal epithelial cells producing IgA secreting component.

MDA-MB231 is a mammary adenocarcinoma cell line. MDA-MB231 cells are epithelial cells.

A-549 is an adherent cell line of human alveolar carcinoma.

Human mesothelioma (**M14K** line) is a very aggressive malignant tumor. Of the 3 histological mesothelioma subtypes, the M14K mesothelial cell line of the epithelial type was used.

All tumor cell lines were cultured in RPMI-1640 medium (*Sigma Aldrich*) with 10% SFV (*Faecal Bovine Serum*, GIBCO) [Hilchie, 2011]. Only cells with viability greater than 97% were used. The viability study was performed after 24 hours of incubation at 37 ° C, 5% CO2, using the vital MTT dye.

Methods and techniques for testing the viability of cell cultures

The COLORIMETRIC viability assay using MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] is a cellular metabolism assay based on the capacity of succinate mitochondrial dehydrogenases in living cells to reduces soluble tetrazolium salts from **MTT** (*yellow*) and form insoluble crystals of formazan (dark violet). Exposure to cytotoxic peptide was performed for 24h, 48h, 72h. Work in triplicate for each cell line. **Negative control** was constituted by cells in the wells in which no cytotoxic peptide was added, so that a

comparison could be made between the evolution of peptide-treated and untreated cells.

Evaluation of cellular apoptosis by flow cytometry technique. Flow cytometry is a technique of counting and examining cells by suspending them in a flow of fluid and passing them individually through a concentrated laser beam over an extremely small surface. As the cells pass through the laser beam, each particle will disperse the incident light and emit fluorescence depending on the fluorescent organic compounds used.

The flow cytometry technique is also used for the detection and measurement of the apoptotic process [Vermes et al, 2000]. One of the early events of apoptosis is the translocation of phosphatidylserine (PS), membrane phospholipid, from the inner lipid layer into the outer layer of the lipid, which can be highlighted by flow cytometry technique using Anexine V that will form a complex fluorescence with phosphatidylserine [Vermes et al, 2000; Koopman et al., 1994]. Therefore, ANEXINE V and 7-AAD or propidium iodide (PI) were used as flow cell cytotoxicity tracking markers. Cells in early apoptosis are positive for Annexin V and negative for PI because, although PS translocations have occurred, the plasma membrane is intact so that PI fluorochrome was unable to penetrate the cell. If the cells are in late or dead apoptosis, they are positive for both Annexin V and PI because the plasma membrane has lost its integrity, and PI flurocromate has reached a nuclear level [Vermes et al, 2000; Raynal et al., 1994; Schmid et al., 1992].

Molecular biology techniques for detecting metabolic changes in tumor cells by evaluating gene expression for certain molecular targets.

RNA extraction from tumor cell cultures was performed by automated methods with RNA extraction kits appropriate for the Magnesia 16 automatic extractor from the molecular biology laboratory. The RNA solution was stored at -20 ° C until reverse-transcription was performed in the cDNA. Reverse transcription is a process of copying the RNA template into cDNA (complementary DNA) that will then be amplified. This process was performed using the **Thermo Scientific - RevertAid First Strand cDNA Synthesis Kit**

and the **SuperScript ™ IV First-Strand Synthesis System**. The complementary DNA thus obtained can be used immediately or stored for a shorter time at -20 ° C or long-term at -70 ° C. The purity of the introduced DNA as a template is an important parameter for the success of a PCR reaction.

Real-Time PCR - RT-PCR is also called quantitative real time PCR (Q-PCR / qPCR / qrt-PCR) or kinetic PCR (KPCR), a PCR variant in which amplification (increase in number of amplicons) real time using fluorescence.

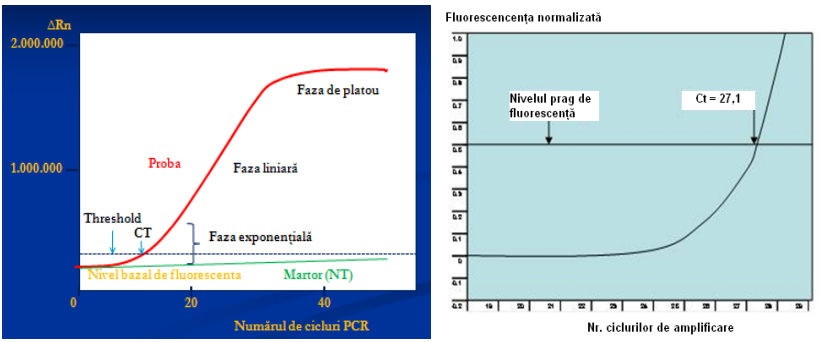


Fig.20: *The quantification can be absolute (Ct or number of molecules) or relative (the result obtained is reported as a constant value).*

To interpret the results obtained by this method, two parameters are needed:

♣ *Threshold* (threshold value) - is set during the exponential phase of the amplification and represents the line joining the points in which the efficiency of the reaction is maximal from one cycle to another.

♣ The threshold cycle is calculated according to the threshold value and represents the number of cycles needed to make the fluorescent signal exceed a predetermined threshold value. The value of the Ct is inversely proportional to the number of copies of DNA.

Relative quantification consists in tandem amplification (standards and sample of interest) of a house-keeping gene that is expressed equally in all cells of the body.

Quantitative detection by Real-Time PCR using the SYBR green method. SYBR GREEN - is a fluorochrome that binds nonspecifically to double-stranded DNA.

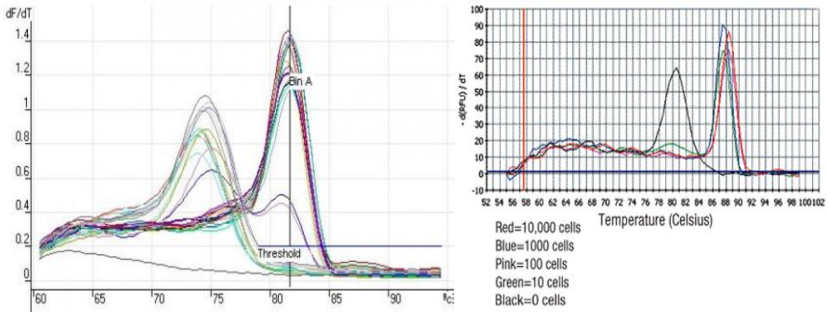


Fig.21: *Melting curve for detection of positive samples having a melting point of 83 ° C. At 75 ° C, the peaks reflect primer dimers.*

This method requires a melting curve at the end of the amplification, which translates into a slow temperature gradient (1 ° C to ~ 2-3 seconds) starting from the hybridization temperature of the primers to the temperature denaturing the entire reaction mixture. In each denaturation step, the fluorescence obtained by melting the reaction products (Fig. 21) will be read. Each product resulting in the amplification reaction thus produces a melting peak. Depending on the melting temperature of the product (s) and the number of peaks obtained, the specificity of the reaction can be interpreted.

RESULTS

Determination of cytotoxicity of dermaseptin and lycotoxin

For the **HT-29** tumor line, a statistically intense statistically significant cytotoxicity ($p < 0.001$) was observed at the 4 μM peptide concentration by the MTT colorimetric technique, which was demonstrated by repeating the experiment (in triplicate) and determining cell viability by the flow cytometry technique , when a significant 21% apotose was obtained for incubating these cells for 48h with dermaseptin. By incubating cells with peptide at high concentrations of 15μM and 20μM, the cytotoxicity was also statistically intense, being demonstrated by both the MTT ($p < 0.001$) and the flow cytometry method, demonstrating an apoptosis of 57 % at a 15 μM peptide concentration with a 10% cell death for the control (cells that were not incubated with the peptide).

For the A549 lung alveolar carcinoma tumor cell, a statistically intense statistically significant cytotoxicity ($p < 0.001$) was observed at the 4 μM peptide concentration by MTT colorimetry technique, which was demonstrated by the flow cytometry technique when a significant apoptosis of 12 % for incubating these cells for 48h with dermaseptin. By incubating cells with peptide at high concentrations of 15μM and 20μM, the cytotoxicity was also statistically intense, being demonstrated by both the MTT ($p < 0.001$) and the flow cytometry method, which demonstrated a 32-fold apoptosis % with a 10% cell death at a peptide concentration of 15 μM.

For the MDA MB-231 breast adenocarcinoma tumor line and for the M14K tumor line - the cytotoxicity of dermaseptin was statistically weakly significant at peptide concentrations of 8 μM ($p < 0.05$) and insignificant at 4 μM, respectively 15 μM and due to reasons not continued with peptide testing on these tumor cell lines.

In the case of lycotoxin 1, after a 48-hour incubation, a significant apoptosis was observed by flow cytometry technique only at high peptide concentrations (15 μM) compared to the result obtained for untreated cells. It is worth noting that lycotoxin 1 has seen a rise in the mean values of their abilities in the sense of their duplication. This suggested increasing the number of cells, but flow cytometry revealed

that approximately 83.67% of these cells were in late apoptosis compared to incubating these cells at 4uM concentrations for which the behavior of the cells was similar to that of the negative witness. For this reason, it is appropriate to evaluate cell viability by at least two different methods.

Gene expression modifications of selected molecular targets under dermaseptin

In this chapter are presented experimental results obtained for HT-29 and A549 tumor cell lines for which molecular expression was determined by molecular biology techniques of molecular targets involved in tumor cell metabolism reprogramming both in the presence and in the absence of peptides dermaseptin and lycotoxin) with tumoricidal potential. These molecular targets refer to pro-apoptotic (CHOP, XBP1, IRE1alfa, PERK) and anti-apoptotic (BCL2) genes involved in proliferation (NRF2) and survival (AKT, HIFα, PIK3) cells.

All biomolecular techniques have been developed in the laboratory, being home optimized techniques. The technique was Real Time (RT) -PCRsauq RT-PCR, reporting the results obtained with the GAPDH and ABL reference gene. In order to quantify the results, standards (known concentrations of DNA) from the genetic material extracted from the control cells (untreated with peptide) were established and the results were reported as percentages of increase or decrease of the gene expression compared to the control, reported to the reference gene (ABL or GAPDH).

By RT-PCR amplification, amplicons of interest of different concentrations were obtained which were reported at the concentration of ABL extracted from each cell line used in the experiment.

Analysis of the obtained results revealed a significant increase (80%) for the **CHOP gene** on the HT29 line with 4 μM incubated dermaseptin 48h (HT4) compared to the control (HT0) and a 2% increase for the A549 line with 4 μM incubated dermaseptin 48h (A4) vs. control (A0). There was a 16% increase in CHOP gene expression in non-peptide cells at 24 hours of incubation without a change in cells incubated with 15 μM / 24h peptide.

The analysis of the obtained results revealed a significant increase (302.67%) for the **AKT gene** on the HT29 line with 15 μM dermaseptin incubated 48h (HT15) compared to the control (HT0) for which the increase was 92.04% relative to the gene reference ABL). Therefore, a 3-fold increase in AKT gene expression in the HT29 line treated with dermaseptin versus the untreated line. For the A549-incubated line with dermaseptin there was an increase of 76.48% (A15) compared to the control (A0).

The **NRF gene** at the HT29 line revealed a 2-fold decrease in gene expression in the 15 μM dermaseptin-treated tumor versus the untreated line and a decrease of about 4-fold for the A549 treated with 4 μM comparative incubated dermaseptin 48h (A4) with the witness (A0).

For the **XBP gene**, a high 45-fold increase in gene expression in the HT29 line treated with dermaseptin 4 μM and 15 fold at a concentration of 15 μM dermaseptin over the untreated line was noted. Also, an increase of 248.32% for line A549 with 15μM incubated 48h (A15) dermaseptin compared to control (A0 for which the increase was 94.2% relative to the ABL reference gene) was noted. That is, a 3-fold increase in gene expression for XBP.

The experimental results obtained for the PERK gene, whose gene expression was evaluated at the cell line HT29 and A549,
revealed a 2-fold decrease in gene expression for the peptide-linked A549 line at a concentration of 4 μM, with no expression of this at higher peptide concentrations. Gene expression was absent for the HT29 line incubated with dermaseptin compared to the control that expressed this gene.

Modifications of Gene Expression of Selected Molecular Targets for HT29 and A549 by Lycotoxin 1 and 2

CHOP gene expression for the HT29 cell line decreased 1.5-fold over the control and 300-fold as a percentage relative to the reference gene at 4 μM lycotoxin 1 concentrations. It also decreased significantly from the control and at the concentration of 15 μM, but similar to the lower concentration of peptide. This indicates that under the conditions of toxicity the CHOP gene that is involved in the proapothotic pathway of the cell decreases expression with antiapoptotic effect. Compared with

line A549, for which CHOP gene expression is 3-fold greater at 4 μM and only 2-fold increased to 15 μM. This indicates that at lower cytotoxic peptide concentrations the cell evolves to apoptosis faster than at high concentrations. Therefore, lycotoxin 1 has antiapoptotic effect for the HT29 line and proapoptotic effect at low concentrations (4 μM) for line A549.

The **BCL2 gene** is involved in the antiapoptotic pathway of the cell, i.e. in maintaining its survival, and therefore, under cytotoxic conditions, its gene expression should decrease.

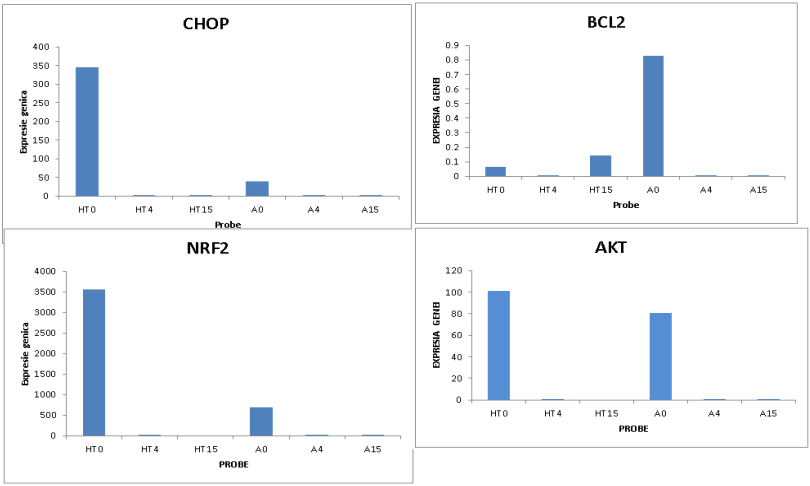


Fig.76: *Quantification of gene expression for CHOP, BCL2, NRF2, AKT compared to the GAPDH reference gene.*

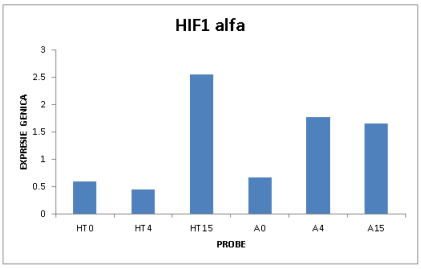
When lycotoxin 1 was introduced into its living environment, the HT29 line showed an increase of 4.5-fold to 4 μM and 16-fold to 15 μM of gene expression compared to the control, which means an increase in defense cell phones.

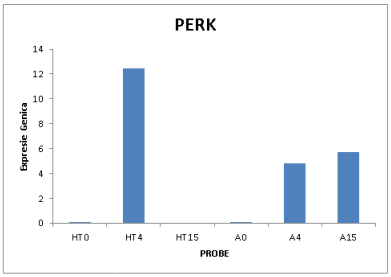
For line A549, there was a 13-fold significant decrease in gene expression at 4 μM and 19 fold at 15 μM, which meant a decrease in cellular defense. Thus, lycotoxin 1 has a tumoricidal effect on the A549 cell line.

The manner in which lycotoxin influenced cell proliferation was determined by evaluating gene expressions for the NRF2 gene, for which a decrease of 274-fold to 4 μM was observed and total suppression at 15 μM for the HT29 line. Therefore, even if there is an increase in cellular defense by modifying expressions for pro and antiapoptotic genes, this peptide significantly reduces or blocks tumor cell proliferation. For line A549, a similar evolution was noted, the expression of the NRF2 gene decreasing 58-fold at 4 μM and 46-fold at 15 μM.

The expression for the **AKT gene** decreased significantly for both cell lines, over 80 times whatever the peptide concentration. This meant that cell survival and proliferation was greatly inhibited irrespective of the concentration of the peptide for both cell lines. However, on the other hand, expression of the HIF 1 alpha and PERK gene is strongly increased for the two lines, which means that cell survival and proliferation is sustained by increasing gene expression for HIF 1 alpha and PERK.

The results also showed the presence of RE stress in tumor cells. The expression of **IRE1 alpha and PI3K genes** is not increased for control tumor cells but only for those incubated with peptide (lycotoxin 1), indicating the presence of stress in tumor cells incubated with the peptide.





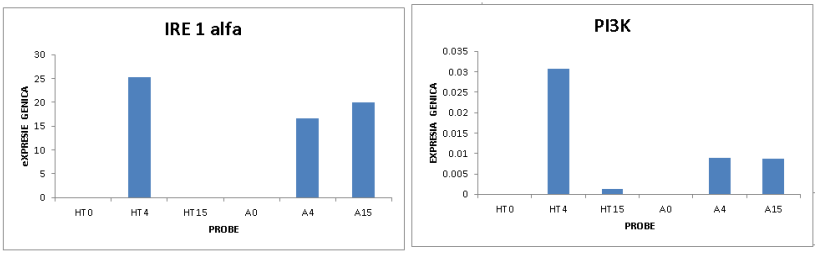


Fig.77: *Quantification of gene expression for HIF α , PERK, PI3K, IRE reported to the GAPDH reference gene.*

In the 48 hours incubation of the two cell lines (HT29, A549) with Lycotoxin 2, it was found that the expression of the **CHOP gene** is greatly diminished, which means inhibition of tumor cell proapoetosis, i.e. a cell defense effect. However, it was noted that expression of the BCL2 gene did not undergo significant alterations for the HT29 line, and for A549 its expression decreased.

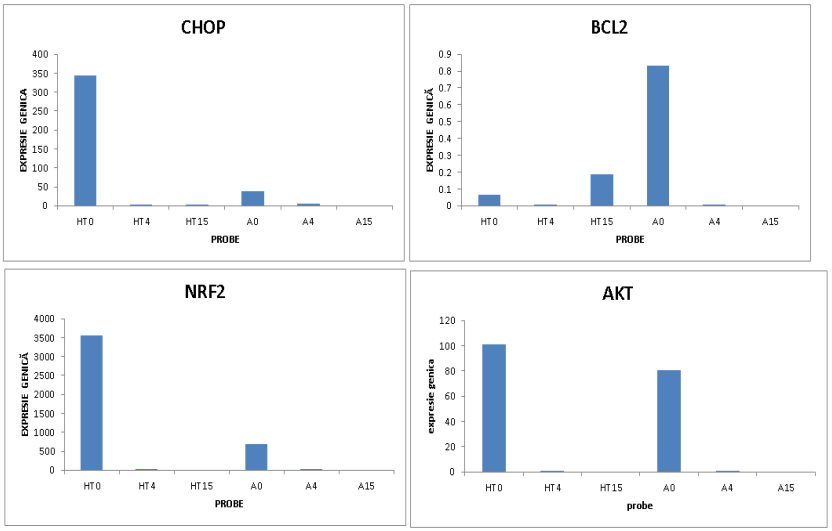


Fig.78: *Quantitation of expression for the CHOP, AKT, BCL2, NRF2 genes relative to the GAPDH gene.*

Cell proliferation evaluated by expression of the **NRF2 gene** indicates a decrease in its expression, a sign that cell proliferation is reduced in the presence of Lyctotoxin 2 for both cell lines.

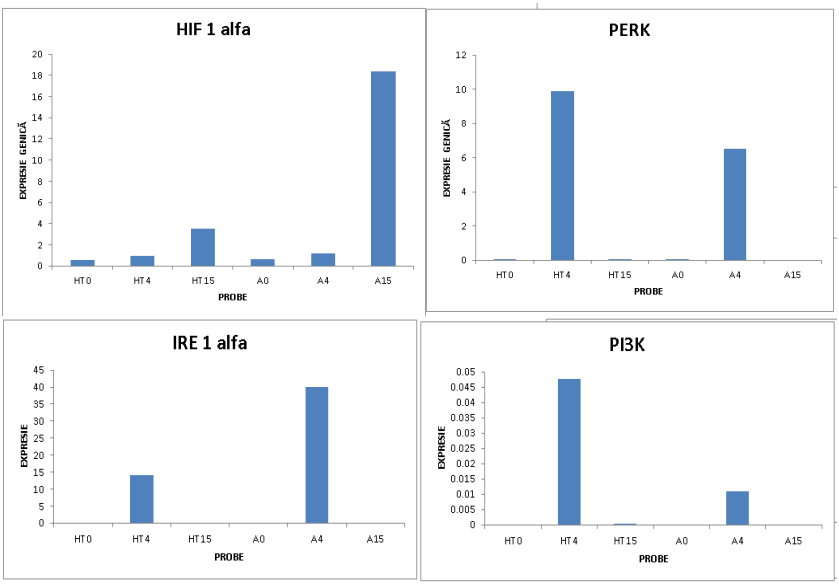


Fig.79: *Quantification of gene expression for HIF1 alpha, PERK, PI3K, IRE1 alpha compared to control cell lines and reported for the GAPDH reference gene.*

The results obtained for the HIF1 alpha and PERK genes showed an increase in expression for the two lines, being more intensively increased for HIF 1 alpha at the 15 μM concentration and for PERK at 4 μM. In the case of the AKT gene expression decreased for both cell lines incubated with this peptide compared to the control lines.

Also noted was the increase in gene expression (IRE 1 alpha, PI3K) indicating the presence of stress in the two lines in the presence of the lycopotoxin 2 peptide.

DISCUSSIONS

In these studies, the tumorigenic potential of dermaseptin and lycotoxin 1 and 2 was determined to measure cell viability by the flow cytometry method for peptide concentrations at which significant apoptosis was produced by the MTT method. The following adherent cell lines were used: HT-29 - colorectal adenocarcinoma and A-549 - human alveolar carcinoma.

For dermatase viability test, concentrations of dermaseptin: 4μM ($p < 0.01$) and 15-20μM ($p < 0.001$) were selected. The results obtained for the two cell lines HT-29 and A549 are different under the action of dermaseptin; at the 15μM concentration, cell apoptosis is twice as high as the colorectal adenocarcinoma line compared to the alveolar carcinoma. In lycotoxin 1 and 2, the viability of HT29 tumor cells was significantly influenced only by lycotoxin 2, and for the A549 line, toxicity was significant for lycotoxin ($p < 0.01$).

Also, the optimization of the Akt, Hif-1α, XBP, Nrf2, PERK primers was evaluated in order to assess the tumor cell metabolism by determining the gene expression of the genes: Akt, Hif-1α, XBP, Nrf2 and CHOP under the cytotoxic peptide used . For this purpose, RNA was extracted from cell cultures and reverse transcripts into cDNA which was subsequently amplified by the qRT-PCR technique. Evaluation of tumor cell metabolism was performed by determining the gene expression for Akt, Hif-1alpha XBP, Nrf2, and PERK under the action of the cytotoxic peptides used.

For the **CHOP gene**: **a significant increase** (80%) of the gene expression for the incubated 48h (HT4) line with 4μM dermaseptin compared to the control (HT0) and a 2% increase for the A549 (A4) A0). Only 16% increase in CHOP gene expression in peptide-free cells was noted.

For the **Akt gene**: **a 3-fold increase** in AKT gene expression in the HT29 line treated with 15 μM dermaseptin and an increase of 76.48% for the A549 48h (A15) incubation line compared to control (A0).

For the **Nrf2 gene**: **a 2-fold decrease** of NRF gene expression on the 15 μM dermaseptin-treated HT29 line relative to the untreated

peptide line and a decrease of approximately 4-fold for the A549 line with 4 μM incubated 48h (A4) dermaseptin versus witness (A0).

For the **XBP gene**: **a 45-fold increase** in XBP gene expression in the HT29 line treated with dermaseptin 4 μM and 15 fold at a concentration of 15 μM dermaseptin relative to the peptide untreated line. Also, a 3-fold increase in gene expression for XBP in the 15 μM treated peptide line compared to control was noted.

For the **Bcl2 gene**: **a 3-fold decrease** in BCL2 gene expression on the HT29 line treated with 15 μM dermaseptin and 2 times at a concentration of 4 μM dermaseptin versus the untreated peptide line. Also, a 2-fold increase in gene expression for BCL2 was observed in the 15 μM treatment-treated peptide versus control line.

HIF 1 alpha evaluated as gene expression with implications for cell survival, growth and proliferation indicated a toxic effect for the HT29 cell line with a decrease in expression of 8-fold at 4 μM and 15-fold at higher concentrations (15 μM) of dermaseptin. In the A549 line, an increase in gene expression was noted 3-fold at 4 μM and a minor change from the control at 15 μM. These changes mean that cell line defense is stimulated in the A549 line at small peptide concentrations.
The PERK gene did not significantly alter expression for any cell line at any peptide concentration.

Cell growth, proliferation and survival of tumor cells can be modulated on metabolic signaling pathways, controlling the expression of tyrosine kinase receptor (RTK) gene for various cell growth factors. The two pathways are PI3K / Akt (phosphatidyl inositol 3 kinase / serine / threonine kinase) and MAP kinase (Ras-BRAF-MEK-ERK). Several classes of PI3K proteins are known, with Class I being best characterized. The p110 subunit has two important components, namely alpha (PI3KCA) and the other beta (PI3KCB) with different tissue-specific expression, with an important and specific role in the human cancer cell. Ras plays an important role in activating the MAP kinase pathway but also signaling the PI3K / Akt pathway, stimulating the appearance of membrane receptors on the surface of the tumor cell for various growth factors including receptors such as VEGFR (vascular epithelial growth factor receptor), EGFR (epidermal growth factor

receptor), PDGFR (platelet-derived growth factor receptor) [Fresno Vara et al, 2004; Courtney et al., 2010]. The extracellular signal that binds to RTK (the tyrosine kinase receptor) activates the alpha catalytic subunit of p110 (PI3KCA), activating the phosphorylation cascade with the formation of phosphatidyl inositol 4,5-bisphosphate and subsequently PIP3 (phosphatidyl inositol 3,4,5- triphosphate) secondary signal for the binding of Akt (protein kinase) to the cell membrane and its phosphorylation, a phosphoinositide kinase (PDK) dependent phenotype, the PDK1 isoform being predominantly involved. Akt is a serine / threonine kinase, in the human tissue being identified 3 isoforms not rated Akt 1, Akt2. Act 3 [Dummler et al, 2007]. Activation of Akt by phosphorylation determines the decrease in synthesis for some effector proteins, which are activation signals for mTOR (rapamycin) with a well-defined role in human cancer, so that Akt becomes an important molecular target in cancer therapy [Meric-Bernstam et Gonzalez -Angulo, 2009].

Activation of these cellular and molecular signaling cascades has significant implications for the functioning of the cancer cell, favoring its proliferation and inhibiting cellular apoptosis. A PI3K / Akt pathway inhibitor (negative regulator) is a PTEN protein encoded by the gene of the same name (the gene for the deletion on chromosome 10 of the phosphatase and the homologous strain). PTEN is an important and major tumor suppressor, being a phosphatase that dephosphorylates PIP3 by blocking the PI3K / Akt pathway [Cantley et al, 1999]. If PTEN accumulates genetic alterations and loses its function, the cell suffers serious consequences with evolving malignancy [Sansal et Sellers, 2004].

Many types of such recombinant proteins are known to play oncogenic activators on the MAP-kinase pathway involved in the proliferation and survival of human tumor cells. There are numerous studies showing that these activators also influence the PI3P / Akt cellular and molecular signaling pathway by activating the p85 subunit, playing an important role in the existence of cancer cells [Miyagi et al, 2004]. Elemental activation of RET tyrosine kinase or direct

phosphorylation of PDK and Akt with RET / PTC may occur [Jung et al, 2005].
The coexistence of these genetic alterations would appear to play an important role in tumor aggression with others that lead to activation of the two signaling pathways involved in the proliferation and survival of cancer cells, increase their survival and inhibit cellular apoptosis [Rhoden et al., 2006]. Thus, by accumulation of genetic alterations, the tumor on the activated PI3K / Akt pathway amplifies its invasiveness and aggression [Hou et al, 2007].

In the studies performed, the gene expression for Akt increased significantly. The results obtained for the two cell lines HT-29 and A549 are significantly different under the action of dermaseptin at 15 μM concentrations, cell apoptosis being twice as large for the colorectal adenocarcinoma line compared to the alveolar carcinoma. This explains the increase in gene expression for Akt in HT29 cells whose survival battle was much more intense. A similar evolution was also noted in the 48-hour incubation with lycotoxin 1 and 2 of the two cell lines.

Endoplasmic reticulum (RE) is an important organ in the maturation and packaging of proteins by making secondary, tertiary and quaternary structures for proteins to function. On the other hand, this organite plays an important role in cellular homeostasis and its death. Exposure of the cell to various stress factors allowed RE to be studied under stress conditions in both normal and cancerous cells. RE is responsible for UPR (unfolded protein response) and there are studies that have highlighted the link between RE stress and cancer involving UPR proteins in both carcinogenesis and resistance to chemotherapy. Under normal circumstances, stress exposure to RE leads to apoptosis [Tabas et al, 2011]. The imbalance between Bcl2 pro and anti-apoptotic proteins under RE conditions may result in increased Bcl2-like 1 (BIM) transcription for PUMA (p53 as an irregular apoptosis modulator interacting with Bax with cytochrome c formation and activation of apoptosis dependent caspase, by cleavage of p53), for NOXA (NADPH activator) and for BH3 proteins.
RE stress in tumor cells can be produced under certain conditions such as hypoxia, nutrition deprivation, pH modulation, poor vascularisation,

conditions in which UPR formation is activated [Martinon, 2012; Tsai et al., 2010].

Metabolic and inflammatory changes are important in the carcinogenesis process as they increase the activity in RE for assembling and transporting proteins, thus leading to stress in RE. The stress response of RE is cytoprotector and involved in the growth and adaptation of the cancer cell to the aggressiveness of the environment in which it exists [Healy et al, 2009]. Three signaling pathways are known for endoplasmic reticulum stress:

- Inhibitory Inositol 1α (IRE1α) enzyme that contributes to cancer progression and decreased expression of the X-bax binding protein gene (XBP1) [Koong et al, 2006].
- Transcriptional activation factor 6 (ATF6).
- Pancreatic RE-kinase (PERK) located in RE and involved in carcinogenesis.

XBP1 is increased in many human cancers such as breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma [Koong et al, 2006]. RE can be a potential target for the development of new drugs in cancer therapy to reduce the hypoxia adaptation , to inflammation and angiogenesis of tumor cells so that resistance to cytostatic treatment can be prevented [Schleicher et al, 2010; Kraskiewicz et Fitzgerald 2012].

Synthesis, proliferation, and glycolysis processes are much more intense in tumor cells than normal [Osthus et al, 2000]. Factor 1a induced hypoxia (HIF1α) plays an important role in tumor development mediating angiogenesis, proliferation and tumor invasion, regulating the expression and activity of glycolytic enzymes. Blocking HIF 1 alpha expression may be a promise as a therapeutic target for the treatment of tumors [Kong et al, 2005].

Also, the decrease or inhibition of protein synthesis of UPR components such as ATF4, XBP1, PERK may be potential targets in cancer therapy [Wang et al, 2012; Luo et Lee, 2013]. In many types of cancer such as breast, hepatocellular, cerebral, colon, ovarian, pancreatic, glioblastoma, are over-expressed UPR components, chaperone RE and glucose receptor regulation receptor (GRP78) in

correlation with RE stress, being highlighted in animal models [Nagelkerke et al, 2013; Pike et al., 2013; Clarke et al., 2014].
On the other hand, CHOP has been shown to induce tumor cell death by stimulating the synthesis of pro-apoptotic proteins and by stimulating oxidative processes in cancer cells exposed to stress for RE [Zinszner et al, 2008]. Under RE's stressful conditions, the cancer cell increases the expression of COX2 via the NF-κB pathway, which plays an important anti-apoptotic role. Also, activation of the NF-kB pathway plays an important pro-inflammatory role through CHOP, and stimulates IL-8 synthesis as is the case with human epithelial cells [Park et al, 2010].

In our studies, gene expression for HIF 1αa decreased significantly for the HT 29 line incubated with dermaseptin, indicating a toxic effect, but for the A549 line the expression of this gene increased the same as for lycotoxin 1 and 2 for the two cell lines (HT29, A549). For PERK, the modifications were insignificant for dermaseptin but there was an increase in lycotoxin 1 and 2 expression for both cell lines.

Gene expression for XBP increased significantly at the 15μM dermaseptin concentration in the culture medium for colorectal cancer tumor cell lines (HT29) against the lung tumor cell line (A549). This means that HT29 cells are more sensitive to the action of dermaseptin compared to A549. The results obtained for the two cell lines are significantly different under the action of dermaseptin at concentrations of 15μM, cell apoptosis being twice as large for the colorectal adenocarcinoma line compared to the alveolar carcinoma. This explains the increase in gene expression for XBP in HT29 cells whose survival was much more intense.

NRF2 is the nuclear factor erythroid 2 (NF-E2) and is one of the most important signaling pathways involved in the defense and survival of the cell and tissue in the aggression of various toxic or carcinogenic substances. In this way, the expression of many cytoprotective genes is increased. In Nrf2 tumor cell plays a protective role being involved in cytostatic chemorezistence and tumor progression. Also, increased gene expression of Nrf2 is associated with low prognosis and multiple chemoresistance.

There are studies that have demonstrated the stimulation of cellular proliferation by NRF2 for the lung carcinoma cell line A549 [Mitsuishi et al, 2012] by the involvement of enzymes such as glucose-6-phosphate dehydrogenase (G6PDH), transcetolase (TKT) and transaldolase 1 (TALDO1) that are involved in the recovery of NADPH (nicotinamide adenine dinucleotide phosphate). Nrf2 is responsible for the direct activation of G6PDH, TKT, TALDO1, ME1 (malic enzyme1), IDH1 (isotretrate dehydrogenase 1), with proteins involved in increasing intracellular glucose influx and stimulation of metabolic processes, stimulation of purine synthesis, blocking the processes destruction of DNA and RNA, resulting in stimulation of tumor cell proliferation. Nrf2 can stimulate cancer cell proliferation and regulate the redox balance, ie by generating antioxidants such as stimulating glutathione synthesis [Reddy et al, 2007]. For A549 cells [Mitsuishi et al., 2012] it was observed that they transformed glutamine from the culture medium into glutathione under the action of Nrf2, thereby accelerating the proliferation of A549 cells. Nrf2 could be a molecular target for cancer therapy by molecules that would inhibit Nrf2 activity in tumor cells [Ren et al, 2011; Magesh et al., 2012].

Dermaseptin significantly inhibits the gene expression of Nrf2 for the HT29 line at concentrations of 15μM dermaseptin, and for line A549 by exposure to 4μM dermaseptin for 48h. For A549 cells to 15μM dermaseptin, the Nrf2 gene expression is insignificantly high. It can be mentioned that in view of this molecular target, presumably over a time span of more than 48 hours, cell apoptosis would be greater at the concentration of 4 μM and it would be interesting to follow which is the variation of the gene expression for Nrf2 to 72 hours compared to cell viability by flow cytometry. Gene expression for lycotoxin 1 and 2a was similarly altered.

Accumulation of defects through the apoptotic pathway of tumor cells can lead to increased survival and resistance to chemotherapy. The Bcl2 family of proteins (B cell lymphoma 2) plays an important role in this direction by inactivating the BH3 domain of pro-apoptotic proteins. The Bcl2 family consists of approximately 25 pro-and anti-apoptotic members involved in cell survival and death. The increase in gene

expression for members of the Bcl2 family having an anti-apoptotic role has been associated with resistance to chemotherapy in various cancers [Del Poeta et al, 2003]. For HT29 line, dermaseptin in both 15 μM and 4 μM concentration blocks Bcl2 gene expression, resulting in a decrease in anti-apoptotic protein synthesis. In case of A549 line, Bcl2 gene expression is increased by exposure to dermaseptin 15μM.

In these studies, dermaseptin at a concentration of 15 μM in the culture medium (48h) was observed to determine apoptosis of the HT29 cell lines by increasing expression of the Akt, XBP, CHOP genes, and decreasing gene expression for Bcl2 and Nrf2. The evolution of line A549 is similar to that of the HT29 line with the exception of the Bcl2 and Nrf2 genes for which an increase in expression of these genes was observed at 15 μM, and at dermaseptin concentrations of 4 μM a decrease in their expression was observed. A possible explanation would be that at high cytotoxic concentrations, the fight of cells is much more intense by increasing the synthesis of anti-apoptotic proteins and reducing oxidative stress by increased synthesis of glutathione, increased glucose influx, purine synthesis and DNA repair in the tumor cell. It appears that these mechanisms are not activated at 4μM concentrations at which CHOP gene expression is increased, i.e., the pro-apoptotic protein synthesis is stimulated in the cancer cell. The expression of the CHOP gene is therefore influenced by both XBP and PERK. And XBP activation of IRE 1 alpha. These peptides have been found to induce a state of stress and are toxic, but the cells activate their defense resources differently

Therefore, exposure of the two tumor cell lines (HT29, A549) to dermaseptin or lycotoxin 1 and 2 for 48 hours was cytotoxic at low peptide concentrations (4μM).

CONCLUSIONS

1. HT29 (colorectal carcinoma) and A549 (lung alveolar carcinoma) were selected from the cell lines tested which showed significant cytotoxicity at low (4 μM) / large (15 μM) peptide concentrations, viability being tested by MTT and flow cytometry .
2. For the CHOP gene, an increase in gene expression was observed by exposing the two cell lines to dermaseptin at a concentration of 4 μM, which may be explained by increased pro-apoptotic protein synthesis or oxidative stress in the cancer cell (IRE 1 gene activation alpha that increases XBP gene activity). The activity of the CHOP gene is also influenced by the activation of the PERK gene. In this case, expression of the PERK gene has not changed under the action of dermaseptin, which means that only the increase in oxidative stress has augmented CHOP gene activity.
3. Increased gene expression for Bcl2 family members with anti-apoptotic role has been associated with resistance to chemotherapy in various cancers. In our studies, low concentrations of dermaseptin (4μM) decreased for both tumor cell lines, demonstrating a toxic effect. Only for the HT-29 line at high peptide concentrations (15 μM), the gene expression of BCL2 was probably increased by increased anti-apoptotic effect.
4. Dermaseptin significantly inhibits gene expression for Nrf2 for both the HT29 line and the A549 line which is a molecular marker for cell proliferation and which decreases significantly under the action of dermaseptin. Therefore, we can say that Bcl2, Nrf2 and CHOP may be molecular targets for A549 and HT29 cells in order to monitor the toxicity of dermaseptin.
5. Gene expression for HIF 1 alpha may be a marker for dermaseptin toxicity monitoring for colorectal cancer (HT29).
6. In the A549 48 hour incubation line with dermaseptin, growth, proliferation and cell survival can be monitored by decreasing AKT gene expression (Ras-PI3K-AKT axis).
7. Incubation of HT-29 and A549 tumor cell lines with Lycotoxin 1 allowed the establishment of molecular markers for toxicity monitoring and pro-apoptotic pathway, by expressing the CHOP

gene and the anti-apoptotic pathway, respectively, with the BCL2 gene. These genes underwent changes in CHOP and BOP2 decrease in the HT29 line. This phenomenon demonstrated resistance of colorectal cancer cells to lycotoxin 1 activity.

8. Cellular proliferation, followed by NRF2 gene expression, significantly decreased for both cell lines under the action of the peptide, and cell growth and survival, only evidenced by a decrease in AKT gene expression. The increase in cellular defense was evidenced by the increase of gene expression for HIF1 alpha and PERK for both cell lines.
9. Expression of IRE1 alpha and PI3K genes, grown only for peptide-incubated tumor cells (lycotoxin 1), indicates the presence of RE stress in tumor cells.
10. Expression of the CHOP gene much diminished by incubation of the two cell lines with Lycotoxin 2 signifies inhibition of pro-apoptosis of tumor cells. However, expression of the BCL2 gene, with no significant alterations for the HT 29 line, and low for A549, indicates a low anti-apoptotic effect in the presence of the peptide.
11. Decrease in expression of NRF2, which evaluates cell proliferation, indicates the cytotoxic effect of Lycotoxin 2 on both cell lines.
12. Increase in expression of IRE1alfa genes, PI3K indicates the presence of stress at the level of the two tumor cell lines under the action of Lycotoxin 2.
13. Studies conducted in this PhD thesis have demonstrated that dermaseptin and lycotoxin have toxic effects on the HT29 and A549 tumor cell lines and allowed the establishment of molecular markers for growth, proliferation, survival, pro-apotosis, anti- apoptosis as well as methods for evaluating and monitoring tumor cell cytotoxicity by high-performance molecular biology techniques.

List of articles
published in "extenso" by the author of the doctorate

ISI Articles

1. **Diaconescu BM**, Jitaru D, Dragoş ML, Bădescu M, Ştefanache T, Ciocoiu M, Mocanu M, Bădescu C. The effect of some natural cytotoxic peptides on tumor cells. REV.CHIM. 2018; 69(3): 597-601.
2. Ştefanache T, Jitaru D, Dragoş ML, Bădescu M, **Diaconescu BM**, Ciocoiu M, Mocanu M, Bădescu C. Tumor immune response in the presence of a cytotoxic peptide. REV.CHIM. 2018; 69(5); 1179-1186 (autor corespondent).

BDI Articles

1. **Diaconescu BM**, Jitaru D, Bădescu M, Ciocoiu M, Bădescu L. Highlighting the effects of cytotoxic natural peptides. Annals of R.S.C.B. 2017; 2: 16-22.
2. **Diaconescu BM**, Jitaru D, Bădescu M, Ştefanache T, Ciocoiu M, Bădescu C. Experimental testing of Dermaseptin cytotoxicity on tumor cells. Annals of R.S.C.B. 2017; 3: 13-19.
3. Ştefanache T, Jitaru D, Bădescu M, **Diaconescu BM**, Ciocoiu M, Bădescu C. Evolution of some natural cell culture under different concentrations of DEFENSIN and CATHELICIDIN – LL37 by MTT viability evaluation. Annals of R.S.C.B. 2017; 3: 20-28 (autor corespondent).

BIBLIOGRAPHY

1. Albihn A, Johnsen JI, Henriksson MA. MYC in oncogenesis and as a target for cancer therapies. *Adv Cancer Res* 2010; 107: 163–224.
2. Bao A, Zhong J, Zeng XC, et al. A novel cysteinefree venom peptide with strong antimicrobial activity against antibioticsresistant pathogens from the scorpion Opistophthalmus glabrifrons. *J Pept Sci* 2015; 21: 758-764.
3. Beckner ME, Fellows-Mayle W, Zhang Z, et al. Identification of ATP citrate lyase as a positive regulator of glycolytic function in glioblastomas. *Int J Cancer* 2010 ; 126: 2282–2295.
4. Benesch C, Schneider C, Voelker HU, et al. The clinicopathological and prognostic relevance of pyruvate kinase M2 and pAkt expression in breast cancer. *Anticancer Res* 2010; 30: 1689–1694.
5. Brahimi-Horn C, Pouysségur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion.*Bull Cancer* 2006; 93:E73–80.
6. Chiche J, Brahimi-Horn MC, Pouysségur J. Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. *J Cell Mol Med* 2010; 14: 771–794.
7. Clarke HJ, Chambers JE, Liniker E, Marciniak SJ. Endoplasmic Reticulum Stress in Malignancy. *Cancer Cell* 2014; 25: 563-573.
8. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol* 2010; 28: 1075–1083.
9. Del Poeta G, Venditti A, Del Principe MI, et al. Amount of spontaneous apoptosis detected by Bax/ Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). *Blood* 2003; 101: 2125–2131.
10. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer* 2008; 8: 705–713.
11. Dos Santos C, Hamadat S, Le Saux K, et al. Studies of the antitumor mechanism of action of dermaseptin B2, a multifunctional cationic antimicrobial peptide, reveal a partial implication of cell surface glycosaminoglycans. *PLoS ONE* 2017; 12(8): e0182926.
12. Dummler B, Hemmings BA. Physiological roles of PKB/Akt isoforms in development and disease. *Biochem Soc Trans* 2007; 35: 231–235.
13. Felício MR, Silva ON, Gonçalves S, et al. Peptides with Dual Antimicrobial and Anticancer Activities. *Front Chem* 2017, 5: 5.
14. Galanth C, Abbassi F, Lequin O, et al. Mechanism of antibacterial action of dermaseptin B2: interplay between helix-hinge-helix structure and membrane curvature strain. *Biochemistry* 2009; 48(2): 313–327.

15. Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. *J Nucl Med* 2008; 49: 24S–42S.
16. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646–674.
17. Healy SJ, Gorman AM, Mousavi-Shafaei P, et al. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. *Eur J Pharmacol* 2009; 625: 234- 246.
18. Hoskin DW, Ramamoorthy A. Studies on Anticancer Activities of Anti microbial Peptides. *Biochimica et Biophysica Acta* 2008; 1778: 357–375.
19. Hu J, Chen C, Zhang S, et al. Designed antimicrobial and antitumor peptides with high selectivity. *Biomacromolecules* 2011; 12: 3839–3843.
20. Huber AL, Lebeau J, Guillaumot P et al. p58(IPK)-mediated attenuation of the proapoptotic PERK-CHOP pathway allows malignant progression upon low glucose. *Mol Cell* 2013; 49: 1049-1059.
21. Koong AC, Chauhan V, Romero-Ramirez L. Targeting XBP-1 as a novel anti-cancer strategy. *Cancer Biol Ther* 2006; 5: 756-759.
22. Luo B, Lee AS. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene* 2013; 32: 805-818.
23. Marciniak SJ, Yun CY, Oyadomari S, et al. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 2004; 18: 3066-3077.
24. Marquette A, Bechinger B. Biophysical Investigations Elucidating the Mechanisms of Action of Antimicrobial Peptides and Their Synergism. *Biomolecules* 2018; 8(2): 18.
25. Mitsuishi Y, Taguchi K, Kawatani Y, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* 2012; 22: 66-79.
26. Van Zoggel H, Carpentier G, Dos Santos C, et al. Antitumor and angiostatic activities of the antimicrobial Peptide dermaseptin b2. *PLoS One* 2012; 7(9): e44351.
27. Viźn P, Alcarraz-Viźn G, Díaz-Moralli S, et al. Modulation of pentose phosphate pathway during cell cycle progression in human colon adenocarcinoma cell line HT29. *Int J Cancer* 2009; 124: 2789–2796.
28. Wang K, Dang W, Xie J, et al. Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. *Biochim Biophys Acta* 2015; 1848: 2365-2373. ,