UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
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THE IMMUNE RESPONSE MODULATION OF CULTURED TUMOR CELLS IN THE PRESENCE OF CYTOTOXIC PEPTIDES AND THE APPLICABILITY TO ORAL NEOPLASIA

DOCTORAL THESIS SUMMARY

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CONTENTS

CURRENT STATE OF KNOWLEDGE

Oral cancer is a serious public health problem, with over 200,000 new cases reported annually worldwide, of which two thirds occur in developing countries. Due to the advanced stage of the disease at the time of diagnosis the overall mortality rate for oral cancer remains high at about 50%, even with modern medical services [Johar et al, 2017].

The high incidence is correlated with the behaviors associated with oral cancer, such as alcohol and tobacco use. The researchers reported that these behaviors lead to genetic variations of tumor suppressor genes (APC, p53), proto-oncogene (Myc), oncogene (Ras) and genes that control normal cellular processes (EIF3E, GSTM1). Processes such as chromosome segregation, number of genomic copies, loss of heterozygosity, telomere stability, regulation of cell cycle checkpoints, DNA damage repair, and defects in growth signaling pathways are involved in the production of oral cancer [Johar et al, 2017]. In order to develop preventive and therapeutic options, it is necessary to understand the basic molecular mechanisms.

Several studies have reported an association between chronic periodontitis and cancer in both the oral and distant cavities [Fitzpatrick and Katz, 2010; Mai et al, 2014]. Tezal and co-workers reported that patients with periodontitis had a 5.33-fold increased risk of tongue cancer for every millimeter of alveolar bone loss [Tezal, 2007].

Chronic inflammation promotes cancer through multiple mechanisms. Genomic instability and DNA damage, partially mediated by reactive oxygen species (ROS), can cause genetic and epigenetic mutations that initiate cell transformation and cause cancer [Schetter et al, 2010].

In all tumors, regardless of their etiopathogenesis, cancer-associated inflammation is present and maintains a tumor-promoting and immunosuppressive environment, allowing the tumor to elude immune surveillance [Grivennikov et al, 2010].

Despite progress in reducing cancer mortality rates and transformative changes in therapeutic paradigms in recent years, the development of new therapeutic approaches remains an urgent priority, particularly in treatment-refractory malignancies.

Due to the clinical success of cancer immunotherapy, there is a renewed interest in developing immunomodulatory strategies in oncological treatment [Syn et al, 2016]. Antimicrobial peptides (AMPs) are structurally diverse molecules, essential effectors of innate immunity, which act quickly to inactivate invading microorganisms, particularly at the surfaces of mucous membranes and epithelial barriers. These peptides demonstrate strong antimicrobial activity and are rapidly mobilized to neutralize a wide range of biological factors, including viruses, bacteria, protozoa and fungi [Shai, 2002]. As both microbial resistance to classical antimicrobial agents and the lack of response of some tumors to conventional chemotherapy are increasing, the need for unconventional therapeutic options has become urgent.

AMPs have a number of potential benefits as future therapies; in addition to broad-spectrum antimicrobial activity, they neutralize endotoxin and are unaffected by the classic mechanisms of antibiotic resistance [Peschel et Sahl, 2006; Harris et al, 2009].

AMPs are increasingly recognized to interact with host cells to influence different signaling cascades that can increase the resolution of infections. For example, β-defensins, a peptide active against many

viruses and gram-negative and positive bacteria, also manifest as a ligand for the CCR6 chemokine receptor that is expressed on T lymphocytes and dendritic cells, thus serving as a bridge between adaptive and innate branches of host immunity. Other previously unappreciated consequences on host immune cells have been described, including alteration of host gene expression, induction of chemokine secretion, modulating the activation or destruction of neutrophils, T lymphocytes and dendritic cells, regulating cellular differentiation pathways and promoting immune-mediated wound healing [Territo et al, 1989; Yang et al., 2002; Heilborn et al, 2003].

Defensins are cationic peptides produced by eukaryotes and comprise two superfamilies that have undergone divergent evolution in terms of sequence, structure and function. The antitumoral properties of human defensins are presented in a group of fast-growing findings and encouraging preclinical results were obtained by treating cancer cells or xenograft cancer models with different natural or synthetic defensins. LL-37 is a α-helix derivative AMP derived from human cathelicidin. Its precursor is found in body fluids and functions as a peptide antibiotic and signaling molecule [Dürr et al, 2006]. In a previous study, human colon cancer cells treated with FK-16, a fragment with 16 residues of LL-37, underwent caspase-independent apoptosis and autophagy as a result of p53-Bcl-2/Bax cascade activation [Ren et al , 2013].

The selectivity, efficacy and major requirements for antitumoral activity are discussed. As the literature is extensive in this regard, this analysis focuses on a period that covers nearly 20 years of treatment of cancer cells with AMPs.

Hydrophobicity of the peptides is an important property and can be easily modulated to increase antitumoral activity [Huang et al, 2011].

In a recent study, the authors demonstrated that manipulating the hydrophobicity of an amphipathic peptide by switching alanine-leucine residues was possible to increase the activity against cancer cells and thus indicated a correlation between hydrophobicity and antineoplastic activity [Huang et al. 2011]. The hydrophobicity of the amino acids increases with the length of the carbon chain. Also, a branched chain may have a lower hydrophobicity than a linear chain with the same number of carbon atoms, because it has a low exposed surface.

Tumorigenesis is a multi-step process involving many factors for tumor growth and progression, as well as for metastatic and angiogenic events. For effective targeting of each stage, new therapeutic agents with the ability to destroy slow-growing and drug-resistant cancer cells are needed, despite their proliferative capacity.

THE PERSONAL PART

PURPOSE

Based on the data from the literature, according to which antimicrobial peptides have antitumor potential, in the experiments carried out within this PhD thesis, the tumoricidal potential of defensin beta1 and cathelicidine LL37 on some adherent tumor cell lines was evaluated in vitro (A549, MDA-MB231, HT-29, M14K, A375, HOS), but also the effect on normal cell lines (HMLE, HOB), to determine the selectivity of the two peptides and to determine the lowest peptide concentration at which there is significant tumoricidal effect. This was possible by determining the gene expression of some molecular targets involved in the pro-apoptotic (CHOP, XBP1, IRE1a, PERK), anti-apoptotic (BCL2), genes that stimulate cell proliferation (NRF2) or are involved in their survival (AKT, HIF1a, PIK3).

GENERAL OBJECTIVES

1. Selection of adherent tumor cell lines that will be the in vitro experimental model for the cytotoxicity testing of the two studied peptides (human β1 defensin and cathelicidin LL37).

2. Establish the peptide testing model by performing a working concentration algorithm from stock solutions, as well as the cell line incubation protocol.

3. Choosing and optimizing at least two methods for determining cell viability under the action of cytotoxic peptides.

4. Determination of molecular targets and optimization of molecular biology techniques for determining the expressions of genes involved in the activation or inhibition of molecular pathways for survival, growth, proliferation and apoptosis, in the presence or absence of cationic peptides (defensin and cathelicidin).

MATERIALS, METHODS AND TECHNIQUES

The peptides used to carry out the studies in this doctoral test were: Beta Defensin 1 (human), Cathelicidin LL-37, to evaluate their effects on tumor or normal cell lines: A549, MDA-MB231, HT-29, M14K, A375, HMLE, HOS, HOB. Cell cultures were maintained in incubator with controlled medium (temperature of 37°C and 5% CO_2).

1. Beta defensin 1 - (Abcam product) lyophilized form, 97% purified HPLC peptide with 1070kDa molecular weight. Rehydration was performed with a volume of 934 μL RPMI 1640 to reach a stock concentration of 100 mM.

2. Cathelicidin LL-37 - (Eurogentec product) in lyophilized form, 97% purified HPLC peptide with a molecular weight of 4493.6 $g.mol^{-1}$. The rehydration was done with a volume of 1000 μL RPMI 1640 to reach a stock concentration of 222.46 μM.

In these experimental studies, aqueous solutions of peptides were used in successive concentrations in a final volume of 200 μL culture medium. The control to which the results were reported consisted of the studied cell line, incubated without peptide (0 μM).

Cell lines used - adherent tumor cell lines: A549 - pulmonary alveolar carcinoma, MDA – MB231 - breast adenocarcinoma, HT29 - colorectal adenocarcinoma, M14K - mesolithelioma, A375 - malignant melanoma, HOS - osteosarcoma and two normal cell lines (HMLE – epithelial cells, HOB - primary osteoblasts).

Statistical processing and interpretation of results

The statistical software PRISM version 5, the Sigma Plot graph pad, the t-Test and Excel were used as statistical methods for calculating gene expressions for statistical processing. The results of the statistical analysis of the experimental data were reported as mean ± SD (standard deviations) / SE (standard errors). The averages for 2 variables with normal and continuous distribution were compared as independent samples in the t-Student test. A statistically significant difference for a significance threshold $p < 0.05$ was considered.

The level of statistical significance (p) represents the maximum probability of an error occurring. In practice, a significance level of 0.05 (5%) is sufficiently accurate, the probability (confidence level) of 95% showing that the decision is fair. In these cases we affirm that the H_0 hypothesis was rejected at a level (significance threshold) of 0.05,

the probability of error is 0.05 (5%) and the confidence interval is 95%.

RESULTS

Evolution of cell cultures under the action of β1 defensin and cathelicidin LL37 by MTT method and flow cytometry

To test the activity of the two cationic peptides (defensin and cathelicidin LL37), in vitro experiments were performed on optimized cell models, in the „IRO" Iasi molecular biology laboratory. Thus, six adherent tumor cell lines and 2 normal cell lines were used, namely:

1. HT-29 is an adherent cell line of colorectal adenocarcinoma.
2. M14K is a very aggressive malignant tumor that appears on surfaces lined with mesontellial cells.
3. A549 is a basal epithelial adenocarcinoma cell line.
4. MDA-MB231 is an adherent cell line of breast adenocarcinoma.
5. A375 is an adherent cell line for malignant melanoma.
6. HMLE is a cell line of the normal epithelium of the human breast.
7. HOS is an adherent cell line of human osteosarcoma.
8. HOB is a cell line of normal human primary osteoblasts.

For each working concentration two or three wells were used to determine viability with MTT, therefore duplicate or triplicate readings were taken. The reference range to which the read absorptions were reported was between 595 and 620 nm.

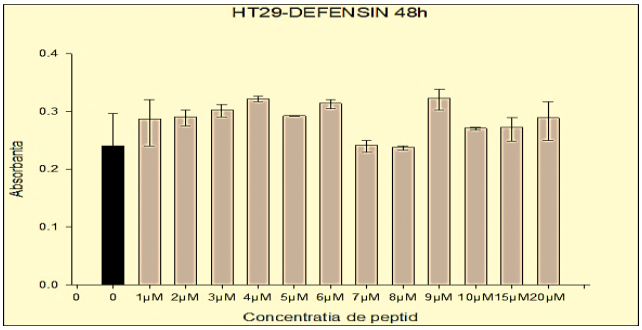


Fig. 1. Determination of viability of HT29 cells with plaque spin at 48 hours of incubation with defensin

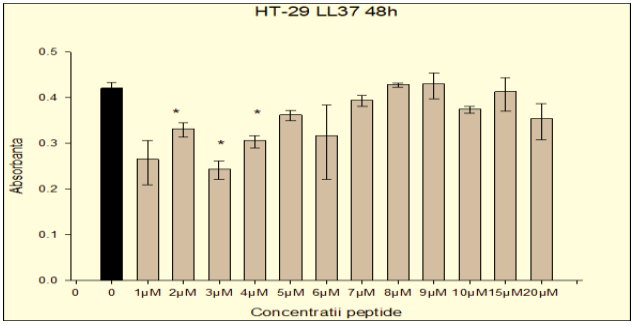


Fig. 2. Determination of viability of HT29 cells with plaque spin at 48 hours incubation with cathelicidin LL37

The number of cells incubated for 48 hours with catelicidin was $1.2x10^5$ cells/well in a final volume of 500μL. After fluorochrome labeling, the affinity of the cells for Annexin V and propidium iodide (PI) was determined. Approximately 10^4 events (cells) were acquired and analyzed by flow cytometry technique. About 19% of the cells

were in apoptosis compared to the control for which the viability was 89% and the apotosis 11%.

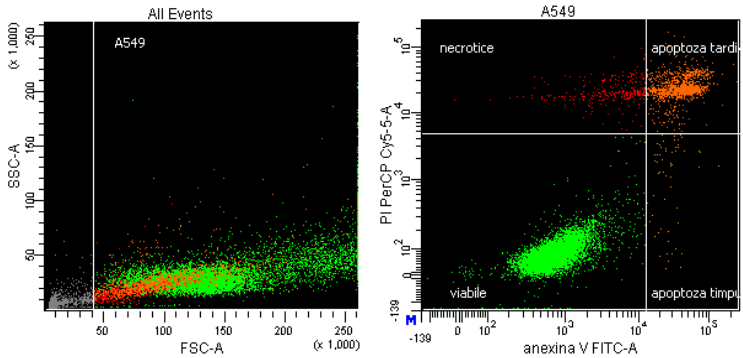


Fig. 3. Analysis by flow cytometry of the viability of the HT29 cell line treated with 4μM cathelicidin LL37 (48h incubation) (Q1 = necrotic cells, Q2 = cells in late apoptosis, Q3 = living cells, Q4 = cells in early apoptosis)

Incubation of A549 line cells with cathelicidin LL37 at a concentration of 20 μM for 48h led to a 33.4% apoptosis evidenced by flow cytometry technique; it is important to mention that 28% of these cells were necrotic. For lower concentrations (9 μM), incubated for longer time (72h), mortality was significant (16%) of which most cells were in late apoptosis (11%). Cellular viability for the control was significant (95%) at both 48 hours and 72 hours.

The results obtained for the 2 cell lines HT-29 and A549 are significantly different under the action of catelicidine LL37. At high concentrations of 20 μM, cell apoptosis is over 30% higher for lung cancer compared to exposure through lower concentrations but longer

time (16%). The results obtained for the A549 line incubated with defensin indicated an insignificant cytotoxicity on this type of cells.

For the lines HOS (tumor cell line, adherent, human osteosarcoma type) and HOB (normal cell line, adherent, normal human osteoblasts type) - after thawing, they were placed in culture medium with RPMI and after 14 days when proliferation provided a sufficient number of cells to perform the experiments, the cells were distributed in approximately equal numbers in all working wells (Fig. 4).

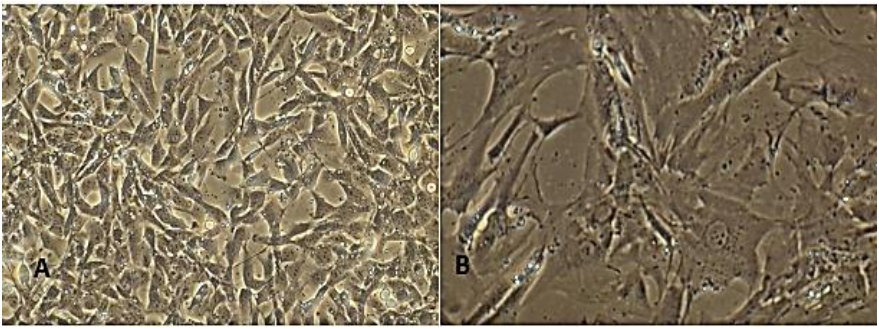


Fig. 4. Osteosarcoma HOS cell line (A) and normal osteoblast HOB cell line 14 days after thawing

Incubation of the osteosarcoma cell line (HOS) with cathelicidin LL37 and evaluation of cytotoxicity at 48 hours by determining viability with MTT method, showed a statistically significant tumoricidal effect for both low peptide concentrations (1-6μM) and high concentrations of 15 μM ($p < 0.001$), respectively 20 μM ($p < 0.001$) LL37 (Fig. 5).

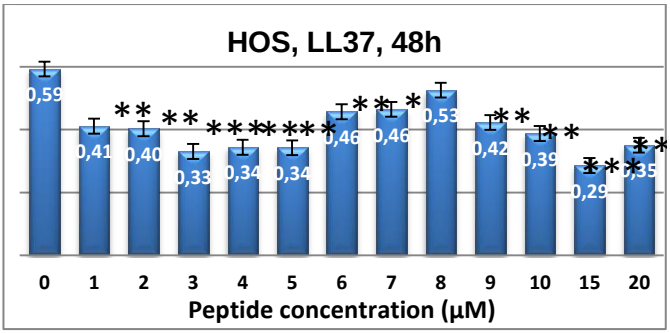


Fig 5. Variation of absorbance averages by MTT technique for HOS line incubated with LL37 for 48h (p <0.05; ** p <0.01; *** p <0.001)*

After evaluating the cell viability under the action of the two peptides, the experiments were resumed to determine the cytotoxicity of cathelicidine and defensin on the HOS and HOB line after an incubation of 48 h, at low concentrations of 4 µM compared to high concentrations of 15 µM, evaluating cell apoptosis by flow cytometry.

The toxicity of defensin for the normal osteoblast line was insignificant (less than 5%) and in the case of cathelicidin, the cytotoxicity was higher but did not exceed 10% for 4µM concentrations and for high peptide concentrations (15µM) it was only 16.64%.

In conclusion, the cytotoxicity of cathelicidine LL37 was significant for the osteosarcoma tumor cell line (HOS) and did not significantly affect the normal osteoblastoma cell line (HOB), for which cell apoptosis was 10% at low concentrations of peptide (4µM) and 16.64% at high concentrations after a 48h incubation.

A375 lines (tumor cell line, adherent, human malignant melanoma type) and HMLE (normal, adherent cell line, normal human epithelial cell type) - according to a protocol similar to that performed in the cases of previously studied lines, after thawing, were placed in culture medium with RPMI and after 14 days when it was verified that there was a sufficient number of cells to perform the experiments, the cells were distributed in approximately equal numbers in all working wells (Fig. 6).

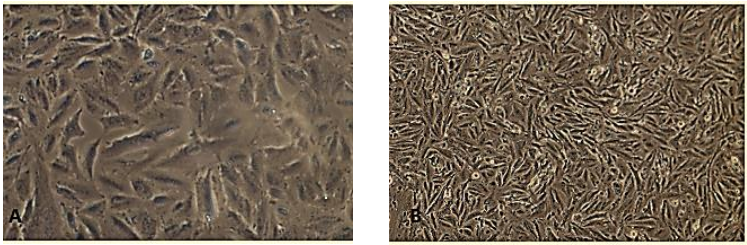


Fig. 6. Malignant melanoma cell line A375 (A) and normal epithelial cell type HMLE cell line 14 days after thawing

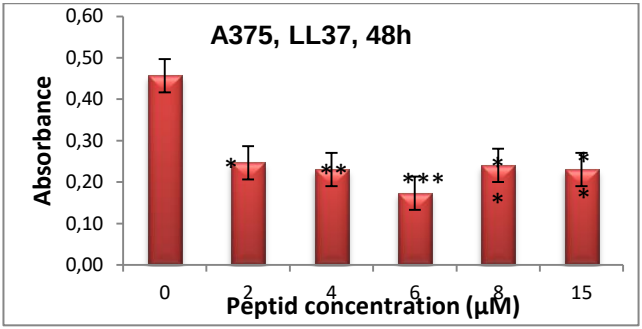


Fig. 7. Variation of absorbance averages obtained by MTT technique for A375 malignant melanoma cell line incubated with LL37 for 48 h (p <0.05; ** p <0.01; *** p <0.001)*

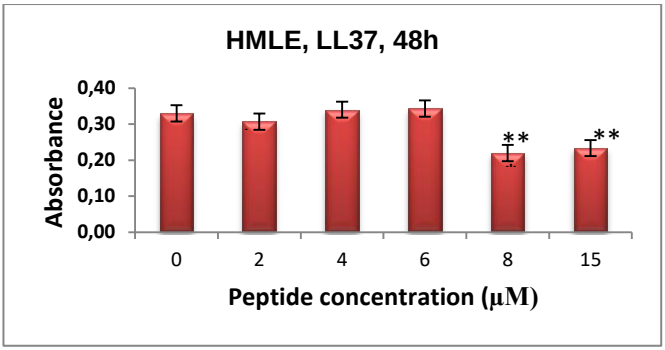


Fig. 8. Variation of absorbance averages obtained by MTT technique for normal epithelial cell type HMLE incubated with LL37 for 48 h (p <0.05; ** p <0.01; *** p <0.001)*

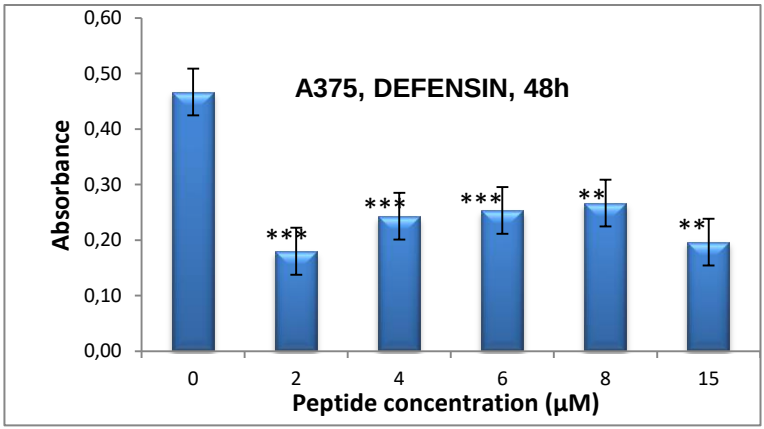


Fig.9.Variation of absorbance averages obtained by MTT technique for malignant melanoma cell line A375 incubated with defensin for 48 h (p <0.05; ** p <0.01; *** p <0.001)*

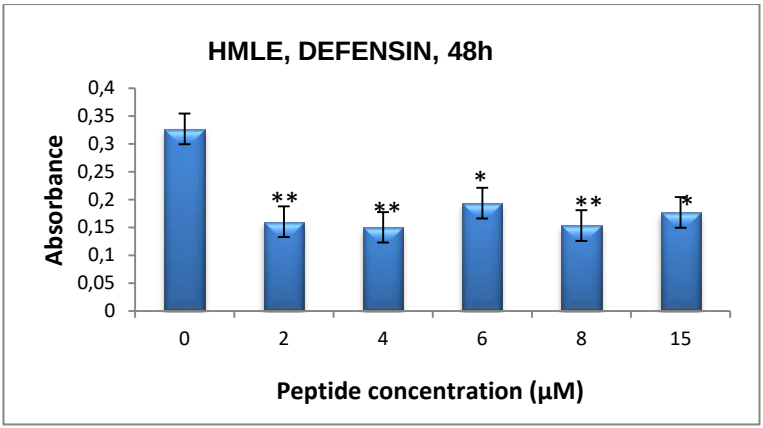


Fig.10. Variation of absorbances obtained by the MTT technique for the HMLE cell line of the noemal epithelium type incubated with defensin for 48 h (p <0.05; ** p <0.01; *** p <0.001).*

In conclusion, the cytotoxicity of cathelicidin LL37 and defensin was significant for the malignant melanoma tumor cell line (A375) and did not significantly affect the normal epithelial cell line (HMLE), for which cell apoptosis was 4% at low concentrations (4 µM) of LL37 and 16% for defensin. At high concentrations (15 µM) after a 48h incubation, the apoptosis of the HMLE line was below 30% for both defensin and cathelicidin LL37.

Evaluation of gene expression of molecular targets by molecular biology techniques (qRT-PCR)

In order to determine how the studied peptides (defensin and cathelicidin LL37) act on tumor cells, the expressions of some genes involved in different cellular signaling pathways were evaluated by molecular biology techniques, such as:

• the pro-apoptotic pathway (CHOP, XBP1, IRE1a, PERK),

• anti-apoptotic pathway (BCL2),

• genes that stimulate cell proliferation (NRF2),

• genes that stimulate cell survival (AKT, HIF1a, PIK3).

Molecular biology technique used: Real Time (qRT) –PCR is a quantitative method and has been developed and optimized in the laboratory.

Optimization of primers specific for the molecular targets pursued

In order to be able to establish the quantitative amplification protocols, it was necessary to optimize, the sets of primers necessary for the gene expression evaluation of the molecular targets.

The results obtained for the optimization of primers in order to use them for the evaluation of gene expression on complementary DNA (cDNA) obtained from tumor cell cultures (extracted RNA) were achieved by two methods:

1. Classical PCR and 2% agarose gel migration and
2. qRT - PCR with SYBR green.

Primers that could not be optimized at the specific temperature, led to the formation of primer dimers, which overlap with amplification products, leading to altered results (intense amplification signal, falsely increased due to primer dimers). Other optimal primer priming temperatures were 56°C and 72°C. The following primers were used for the ABL reference gene:

Fw: 5 ’TGTGATTATAGCCTAAGACCCGGAGCTTTT 3’
Rev: 5 'TTCAGCGGCCAGTAGCATCTGACTT-3
For the GAPDH reference gene, the optimal priming temperature was 58°C and the melting temperature was 86.9°C (Fig.11).

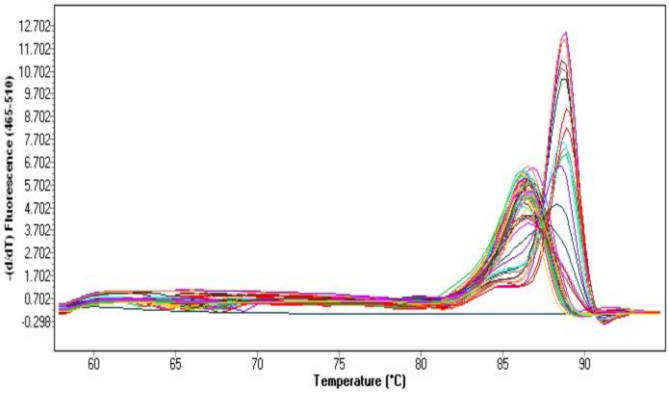
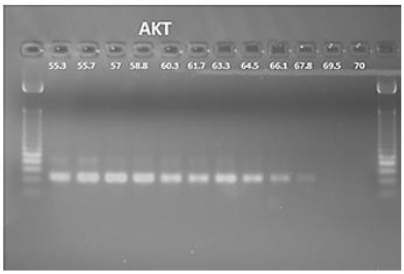
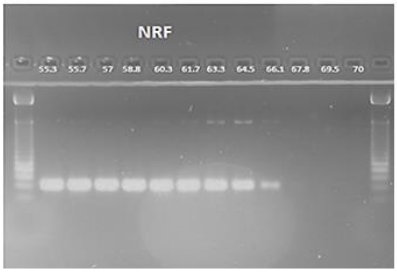


Fig.11. Standard curves for the GAPDH reference gene and melting temperatures by RT-PCR method with Sybr green

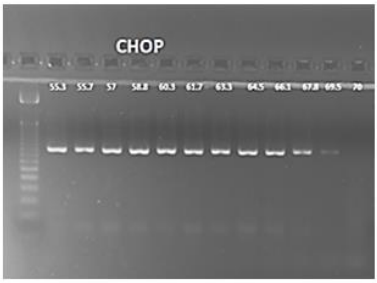
The PCR amplification products were subsequently migrated in 2% agarose gel and the alignment temperatures for which specific products were obtained were selected.



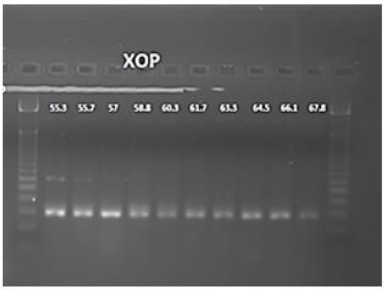
AKT
Primer Forward: 60.0°C
tctatggcgctgagattgtg



NRF2
Forward: 54.3 °C
agtggatctgccaactactc



XBP
Primer Forward: 62.3 °C
cctggttgctgaagaggagg
Primer Reverse: 64.0 °C
ccatggggagatgttctggag



CHOP
Primer Forward: 61.7 °C
ttctctggcttggctgactg
Primer Reverse: 61.5 °C
ctgcgtatgtgggattgagg

Molecular biology results for A549 and HT29 incubated with cathelicidin LL37

Analysis of the results showed a significant increase (344% compared to ABL) in CHOP gene expression by HT29 line incubated 48h without cathelicidin, compared to cells incubated with cathelicidin

LL37; an increase of 39% and 26% respectively for A549 line incubated 48h without peptide (A0) compared to cells incubated with cathelicidin 20 μM (48h) and 9 μM (72h), respectively.

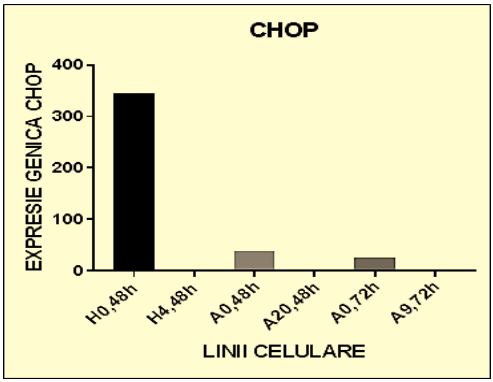


Fig. 12. Quantification of gene expression for the CHOP gene related to the ABL reference gene

The experimental results obtained for the AKT gene, whose expression was evaluated in the HT29 (colorectal carcinoma) and A549 (alveolar carcinoma) cell lines in the presence of the cathelicidin were compared to a control consisting of the peptide-free cell line. The incubation time with peptide was also taken into account, the control (line without peptide) being incubated for the same time.

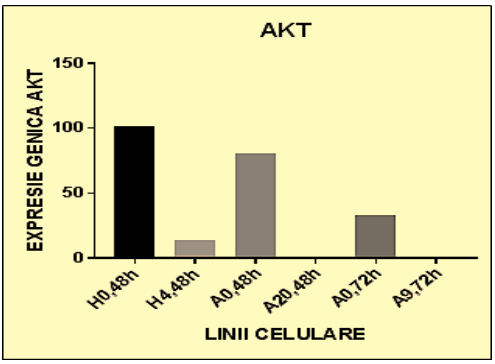


Fig. 13. Quantification of gene expression for the AKT gene related to the ABL reference gene

Gene expression for AKT was increased only for peptide-free tumor cells and totally inhibited for cytotoxic peptide-incubated A549 cells and significantly reduced to 14% for 4uM cathelicidin-incubated HT29 line cells (Fig. 13).

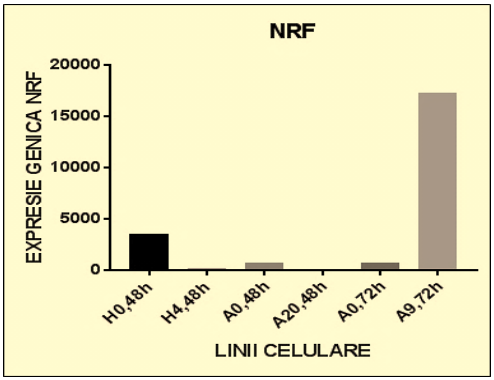


Figure 14. Quantification of gene expression for the NRF gene related to the ABL reference gene

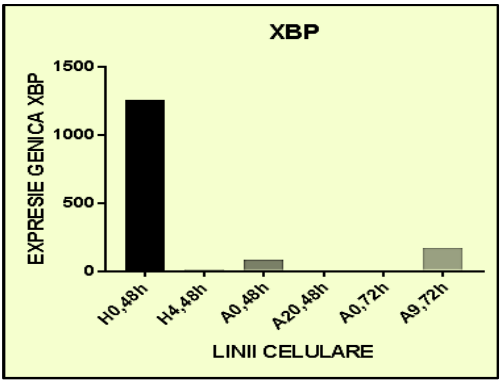


Figure15. Quantification of gene expression for the XBP gene related to the ABL reference gene

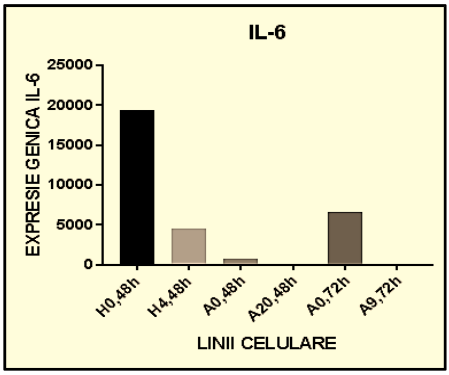


Fig. 16. Quantification of gene expression for the IL-6 gene related to the ABL reference gene

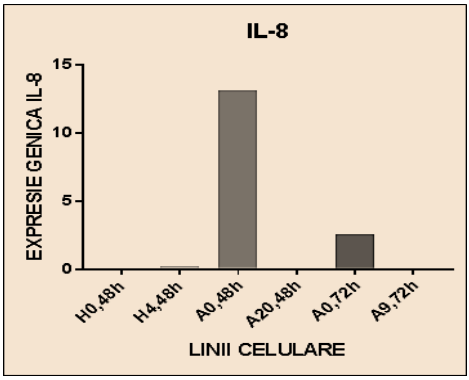


Fig. 17. Quantification of gene expression for the IL-8 gen related to the ABL reference gene

Molecular biology results for A375 and HMLE incubated with cathelicidin LL37

For the A375 malignant melanoma cell lines and HMLE of normal epithelial cells, the GAPDH reference gene was used; the action of

cathelicidine was compared at low concentrations of 4 μM compared to high concentrations of 15 μM.

Gene expression (%) =	A375 - LL37			HMLE - LL37		
Target gene*100/reference gene GAPDH	Changes to control	4uM	15uM	Changes to control	4uM	15uM
CHOP	High	1	1	Low	1	28
XBP 1	-	-	-	-	-	-
PERK	High	3	2	Low	2	5
IRE 1a	High	4	5	High	0	1
AKT	-	-	-	Low	4	4
HIF 1a	High	3	3	Low	2	4
PI3K	High	3	3	Low	2	3
BCL 2	Low	1	1	Low	1	5
NRF 2	Low	2	29	High	2	1

Tabe XLI: Variation in gene expression of molecular targets evaluated for LL37 incubated lines compared to control cells

Gene expression (%) =	A375 - LL37			HMLE - LL37		
Target gene*100/reference gene GAPDH	0uM	4Um	15uM	0uM	4uM	15uM
CHOP	7,80	9,40	11,00	6,83	4,95	0,24
XBP1	0,00	0,00	0,00	0,00	0,00	0,00
PERK	35,77	92,34	73,09	135,86	87,72	25,73
IRE1a	38,11	137,24	189,13	0,00	0,34	1,08

Tabel XLII: Calculation of gene exposures of molecular targets CHOP, XBP1, PERK, IRE 1a evaluated for lines incubated with LL37, by reference to GAPDH as reference gene

Cell survival was also assessed by PI3K gene expression, which correlates with expression for AKT, and the HIF 1α gene, which expresses the degree of hypoxia, and which correlates with the IRE1α gene involved in oxidative stress. A 3-fold increase in the expression of these genes was found for the melanoma line compared to the normal epithelial cell line, where cell survival was not affected. Although there was no expression for the AKT gene, there was a 3-fold increase in expression for PI3K which meant hyperphosphorylation with decreased synthesis for some effector proteins. For normal epithelial cells, cell survival was not influenced by the presence of LL37 peptide in their living environment.

Results obtained by molecular biology for HOS and HOB incubated with cathelicidine LL37

For the osteosarcoma and HOB cell lines of normal osteoblast cells, the ABL reference gene was used and the action of cathelicidine was compared at low concentrations of 4 μM compared to high concentrations of 15 μM in the cell living environment.

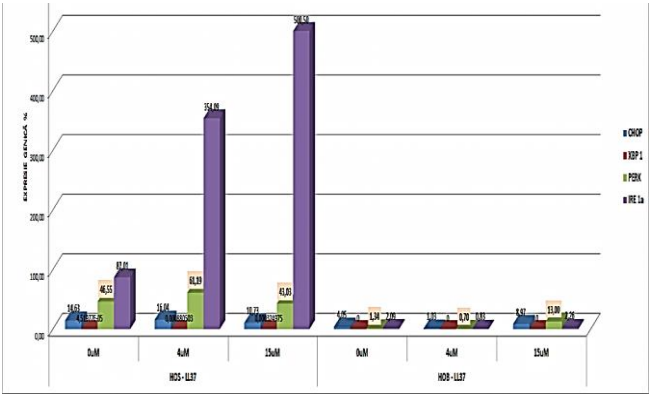


Fig. 18. CHOP, XBP1, PERK, IRE1a gene expression variation relative to ABL reference gene

For XBP 1 gene, expression was significantly increased 19-fold for 4 μM and 7-fold for 15 μM peptide influencing chaperone synthesis and being involved in the generation of oxidative stress. Moreover, the expressions of the pro-apoptotic genes CHOP and PERK were significantly increased for the HOS line at concentrations of 4 μM peptide and insignificantly increased at the line of normal epithelial cells at 4 μM peptide.

Results obtained by molecular biology for HOS and HOB incubated with defensine

It was found that there was a major oxidative stress in the HOS cell line, evidenced by increased gene expression for IRE 1α, 4 times at concentrations of 4 μM peptide, and 5 times at high concentrations (15 μM) of peptide. Given the IRE1α expression values for the HOB line, it was found that defensin did not generate oxidative stress in normal osteoblasts, as did the osteosarcoma line.

Although the XBP 1 gene was not expressed, the expressions of the pro-apoptotic genes CHOP and PERK were significantly increased for the HOS line and significantly decreased in the normal osteoblast line.

Increased CHOP gene expression was correlated with inhibition of cell survival by stimulating apoptosis by oxidative stress in the endoplasmic reticulum, respectively, correlated with increased PERK gene expression, which inhibited the synthesis of effector proteins (Fig. 19).

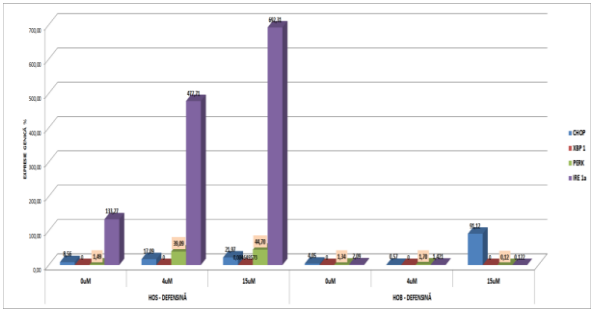


Fig. 19. CHOP, XBP1, PERK, IRE1α gene expression variation relative to ABL reference gene for HOS and HOB lines incubated with defensin

DISCUSSIONS

The experimental studies carried out in this doctoral thesis aimed to investigate the toxicity of the human catinonic peptides defensin β1 and cathelicidin LL37 in correlation with their concentration and the type of cell line used.

In our experimental studies, the tumoricidal potential of CATHELICIDINE - LL37 was determined depending on the type of cell line, measuring viability by the flow cytometry method for peptide concentrations at which a significant apoptosis was observed by the vital colorimetric MTT method.

Flow cytometry viability testing for HT29 line (colorectal adenocarcinoma) was performed at peptide concentrations (cathelicidin LL37) for which statistically significant changes were obtained under the action of the cytotoxic peptide at 48 hours of incubation, evidenced by the MTT method, namely for concentrations of 4 μM ($p < 0.05$) and 15 μM ($p < 0.01$).

Thus, the incubation of HT29 line cells with catelicidin LL37 in a low concentration of 4μM for 48h led to a significant apoptosis of 19% evidenced by the flow cytometry technique, of which 15% of the cells were in late apoptosis, meaning a significant cell mortality. Compared with the HT29 line treated with LL37, the cell viability for the control was significant at 89%.

DEFENSINS are small cysteine-rich peptides, moderately cationic (with 3-10 net positive charges), with antimicrobial action. In vitro, they have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungi [Ganz et al, 2003].

For the HT29, A549, M14K and MDA-MB231 cell lines incubated with defensin for 48 hours, viability tests by the vital MTT technique showed insignificant cytotoxicity ($p > 0.05$), both for low peptide concentrations and for high concentrations.

At low concentrations of 4 μM peptide, cellular apoptosis of the HOS line was significant, approximately 30% for cathelicidin LL37 and approximately 11% for defensin, compared to control (10%). At high concentrations of 15 μM peptide, cellular apoptosis of the HOS line was over 50% for cathelicidin LL37 and approximately 12% for defensin, compared to control (10%).

The toxicity of defensin for the normal osteoblast line was insignificant (less than 5%) compared to the control values, and in the case of cathelicidine, the cytotoxicity was higher but did not exceed 10% for 4 μM concentrations and for high concentrations of peptide (15μM) was only 16.64%.

The cytotoxicity of cathelicidine LL37 was significant for the osteosarcoma tumor cell line (HOS) and did not significantly affect the normal osteoblastoma cell line (HOB), for which cell apoptosis was 10% at low peptide concentrations (4 μM) and 16.64% at high concentrations after a 48h incubation. Defensin had no significant tumoricidal effect for the HOS line, for which the apotosis was maximal 12%, as it did not have a significant toxic effect for the HOB line at which the maximum apotosis was 4%.

The cytotoxicity of the two peptides, human defensin beta 1 and cathelicidin LL37 on the six tumor cell lines and two normal cell lines, was also evaluated by molecular biology techniques, in which the expressions of some genes involved in different signaling pathways were determined such as: pro-apoptotic pathway (CHOP,

XBP1, IRE1a, PERK), anti-apoptotic pathway (BCL2), genes that stimulate cell proliferation (NRF2), genes that stimulate cell survival (AKT, HIF1a, PIK3). For this purpose, the genetic material (RNA) was extracted from cell cultures and reverse-transcribed into cDNA which was subsequently amplified by the qRT-PCR technique, which is a quantitative method and was developed and optimized in our molecular biology laboratory by the method with Sybr green.

• For the CHOP gene: a significant increase (344% compared to ABL) in CHOP gene expression expressed by the HT29 line incubated for 48h without cathelicidin, compared to cells incubated with cathelicidin LL37 and an increase of 39% and 26% for line A549, respectively incubated 48h without peptide (control) compared to cells incubated with cathelicidin 20 μM (48h) and 9 μM (72h), respectively. That is, gene expression for CHOP was increased only for peptide-free tumor cell lines and totally inhibited for cells incubated with cytotoxic peptide.

• For the Akt gene: analysis of the results showed a significant increase (101% compared to ABL) in gene expression for Akt expressed by HT29 line incubated 48 h without cathelicidin, compared to cells incubated with cathelicidin LL37 and an increase of 80% and respectively 33% for line A549 incubated 48h without peptide compared to cells incubated with cathelicidin 20 μM (48h) and 9 μM (72h), respectively. That is, gene expression for Akt was increased only for peptide-free tumor cells and totally inhibited for cytotoxic peptide-incubated A549 cells and significantly decreased to 14% for 4uM cathelicidin-incubated HT29 line cells.

• For the Nrf2 gene: analysis of the obtained results showed a significant increase (over 3000% compared to ABL) of gene expression for Nrf2 expressed by HT29 line incubated 48h without

cathelicidin, compared to cells incubated with cathelicidin LL37 and an increase of over 600% for line A549 incubated 48h without peptide (control) compared to cells incubated with cathelicidin 20 μM (48h) and 9 μM (72h), respectively. That is, gene expression for Nrf2 was increased only for peptide-free tumor cells and totally inhibited for cytotoxic peptide-incubated A549 cells and significantly decreased to 139% (compared to the ABL reference gene) for HT29 line cells incubated with 4uM cathelicidin. Note that only in the case of A549 line incubated for 72 hours with cathelicidin 9 μM gene expression for Nrf2 was approximately 3-fold higher than for the line without peptide.

The marked decrease in Nrf2 in the cell lines treated with cytotoxic peptides used by us suggests another way of inhibiting tumor cell metabolism: lowering glutathione (GSH), the most abundant intracellular thiol with antioxidant action.

• For the IL-6 gene: analysis of the results obtained by us showed a significant increase (approximately 19000% compared to ABL) of gene expression for IL-6 expressed by the HT29 line incubated 48h without cathelicidin, compared to cells incubated with cathelicidin LL37. Therefore, gene expression for IL-6 was approximately 4-fold lower at the HT29 line incubated with cytotoxic peptide.

IL-6 activation further drives angiogenesis by promoting the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor (bFGF) by tumor cells, supporting the rapid vascularization necessary for tumor growth and metastasis [Van Herpen et al, 2014] . IL-6 has also been reported to promote tumor metastasis by attracting the most aggressive cells circulating back into the primary microenvironment of the tumor, where they contribute to tumor progression and metastasis.

• For the IL-8 gene: analysis of the results showed an insignificant increase in gene expression for IL8 expressed by the HT29 line incubated 48h with cathelicidin, compared to cells incubated without cathelicidin LL37 and a 13% increase for the A549 line incubated 48h without peptide, compared to cells incubated with 20uM cathelicidin (48h).

Immunosuppression in cancer patients is a well-established phenomenon and may include changes in cytokines and in the balance of immune cell populations [Jebreel et al, 2007]. Helper T lymphocytes play a key role in controlling the immune response and are subdivided into T-helper 1 (Th1) and T-helper 2 (Th2). Their differences consist of synthesized mediators and induced responses. The Th1 line is responsible for promoting cell-mediated immunity and acts on anti-tumor pathways, secreting mediators such as interferon-γ (IFN-γ), which activates macrophages, cytotoxic T lymphocytes, and natural killer (NK) cells. The Th2 line induces humoral response and is responsible for the secretion of IL, such as IL-4, which promotes tumor progression by inhibiting mediators secreted by Th1 cells [Agarwal et al, 2010]. The functional imbalance between Th1 and Th2 secreted mediators, which favors IL secreted by the Th2 cell line, is responsible for the progression of SCC of the head and neck. IL-4 has antiangiogenic properties that can inhibit tumor progression. However, the same IL has antiapoptotic properties, which can help protect the tumor immune, in addition to promoting tumor growth. This double action of IL-4 has influenced the increase of its levels and the weaker prognosis of patients with OSCC [Aziz, 2015]. According to the results of the selected studies IL-4, IL-6, IL-8, IL-10 and IL-13 tend to show a response related to the intensification of the carcinogenesis process, while elevated levels of IL-12 were not

associated with prognosis. unfavorable in patients with OSCC [Aziz, 2015].

CONCLUSIONS

1. In vitro experimental models consisting of tumor cells show a rather high heterogeneity in terms of their behavior to certain aggressive factors present in their living environment.

2. The evaluation of gene expressions for molecular targets involved in the pathways of survival, growth, cell proliferation were performed by quantitative molecular biology techniques and optimized *in house* with *Sybr green*. For this purpose, the primers specific for molecular targets were selected: Akt, Hif-1α, XBP, Nrf2, CHOP, PERK, IRE 1alpha, PI3K and the molecular amplification conditions were optimized and the hybridization temperatures of the specific primers were determined.

3. The increase in gene expression for CHOP meant an increase in pro-apoptotic protein synthesis and oxidative stress in the tumor cell, being directly correlated with increased expression of the PERK gene (RE - pancreatic kinase) and IRE1a (inositol-dependent enzyme gene 1 alpha).

4. Cell growth, proliferation and survival, in the case of incubation of HOS lines - osteosarcoma and A375 - melanoma with cathelicidin LL37, was significantly reduced by decreasing gene expression for HIF 1alpha. Therefore, gene expression for HIF 1 alpha (hypoxia-induced factor 1α) may be a marker for tracking the toxicity of cathelicidin LL37 for this type of tumor cell.

5. In the case of HT29 line - colorectal carcinoma incubated for 48 hours with cathelicidin, cell growth, proliferation and survival was

monitored by gene expression decrease for AKT (Ras-PI3K-AKT axis), which encodes a serine/threonine kinase and whose activation by phosphorylation decreases the synthesis of proteins with an effector role. The significant decrease in NRF2 expression indicates a significant decrease in cell proliferation, thus demonstrating the toxicity of this peptide. NRF2 gene expression may be a marker of monitoring the cytotoxic effect of LL37 on this type of tumor cell.

6. Increased gene expression for BCL2 family members with anti-apoptotic role has been associated with resistance to chemotherapy in various cancers. In our studies, at low concentrations of cathelicidin (4 μM) BCL2 gene expression decreased significantly for tumor cell lines A375 and HOS demonstrating a cytostatic effect by stimulating apoptosis.

7. Gene expression for NRF2 (molecular marker for cell proliferation) is significantly inhibited for both the three tumor lines: A375, HOS and HT29, the effect being to drastically limit cell proliferation (cytostatic).

8. Incubation of tumor cell lines with human defensin beta 1, significant cytotoxic effect had only in the case of A375 and HOS lines and did not affect normal HMLE and HOB cell lines.

9. Studies conducted in this doctoral thesis have shown that the cationic antimicrobial peptides cathelicidin LL37 and human defensin 1β have cytotoxic effects on tumor cell lines HOS, A375, HT29, allowing the establishment of molecular markers for the evaluation of growth pathways, proliferation, survival, as well as methods of assessment and monitoring of cytotoxicity by molecular biology techniques.

10. Gene expression for IL-6 increased only for peptide-free tumor cell lines and significantly lower than the ABL reference gene for HT29 and A549 cells incubated with LL37, confirms the protumorigenic role of this cytokine and indirectly the involvement of inflammatory phenomena in tumorigenesis and in promoting the evolution of cancer. Similar results were obtained for IL-8.

11. More and more types of tumors are now associated with inner cellular LL-37 deficiency, and we suggest the need for further exploration of the relationship between LL-37 and other, as yet underinvestigated cancers. Given that LL-37 expression in different cells is inducible, the relationship between these inducible factors and tumorigenesis should be considered and investigated.

12. The antimicrobial peptides cathelicidin LL37 and human defensin 1 beta could have favorable effects on oral neoplasms, localization with a rich microbial flora as well as on mandibular osteosarcoma.

ELEMENTS OF ORIGINALITY

The use of cathelicidin LL37 and human defensin β1 as cytotoxic peptides in various tumor/normal cell cultures has shown both their therapeutic effects on malignant degenerated cells and the absence of negative effects on normal cells. There are no studies in our country with this topic and those in the literature use other cell lines for evaluation.

In order to highlight the mechanism of action of these natural peptides of human origin, the expression of genes involved in cell survival and/or apoptosis was determined in the presence and absence of one of the two AMPs.

Thus, specific primers were chosen for the target genes such as the HIF 1α gene expressing the degree of hypoxia, the IRE1α gene involved in oxidative stress, the pro-apoptotic genes CHOP and PERK, the expression for PI3K and AKT genes, whose increase indicates hyperphosphorylation and secondary decrease in the synthesis of effector proteins, as well as genes that stimulate cell proliferation and survival.

In order to quantitatively determine the expression of the stated genes, the hybridization conditions of the primers were also optimized, the GAPDH and ABL (reference) genes were established and the calculation formula for the quantitative expressions of the selected genes was optimized. Cathelicidin LL37 and human β1 defensin have been shown to have an obvious but different anticarcinomatous effect on tumor cell lines.

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

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1. **Ştefanache T**, Forna N, Bădescu M, Jitaru D et colab. Modulation of the activity of certain genes involved in tumor cell metabolism in the presence of the cytotoxic peptides defensin and cathelicidin LL37, *EXP THER MED*. 2019 Dec; 18(6): 5033-5040, (IF=1,448)
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BDI articles

1. **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.
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