Tumor Immune Response in the Presence of a Cytotoxic Peptide

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We evaluated, by the flow cytometry technique, the viability of two tumor cell lines: colorectal carcinoma (HT-29) and human A549 arcinoma incubated with the cytotoxic peptide LL-37. The results obtained for the two cell lines HT-29 and A549 are significantly different under the action of cathelicidin LL-37. At high concentrations of 20μM, cellular apoptosis was over 30% higher for colorectal adenocarcinoma line compared to that by peptide exposure. Apoptosis was also significant in low-concentration (4uM) catechidine-labeled lung cancer cells for 48 h. Also, optimization of primers was sought to evaluate gene expression for, Bcl2, IL6, IL8. Determination of gene expression for these molecular targets under the action of the cytotoxic peptide was performed in order to evaluate the immune response of tumor cells. For this purpose, the genetic material (RNA) was extracted from cell cultures and reversed in cDNA, which was subsequently amplified by the qRT-PCR technique. Evaluation of tumor cell metabolism was done by determining the gene expression for Bcl2, IL6, IL8 by the action of the cytotoxic peptides used. The cytotoxic effect of cathelicidin LL-37 for the two cell lines HT29 and A549 is supported by the decrease in IL-6 and IL-8 gene expression. The increase in Bcl2 expression for cells exposed to the action of the peptide is explained by the increase in anti-apoptotic protein synthesis that explains the fight of tumor cells for survival and proliferation.

Keywords: colorectal carcinoma, alveolar carcinoma, cytotoxic peptide

In recent years, many cytotoxic peptides having antimicrobial effect have been tested for the possible tumoricidal effect. We investigated the effect of cathelicidin LL-37 on two tumor lines: HT-29 and A549. **The HT-29** cell line is an adherent cell line of colorectal adenocarcinoma. HT-29 cells produce carcinoembryonic antigen and are human intestinal epithelial cells that produce an immunoglobulin A secreting moiety. **A-549** is a cell adherent cell lineage of human alveolar carcinoma. These cells are used for tumorigenic studies.

For the detection and measurement of the apoptotic process we used the flow cytometry technique [1]. Apoptosis is characterized by changes in cell morphology, nuclear condensation, pycnosis, but also by a series of biochemical events that lead to the degradation of the mitochondrial membrane and nuclear DNA. Phosphatidyl-serine normally present in the cell membrane, but not available, is expressed on the surface of cells in various stages of apoptosis. Incubation of cells with the Anexin V complex and their flow-cytometric analysis allow, based on fluorescence analysis, detection of those apoptosis cells, especially those in the early stages of the process. Anexin V can be used to monitor cell viability, early apoptosis, late apoptosis, and cell death. The viability of the cells - the cells are negative for Annexin V and 7-AAD7 (amino-actinomycin D), no signs of apoptosis and the plasma membrane is intact.

Cells in early apoptosis are positive for Anexin V and negative for 7-AAD because, although translocations of PS (phosphatidylserine) have occurred, the plasma membrane is intact so that 7-AAD fluorochrome can not enter the cell. If the cells are in late or dead apoptosis following flow cytometry analysis, they are positive for both Annexin V and 7-AAD because the plasma membrane has lost its integrity, and the 7-AAD fluorocrom has reached the nuclear level. In many studies, Anexin V has been used to detect apoptotic cells *in vitro* and *in vivo* [2, 3].

Tumor cells frequently develop resistance to apoptosis induced by immune cells. One of the simplest mechanisms is overexpression of the antiapoptotic gene product, such as Bcl-2 [4, 5]. The theory of tumor immune surveillance, initially enunciated by Ehrlich, then resumed by Burnett and Thomas, recognizes the major role in controlling the appearance and proliferation of tumors. This theory could explain the increase in tumor frequency, along with the diminishing immune defense effectiveness with age. Interaction between the tumor and the host organism involves the immune system at varying levels and can cause different consequences. These include host protection by the role of immune system surveillance, disruption of immune system functions and favoring tumor development due to chronic inflammation [6]. Moreover, tumors recruit cells of the immune system and stimulate their progression [7]. For this reason, the present study evaluates the immune response of the two types of tumor cells by determining the gene expression of Bcl-2 and some pro-inflammatory cytokines: IL-6 and IL-8 secreted by these cathelicidin LL-37 treated / untreated cells.

Experimental part

Material and methods

The two tumor cell lines were cultivated in RPMI-1640 medium (Sigma Aldrich) with 10% FBS (Faetal Bovine Serum, GIBCO) [8]. Cell expansion was done in 250 mL flasks to obtain a sufficient number of cells (10^5 tumor cells / well) [9]. Cells with a viability greater than 97% were used. The actual testing was performed in 96-well plate plates using a final working volume of 200μL per well.

For the flow cytometry test, the following peptide concentrations (cathelicidin LL-37) were selected for

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which statistically significant changes were obtained under the action of the cytotoxic peptide:

- 4μM ($p < 0.05$) for the HT29 lineage (colorectal adenocarcinoma) at 48 h incubation.
- 9μM ($p < 0.05$) for line A549 (alveolar carcinoma) at 72 h incubation and 20μM ($p < 0.01$) at 48 h incubation.

For the detection and measurement of the apoptotic process [1] we used the flow cytometry technique. Apoptosis is characterized by changes in cell morphology, nuclear condensation, pycnosis, but also by a series of biochemical events that lead to the degradation of the mitochondrial membrane and nuclear DNA. Phosphatidylserine normally present in the cell membrane, but not available, is expressed on the surface of cells in various stages of apoptosis. Compound 7-AAD (7-aminoactinomycin D) is a fluorescent chemical compound with a high affinity for DNA. It is used as a fluorescent marker in flow cytometry and allows the evaluation of cells entering late apoptosis or already dead [10]. It intercalates in double-stranded DNA, having a high affinity for the guanine-rich areas (GC). Compound 7-AAD does not pass through the intact cell membrane, so it is used for cell viability. The compromised membrane cells are stained with 7-AAD, and those with the intact cell membrane remain uncolored.

One of the early events of apoptosis is the translocation in the cell membrane of phosphatidylserine (PS), a membrane phospholipid inside the cell, namely from the inner layer of the lipid bilayer. This event can be evaluated by flow cytometry because PS is highlighted by binding Anexine V coupled with a fluorescent substance such as phycoerythrin (PE), and at the nuclear level, the 7-AAD or propidium iodide (PI) will bind [11].

Molecular Biology Techniques Used by Us - SYBR GREEN RT-PCR Optimizations; Extraction of total RNA with TRIzol or TRIreagent [12].

TRIzol® Reagent is a monophasic solution of phenol, guanidine isothiocyanate and other components that facilitate the isolation of total RNA from biological products while maintaining the integrity of the RNA obtained. Cell suspensions were used as biological samples. The precipitated RNA can be stored in 70% ethanol at -20 ° C for up to one year or 4 ° C for one week. For the quantification of nucleic acids, we used the TermoScientific Nanodrop 2000 Spectrophotometer. We performed Reverse Transcription of RNA extracted from tumor cells into complementary DNA (cDNA). The reverse transcription kit provided both random and oligo (dT) primers. Random primers that hybridize to different regions on the RNA pathway and produce small fragments of all RNA types were used.

PCR technique

The PCR (Polymerase Chain Reaction) is a method of enzymatic amplification in vitro of a particular DNA or RNA sequence whose nucleotide structure is partially known. Amplification is considerable (billions of times) and rapid (within a few hours), allowing sufficient amount of genetic material to be obtained for analysis [13].

High Resolution Melting is a technique for analyzing PCR post-amplification of the melting curves of interest fragments (amplicons). It is an attractive method due to simplicity (only a set of primers) and due to the rapidity of realization [15].

The result consists in obtaining a melting curve characteristic to each amplicon. The HRM reaction can take place in a single step or the PCR reaction can be performed in a standard thermocouple, and only HRM in a Real-Time PCR. The concept of analyzing the PCR product obtained by amplification (amplicon) by making the melting curve is not a new one. It is routinely used in non-specific Real-Time PCR. This process is now called Low Resolution Melting (LRM) [16].

The highest rate of fluorescence decrease in the melt process is at the characteristic melting temperature of the obtained amplicon [17]. The melting temperature is defined as the temperature at which half of the double-stranded DNA molecules are denatured.

The melting temperature is higher for longer and higher CG chains. The easiest way to visualize the melting curve is by plotting the relative fluorescence units (RFUs) relative to time (T) (- d (RFU) / dT) , and on the abscissa the temperature, which causes a peak to rise to the melting temperature (Tm).

The results obtained for the optimization of primers for their use in assessing gene expression on cDNA obtained from tumor cell cultures - was achieved by two methods:

- *Classic PCR and migration to 2% agarose gel* [14].
- *qRT-PCR with SYBR green* [12].

To obtain the optimal temperature of primer alignment to the target cDNA fragments, amplification was performed in qRT-PCR using SYBR green.

Results and discussions

Cell apoptosis for the **A549** line was 16% for the concentration of 9μM cathelicidin LL-37, being significantly increased to 20μM cathelicidin LL-37 (33.4%) (table 1, 2). For the control apoptosis was 5%, with 95% cell viability (table 3).

The behavior of cells incubated with cathelicidin LL-37 20μM for 48h was twice as aggressive as compared to that of cells incubated with cathelicidin LL-37 9μM (incubated 72h) for which apoptosis was 3 times higher than that of the control (table 2, 3).

For assessing the viability of *HT-29 Cell Line* by flow cytometric technique was used citototoxic peptide concentrations (cathelicidin LL-37) which has a significant reduction in viability. Thus, the cell line was incubated with cathelicidin LL37 48h in a concentration of 4μM.

A-549 cell line treated with cathelicidin LL-37 incubated **48h**	A549- cathelicidin LL-37 (**20μM**)	
Cell line type	**A549**	
Type of counted cells and phenotypic marker	A549 cathelicidin LL-37 treated **20μM** PI + Annexin	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	8931	89.3
Necrotic cells	2497	28
Cells in late apoptosis	455	5.1
Live cells	5954	66.7
Cells in early apoptosis	25	0.3

**PI= propidium iodide*

Table 1
CELL VIABILITY OF 48h-INCUBATED A549 LINE WITH CATHELICIDIN LL-37 20μM

A-549 cell line treated with cathelicidin LL-37 incubated **72h**	A549- cathelicidin LL-37 (**9uM**)	
Cell line type	**A549**	
Type of counted cells and phenotypic marker	A549 cathelicidin LL-37 treated **9µM** PI + Annexin	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	9152	91.5
Necrotic cells	104	1.1
Cells in late apoptosis	902	9.9
Live cells	7724	84.4
Cells in early apoptosis	422	4.6

Table 2
CELL VIABILITY OF THE 72h INCUBATED LINE A549 WITH CATHELICIDIN LL-37 9µM

Table 3
CELL VIABILITY OF 48h / 72h INCUBATED LINE A549 WITHOUT CATHELICIDIN LL-37 (WITNESS)

A-549 cell line without cathelicidin LL-37 incubated **48h**	A549- *without peptide* (WITNESS)	
Cell line type	**A549**	
Type of counted cells and phenotypic marker	A-549 cell line without cathelicidin LL-37 _PI+Anexina V	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	9664	96.6
Necrotic cells	129	1.3
Cells in late apoptosis	408	4.2
Live cells	9061	93.8
Cells in early apoptosis	66	0.7
A-549 cell line without cathelicidin LL-37 incubated **72h**	A549- fără peptid (MARTOR)	
Cell line type	**A549**	
Type of counted cells and phenotypic marker	A-549 cell line without cathelicidin LL-37 _PI+Anexina V	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	9742	97.4
Necrotic cells	91	0.9
Cells in late apoptosis	253	2.6
Live cells	9317	95.6
Cells in early apoptosis	81	0.8

Table 4
CELL VIABILITY OF THE HT29 LINE INCUBATED 48h WITH CATHELICIDIN LL-37 4µM

HT-29 cell line without cathelicidin LL-37 incubated **48h**	**HT29**- cathelicidin LL-37 (4µM)	
Cell line type	HT-29	
Type of counted cells and phenotypic marker	HT29- cathelicidin LL-37 treatedH-**4µM** PI+Anexina V	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	9070	90.7
Necrotic cells	303	3.3
Cells in late apoptosis	1329	14.7
Live cells	7378	81.3
Cells in early apoptosis	60	0.7

**PI= propidium iodide*

It was noted that the number of cells significantly decreased after incubation with cathelicidin LL-37 concentration of 4µM compared to the well in which the cells were incubated with peptide (control), given that we started from an approximately equal distribution of cells / well ($2x10^5$ cells in a final volume of 500µL) (fig.1).

Cellular apoptosis for the HT29 line was approximately 20% for the concentration of 4µM cathelicidin LL-37, being 2-fold greater than for peptide untreated cells. For the control, apoptosis was approximately 12%, with a cell viability of 88% (fig. 2). A large number of cells in late apoptosis (about 15%) were also shown against the control for which the percentage of cells in late apoptosis was only 8% (fig. 2, table 5).

In conclusion: Incubation of HT29 lineage cells with cathelicidin LL-37 at a concentration of 4µM for 48h

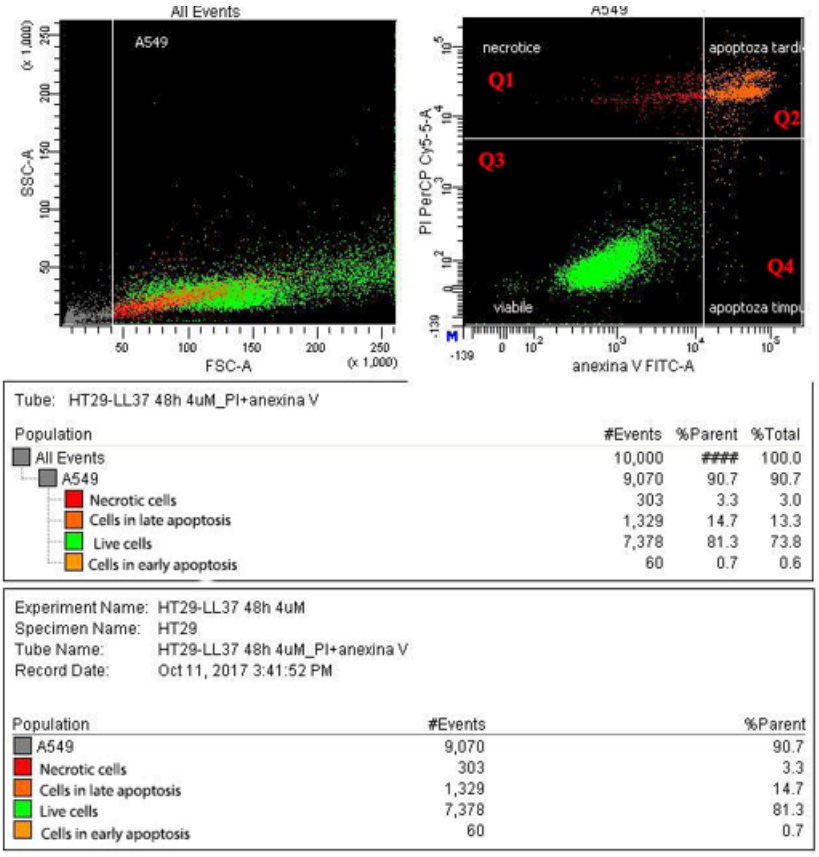


Fig.1. Cellular viability cell line HT29 analysis with 4μM cathelicidin LL-37 (incubation 48h) (Q1 = necrotic cells, Q2 = cells in late apoptosis, Q3 = live cells, Q4 = cells in early apoptosis)

HT-29 cell line treated with cathelicidin LL-37 incubated **48h**	HT-29-WITHOUT PEPTIDE (WITNESS)	
Cell line type	HT-29	
Type of counted cells and phenotypic marker	HT-29-without cathelicidin LL-37 _PI+Anexina V	
Cell populations evaluated	Number of events (cells) in absolute value	Percentage distribution (%) cell populations counted
A549	9634	96.3
Necrotic cells	75	0.8
Cells in late apoptosis	776	8.1
Live cells	8550	88.7
Cells in early apoptosis	233	2.4

Table 5
CELL VIABILITY OF HT29 LINE INCUBATED 48h / 72h WITHOUT CATHELICIDIN LL-37 (WITNESS)

resulted in a 20% significant apoptosis evidenced by the flow cytometric technique, with the indication that 15% of these cells were in late apoptosis (which means a very high cell death rate). Cell viability for the control was significantly increased (88%).

The results obtained for the two cell lines HT-29 and A549 are similar under the action of cathelicidin LL-37. At high concentrations of 20μM, cellular apoptosis was over 30% greater for the colorectal adenocarcinoma line compared to exposure to lower concentrations but longer time. Apoptosis was also significant for catechidine-treated lung cancer cells at low concentrations (4μM) for 48 h.

In the next step, we explored the gene expression of some molecules involved in the immune response. The results obtained for the optimization of primers for their use for assessing gene expression on cDNA obtained from tumor cell cultures - was achieved by two methods:

- Classic PCR and migration to 2% agarose gel
- qRT-PCR with SYBR green

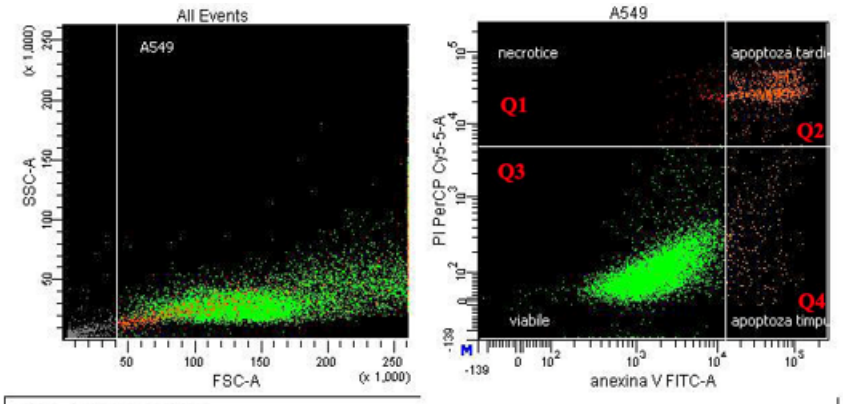
To obtain the optimal temperature of primer alignment to the target cDNA fragments, amplification was performed in qRT-PCR using SYBR green according to the protocol. This protocol describes the detailed experimental procedure for real-time RT-PCR using SYBR Green I as mentioned in Xiaowei Wang and Brian Seed [18]. The results obtained show that not all of the tested primers function optimally at 66 or 72°C (table 6). The primers that could not be optimized at this temperature led to the formation of primers dimers overlapping the amplification products, resulting in altered results (intense amplification signal, i.e. increased false).

The reference gene was chosen for the ABL gene and the following primers were used [47]:

A-5' TGTGATTATAGCCTAAGACCCGGAGCTTTT 3'
B-5' TTCAGCGGCCAGTAGCATCTGACTT-3

ABL (belson murine leukemia viral oncogene homolog is a protein that, in humans, is encoded by the *ABL1* gene (previous symbol *ABL*) located on chromosome 9. The *ABL1* proto-oncogene encodes a cytoplasmic and nuclear protein tyrosine kinase that has been implicated in processes of cell differentiation and cell division [19].

The optimal alignment temperature of these primers (for the ABL gene) was 55°C. Once the optimal temperature of alignment of all primers for the established molecular targets (BCL2, IL-6 and IL-8) has been established, and after establishing the reference gene (ABL) to which the number of copies of amplicons obtained by amplifying qRT- PCR we extracted RNA from cell cultures treated or not with the cytotoxic peptide (cathelicidin LL-37).



Tube: HT29-netratat a+pi

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
A549	9,634	96.3	96.3
Necrotic cells	75	0.8	0.8
Cells in late apoptosis	776	8.1	7.8
Live cells	8,550	88.7	85.5
Cells in early apoptosis	233	2.4	2.3

Experiment Name: HT29-LL37 48h 4uM
Specimen Name: HT29
Tube Name: HT29-netratat a+pi
Record Date: Oct 11, 2017 3:39:12 PM

Population	#Events	%Parent
A549	9,634	96.3
Necrotic cells	75	0.8
Cells in late apoptosis	776	8.1
Lice cells	8,550	88.7
Cells in early apoptosis	233	2.4

Fig.2. Analysis of Cytometry Technique of HT 29 line without cathelicidin LL-37 - Control (incubation 48h) (Q1 = necrotic cells, Q2 = cells in late apoptosis, Q3 = live cells, Q4 = cells in early apoptosis)

	PRIMER TACK TEMPERATURE
Primer IL-8 Fw ATGACTTCCAAGCTGGCCGTGGCT Primer IL-8-Rev TCTCAGCCCTCTTCAAAAACTTCTC	56°C
Primer IL-6 Fw TTCGGTCCAGTTGCCTTCTC Primer IL-6 Rev GAGGTGAGTGGCTGTCTGTG	72°C
Primer Bcl2 –Fw CTGCACCTGACGCCCTTCACC Primer Bcl2-Rev CACATGACCCCACCGAACTCAAAGA	72°C
Primer Bcl2 –Fw CTGCACCTGACGCCCTTCACC Primer Bcl2-Rev CACATGACCCCACCGAACTCAAAGA	72°C

Table 6
OPTIMIZED PRIMERS

After removing the culture medium from each well, 1 mL of triazole was added to the peptide treated / untreated cell pellet, well homogenized and continued with RNA extraction (with AMBION trizole) followed by reverse transcript according to the protocol (Thermo Scientific).

The RNA concentrations listed in the table below (table 7) were obtained, the purity of which was calculated by reading absorbances at 260 and 280nm, respectively. The table also mentions the amount of RNA used in the RT-PCR amplification reaction.

SAMPLE CODE	RNA concentration (ng/uL)	Purity (260 nm/280 nm)	The amount used in RT-PCR (µL)	The amount of nuclease-free H2O added after RT -PCR (µL)
4	127.5	1.76	11	80
5	647.7	1.86	4	109.5
6	772.5	1.85	3	95.9
7	563.4	1.66	4	92.7
8	426.4	1.95	5	86.6

Table7
RNA CONCENTRATIONS OBTAINED BY EXTRACTING IT FROM THE CELL LINES USED

Where the sample codes used are the following

SAMPLE CODE	MEANING
4	HT29 cells treated with LL37 concentration of 4 µM 48h culture
5	HT29 untreated cells, 48h culture
6	A549 untreated cells, 48h culture
7	A549 cells treated with LL37 concentration of 9 µM 72h culture
8	A549 untreated cells, 72h culture

PEPTIDE	LL37					
INCUBATION	48H				72H	
CELL LINE	3	4	5	6	7	8
ABL	6	38800	7020	44500	14	37200
IL-8	0	88.4	0	5850	0	949
IL-6	5	854	36.3	6390	17.6	560
BCL2	0.6	394	4.47	369	0.1	279

SAMPLE CODE	MEANING
4	HT29 cells treated with LL37 concentration of 4 μM 48h culture
5	HT29 untreated cells, 48h culture
6	A549 untreated cells, 48h culture
7	A549 cells treated with LL37 concentration of 9 μM 72h culture
8	A549 untreated cells, 72h culture

Table 8
NUMBER OF TARGET AMPLICON COPIES / μL

RT-PCR amplification amplificons of interest of different concentrations were obtained (table 8) and were reported at the ABL concentration extracted from each cell line used in the experiment, the ratio being multiplied by 100 so that the final result will be one percent (%).

The experimental results obtained for the BCL2 gene whose gene expression was evaluated on the cell line HT29 (colorectal carcinoma) and A549 (alveolar carcinoma) in the presence of the cathelicidin LL-37 were reported in a control consisting of the peptide-free cell line. The analysis of the obtained results revealed a significant increase for the BCL2 gene for the HT29 line with 48μM incubated cathelicidin LL-37 as compared to the control for which the Bcl2 gene expression was inhibited. Also, a 10% increase for line A549 with 20 ìM incubated 48h cathelicidin LL-37 was compared with the control (for which the increase was 0.82% relative to the ABL reference gene). That is, a 10-fold increase in gene expression for BCL2 on the 20μM-treated peptide line compared to the control (Fig. 3, table 9). Note that at 72 h for small concentrations (9μM) of cathelicidin LL37, Bcl2 gene expression remained constant. Therefore, it can be concluded that Bcl2 activity was significantly increased only at high peptide concentrations for A549 cells.

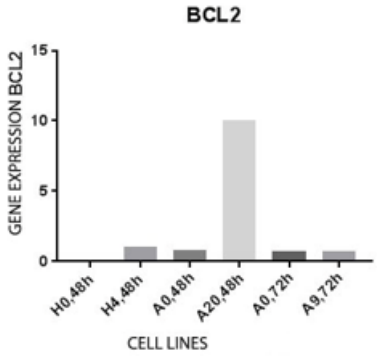


Fig.3. Quantitation of gene expression for the BCL2 gene relative to the ABL reference gene

Analysis of the obtained results revealed an insignificant increase in IL-8 gene expression expressed by the 48h incubated 48h with cathelicidin compared to the cathelicidin LL-37-free incubated cells and a 13% increase for the peptide-free 48h incubated line A549, compared to the cells incubated with cathelicidin 20μM (48h). That is, gene expression for IL-8 was increased only for peptide-free tumor cell lines and totally inhibited for cytotoxic peptide-incubated A549 cells and significantly decreased (relative to the ABL reference gene) for HT29 lineage cells.

The experimental results obtained for the IL6 gene whose gene expression was evaluated on the HT29 cell line (colorectal carcinoma) and A549 (alveolar carcinoma) in the presence / absence of the peptide revealed a significant increase in IL-6 gene expression by the incubated HT29 line 48h without cathelicidin, compared with cathelicidin LL-37-incubated cells. Therefore, gene expression for IL-6 was approximately 4-fold lower in the HT29 line incubated with cytotoxic peptide. On the other hand, in line A549, a significant increase in gene expression was only observed in cells incubated without peptide compared to 20μM (48h) or 9μM (72h) cathelicidin-incubated cells for which the expression of this gene was significantly lower than the ABL reference gene. That is, gene expression for IL-6 was increased only for peptide-

Amplicon copies/μL	H0,48h	H4,48h	A0,48h	A20,48h	A0,72h	A9,72h
	3	4	5	6	7	8
ABL	7020	38800	44500	6	37200	14
BCL2	4.47	394	369	0.6	279	0.1
PERCENT BCL2 = (BCL2/ABL) *100	0.06367	1.01546	0.82921	10	0.75	0.71428

SAMPLE CODE	MEANING
3	HT29 cells treated with LL37 concentration of 4 μM 48h culture
4	HT29 untreated cells, 48h culture
5	A549 untreated cells, 48h culture
6	A549 cells treated with LL37 concentration of 20 μM 48h culture
7	A549 cells treated with LL37 concentration of 9 μM 72h culture
8	A549 untreated cells, 72h culture

Table 9
PERCENTAGE CALCULATION OF BCL2 / μL AMPLICON COUNTS RELATIVE TO THE ABL REFERENCE GENE

Amplicon copies/µL	H0,48h	H4,48h	A0,48h	A20,48h	A0,72h	A9,72h
	3	4	5	6	7	8
ABL	7020	38800	44500	6	37200	14
IL8	0	88.4	5850	0	949	0
PERCENT IL8 = (IL8/ABL) *100	0	0.22783	13.1460	0	2.5510	0
SAMPLE CODE	MEANING					
3	HT29 cells treated with LL37 concentration of 4 µM 48h culture					
4	HT29 untreated cells, 48h culture					
5	A549 untreated cells, 48h culture					
6	A549 cells treated with LL37 concentration of 20 µM 48h culture					
7	A549 cells treated with LL37 concentration of 9 µM 72h culture					
8	A549 untreated cells, 72h culture					

Table 10
PERCENTAGE CALCULATION OF IL8 / µL AMPLICON COUNT RELATIVE TO THE ABL REFERENCE GENE

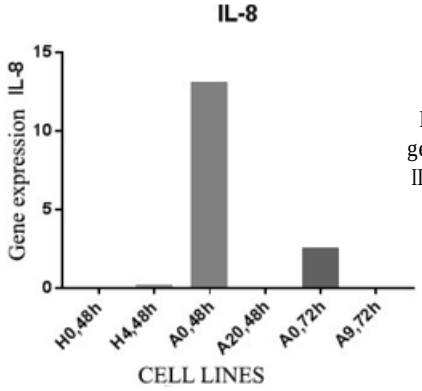


Fig. 4. Quantitation of gene expression for the IL8 gene relative to the ABL reference gene

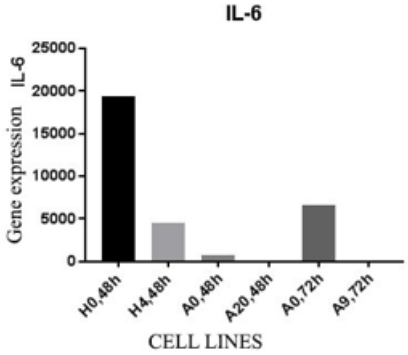


Fig.5. Quantitation of gene expression for the IL-6 gene relative to the ABL reference gene

Table 11
PERCENTAGE CALCULATION OFTHE NUMBER OF IL-6 / µL AMPLICONS RELATIVE TO THE ABL REFERENCE GENE

Amplicon copies/µL	H0,48h	H4,48h	A0,48h	A20,48h	A0,72h	A9,72h
	3	4	5	6	7	8
ABL	7020	38800	44500	6	37200	14
IL6	36.3	854	6390	5	560	17.6
PROCENT IL6= (IL6/ABL) *100	19338.8	4543.32	696.40	120	6642.85	79.545
SAMPLE CODE	MEANING					
3	HT29 cells treated with LL37 concentration of 4 µM 48h culture					
4	HT29 untreated cells, 48h culture					
5	A549 untreated cells, 48h culture					
6	A549 cells treated with LL37 concentration of 20 µM 48h culture					
7	A549 cells treated with LL37 concentration of 9 µM 72h culture					
8	A549 untreated cells, 72h culture					

free tumor cell lines and significantly decreased relative to the ABL reference gene for the HT29 and A549 cell line incubated with catechidine (fig. 5, table 9).

Cancer cells adapt to the tumor micromedium by activating macrophages that will secrete cytokines, growth factors and angiogenesis [20]. Under RE stress, the cancer cell will increase COX2 expression via the NF-kB pathway, which plays an important anti-apoptotic role. Also, activation of the NF-kB pathway plays an important pro-inflammatory role by stimulating IL-8 synthesis as is the case with human epithelial cells [21, 22].

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 was associated with resistance to chemotherapy in various cancers [23, 24]. Also, the accumulation of defects through the apoptotic pathway of tumor cells can lead to their survival and resistance to chemotherapy [26, 27]. For the two lines studied (HT29 and A549), gene expression for Bcl2 was significantly increased when the cells were exposed to the cytotoxic peptide, this being probably explained by the increase in the synthesis of anti-apoptotic proteins involved in cell survival. Note however that at lower concentrations of cathelicidin (9µM) and longer exposure (72h), Bcl2 expression is kept approximately constant compared to the control, which would favor the evolution of tumor cells to apoptosis.

IL-6 is a chemokine that activates the STAT3 transcription factor on the NF-kB-IL6-STAT3 cell signaling pathway, favoring the proliferation and survival of tumor cells. Its overexpression has been reported in almost all types of tumors. The strong association between inflammation and cancer is reflected by elevated IL-6 levels in the tumor micromedium [28]. Furthermore, IL-6 protects cancer cells from therapy-induced DNA damage, oxidative stress and apoptosis by facilitating repair and

induction of pro-survival antioxidant and antiapoptotic pathways). Therefore, blocking IL-6 or inhibiting its associated signaling independently or in combination with conventional anticancer therapies could be a potential therapeutic strategy for the treatment of cancers. We have highlighted that gene expression for IL-6 was increased only for peptide-free tumor cell lines and significantly decreased for the HT29 and A549 cell lines incubated with catechid. The fact that gene expression for IL-6 is increased in cells without peptide exposure and significantly decreased in cathelicidin LL-37-treated cells also proves the tumoricidal effect of the peptide used [25].

IL-8 is a chemokine that stimulates the synthesis of superoxide anion by enhancing oxidative stress. Increased expression of IL-8 and / or its receptors has been described in cancer cells, endothelial cells and tumor-associated macrophages, suggesting that IL-8 may function as a significant regulatory factor in the tumor micro-environment [29] . Induction of IL-8 signaling activates multiple upstream signaling pathways which (a) affect gene expression by regulating numerous transcription factor activities, (b) modulate cellular proteomics and / or (c) cellular cytoskeleton by posttranslational regulation of regulatory proteins. As a consequence of the diversity of downstream effects and targets, IL-8 promotes angiogenic responses in endothelial cells [30], increases the proliferation and survival of endothelial and cancer cells and potentiates the migration of cancer cells. Accordingly, IL-8 expression correlates with angiogenesis, tumorigenesis and tumor metastasis in many in vivo models. Recently, IL-8 signaling has been implicated in regulating the transcriptional activity of the androgen receptor, supporting the transition to androgen-independent proliferation of prostate cancer cells [31]. Therefore, inhibiting the effects of IL-8 signaling may be a significant therapeutic intervention in targeting tumor microclimate to apoptosis. In our experiment, IL-8 gene expression was increased only for peptide-free tumor cell lines and totally inhibited for cytotoxic peptide-incubated A549 cells and significantly decreased for HT29 lineage cells incubated with or without catelicidin. Therefore, cathelicidin LL-37 and possibly other cytotoxic peptides could also be used in cancer therapy given the effect of IL-8 gene expression decrease.

Therefore, inhibiting the effects of IL-8 signaling may be another significant therapeutic intervention in targeting tumor microclimate to neoplastic cell apoptosis.

Conclusions

In these studies the determination of the tumoral potential of cathelicidin LL-37 according to cell line type was measured, measuring the viability by the flow cytometry method for the peptide concentrations at which a significant apoptosis was observed. The cytotoxic effect of cathelicidin LL-37 for the two HT29 and A549 cell lines was evident.

Cell cultures were performed according to the above mentioned protocols and the following adherent cell lines were used: HT-29 - colorectal adenocarcinoma cell line and A-549 - human alveolar carcinoma cell line.

For the two lines studied (HT29 and A549), gene expression for Bcl2 was significantly increased when the cells were exposed to the cytotoxic peptide, this being probably explained by the increase in the synthesis of anti-apoptotic proteins involved in cell survival. However, it should be noted that at low concentrations of cathelicidin (9 μM) and longer exposure (72h), Bcl2 expression is kept at values close to the control, which would favor the evolution of tumor cells to apoptosis.

The cytotoxic effect on cathelicidin LL-37 for the two HT29 and A549 cell lines is supported by the decrease in IL-6 and IL-8 gene expression.

Therefore, blocking IL-6 and IL-8 by cytotoxic peptides such as cathelicidin LL37, or inhibiting their signaling, independently or in combination with conventional anticancer therapies, may be a potential therapeutic strategy for the treatment of cancers.

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Manuscript received: 14.09.2017