

The Effect of Some Natural Cytotoxic Peptides on Tumor Cells

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Many studies have highlighted the anti-tumor properties of some natural peptides known to have antimicrobial virtues. In this study, we evaluated the tumoricidal potential of dermaseptin, defensin, cecropin A and B on tumor cell lines: M14K (human mesothelioma). The viability study was performed using PE V Annexin and 7-AAD (7-amino-actinomycin D) (BD Pharmingen). This test was used to detect and measure apoptosis by flow cytometry technique. The experimental results of our study revealed that the cytotoxic effects of the four peptides depend on their concentration. In this in vitro experimental study, we found that the cytotoxic effect of the four cytotoxic peptides used depended on their concentration in the tumor cell culture medium, being significant at concentrations of 120 μM and maintained at concentrations of 60 μM. At 30 μM concentrations these tumoricidal effects were insignificant. Of all the studied peptides, dermaseptin has the most powerful effect and the weakest effect β - defensin - 1.

Keywords: dermaseptin, defensin, cecropin, human mesothelioma

Human mesothelioma (M14K line) is a very aggressive malignant tumor that appears on mesothelial cell wall surfaces, most often in the pleural cavities, but also in the peritoneum, pericardium, and soft paratesticular tissues. Having metastatic potential in 75% of patients [1] and response below 20% in various chemotherapies [2], mesothelial cells are a good target for evaluating new chemotherapeutic agents. Of the 3 histological mesothelioma subtypes, the M14K mesenchymal cell line of the epithelial type was used in the experiment.

Although the exact mechanism of action of antimicrobial peptides remains a controversial issue, there is a consensus that these peptides selectively disrupt cell membranes, and it is believed that the amphipathic structural arrangement of peptides plays an important role in this mechanism [3,4]. Thus, the biological effect of natural peptides with cytotoxic potential on tumor cells can be traced to both animal experimental model and tumor cell lines [5]. Considering all these aspects of the literature, in this experimental study, we have verified the hypothesis that cytotoxic peptides: defensin, cecropins and dermaseptin have tumoricidal potential, the intensity of which depends both on the nature of the peptide used and its concentration in the medium cell life. In order to achieve this, we have developed an in vitro experimental model in which we have determined the optimum cell concentration to evaluate the cytotoxic potential of the peptides studied as being 10⁵ cells / well in a final volume of 200 μL culture medium.

Experimental part

Material and method

The adherent cell line - M14K (human mesothelioma) was cultured in RPMI 1640 culture medium (RPMI-1640 Medium, Sigma Aldrich) supplemented with 10% FBS (Fetal Bovine Serum, Sigma) [6]. The proper testing was performed in 96-well plate plates, using a volume of 200 μL per well. The work was carried out in triplicate using two

working variants, the cells being incubated in simple RPMI medium or 2% FBS. The viability study was performed after 24, 48, and 72 h incubation at 37°C, 5% CO₂ by flow cytometry, with propidium iodide.

The propidium iodide (PI) viability test by flow cytometry technique is used to detect and measure cell death [7,8]. Propidium iodide (PI) is a vital dye for DNA; it penetrates the dead cells and binds to the nuclear DNA, while the live cell membrane is not permeable to PI. Fluorescence of propidium iodide is read on the second fluorescence channel (FL2). The settings were determined by SSC-side scatter and FSC-forward scatter of unlabeled cells observed at flow cytometry [9,10]. Propidium iodide has the ability to penetrate the cell nuclei and bind to DNA so that the amount of nucleic acid can be quantified by measuring the fluorescence intensity recorded by the FL2 sensor (the wavelength emitted by PI is 562-588 nm when the fluorochrome is excited at 488 nm), the fluorescence intensity being directly proportional to the amount of DNA in the cells [11]. The cell suspension was recovered from the wells and distributed into flow cytometric tubes. For adherent cells, cell disruption was achieved by more vigorous pipetting. After flow cytometric investigation of the unlabeled cell appearance, 10 μL of PI was pipetted into each tube. Data acquisition and interpretation was performed on a FACS Canto II flow cytometer using FACSDiva Software (Becton Dickinson). For the tested cell line, it was determined which is the optimal viability expressed in cells / well, under cultivation conditions with or without 2% FBS.

Results and discussions

The viability of thawing M14K tumor cells was approximately 60% when cultured with RPMI medium (fig.1).

After 21 days the viability of these cell cultures reached 100%, being possible the proposed experiment (fig.2).

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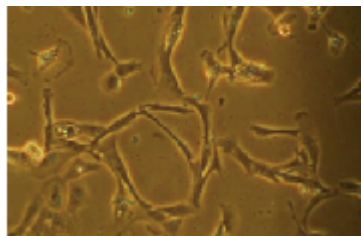


Fig. 1. Distribution of M14K tumor cell culture at defrosting (60% viability)

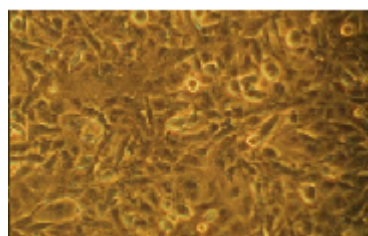


Fig. 2. Distribution of M14K tumor cell culture after 21 days (100% viability)

Table 1
DETERMINATION OF THE CYTOSTASIS (%) FOR THE M14K TUMOR LINE BY MTT TECHNIQUE

Peptide Concentration	M14K tumor cell line % of cytostasis (MTT)							
	120 μ M	60 μ M	30 μ M	15 μ M	7,5 μ M	3,5 μ M	1,8 μ M	0,9 μ M
Cecropin A	26.33	17.793	5.0711	-11.3879	-31.5836	-41.9039	-51.779	-63.07
Cecropin B	22.56	13.920	3.7885	-8.54626	-23.5242	-45.2863	-59.559	-62.55

At 24, 48, and 72 h of incubation with each peptide, cell viability was determined. The percentage of cells that entered apoptosis in the presence of one of the two cecropins was 26.3% for cecropin A and 22.56% in the case of cecropin B for peptide concentrations of 120 μ M in the culture medium of the cells (table 1). The percentage of cytostasis decreased significantly with the decrease in peptide concentration, absent at concentrations of 15 μ M (table 1).

Cell viability was significantly increased with decreasing peptide concentration (at 60 μ M) from culture medium (for all four types of peptides), cellular apoptosis being insignificant at 30 μ M and absent at peptide concentrations of 15 μ M. The highest degree of apoptosis was obtained at the concentration of 120 μ M (table 1).

The experimental results obtained by the flow cytometry technique in which we followed the apoptosis of the M14K line cells incubated with the peptides studied at different concentrations according to the above mentioned work pattern were confirmed by the MTT technique (table 1). For this reason we only compared the results obtained at the concentration of 120 μ M (table 2) for 72 h (maximum effect). The results obtained were always compared with negative CN control (tumor cells incubated in the absence of the peptide).

Flow cytometry allowed the evaluation of cell apoptosis in the presence or absence of the peptide at different concentrations (fig. 3). Cell populations were delimited by four quadrants in the live cell population (Q3), the population of cells that entered early (Q4) and late (Q2) apoptosis and dead cell population (Q1) (fig. 3).

According to this technique, the evolution of M14K cells at their impact with the two cytotoxic peptide types (cecropin A and B) after 72 h of incubation was significantly different (fig. 4).

The evaluation of cellular apoptosis by flow cytometry technique with Anexina V and 7-AAD following the introduction of each cytotoxic peptide studied (cecropin A, cecropin B, β -defensin-1 and dermaseptin) in the culture medium revealed significant cytotoxic effects. Of all the studied peptides, dermaseptin has the most potent effect and the weakest β -defensin-1, human (table 2). For this reason, we aim to continue the research with dermaseptin also on other tumor cell lines.

Dermaseptin is a cationic peptide [12] due to numerous lysine residues, with an α -helix amphiphilic structure, which directly targets certain cell membranes at which they form permeable ion channels that lead to depolarization and irreversible cytolysis, and ultimately to cell death [13]. The tumor cells used by us were lysed at 36.4% in 72 h at a dermaseptin concentration of 120 μ M. Of the lysed cells, 2.8% were dead, 15.2% late apoptosis and 18.4% early apoptosis (table 2). Dermaseptin has 27-34 amino acid residues in its structure and has been shown to have cytotoxic effect in severe fungal infections in vertebrates with immunodeficiency syndromes or with immunosuppressive therapy. Dermaseptin has been proposed as a novel drug, the mechanism of action based on the peptide's affinity for the human red cell membrane without it being toxic to the hematite. The role of erythrocyte is to transport the peptide into the circulatory system. As soon as it reaches the vicinity of the micro-organism (fungi), due to its higher affinity for it, the peptide is spontaneously transferred to the surface of the microbial target over which it will have a lytic action at the membrane level. The same mechanism could also be involved in the therapy of malignant tumors. Of course, it is important that the effect on normal cells is minimal / absent.

To demonstrate that peptides known to be antimicrobial and potentially antitumoral potential did not have cytotoxic effect on normal cells, their mode of action on a normal cell line was followed on the same experimental model. This cell line was composed of normal human epidermal keratinocytes (HEK).

To determine how the physiological serum could influence cell viability and proliferation, in our studies, we used a culture well containing: 150 μ L of physiological saline with 10⁵ tumor cells and 50 μ L of culture medium. The culture medium with saline and cells was incubated for 72 h at 37°C (table 3).

Cell viability was tested by MTT technique and flow cytometry. The experimental results revealed that after a 72 h incubation with the four types of cytotoxic peptides at concentrations of 120 μ M for each well in triplicate, the mean absorbance values were similar to the mean absorbance for NC. The percentage of cytostasis was calculated and found to be no greater than 11%, i.e. cellular apoptosis for HEK cell lines was insignificant in both the presence and absence of peptides.

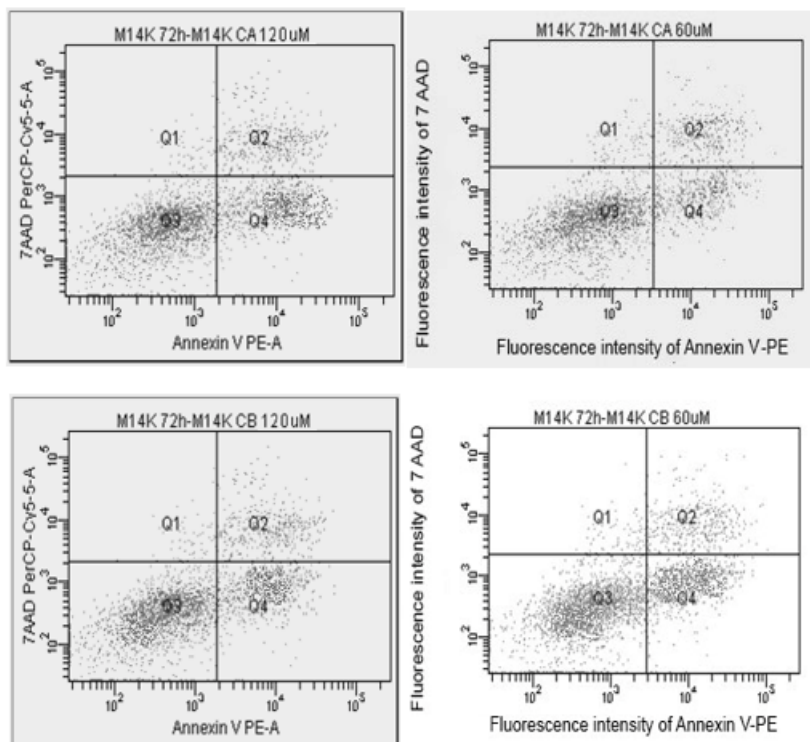
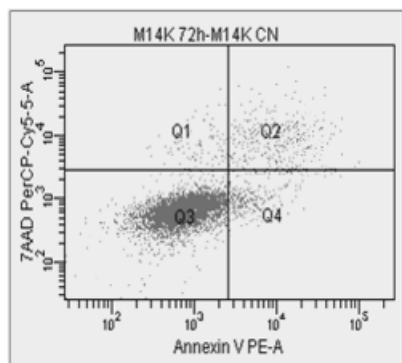
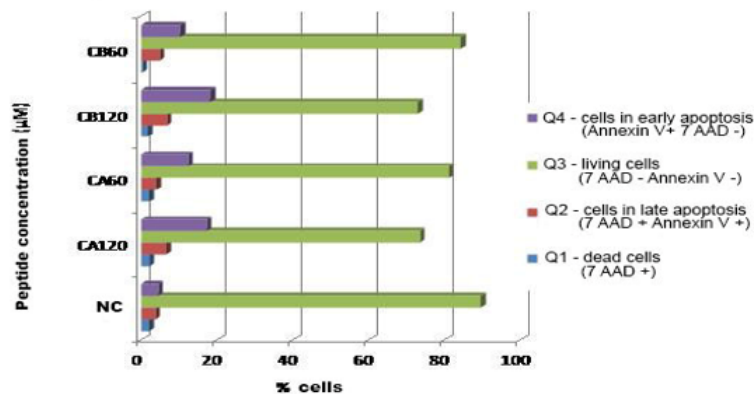


Fig.3. Fluorescence intensity distribution of the 7-AAD and Annexin V markers of M14K cells in the absence and presence of the cytotoxic peptide

Fig.4. Evaluation of apoptosis of tumor cells in the presence or absence of studied cytotoxic peptides: cecropin A (CA) and cecropin B (CB) at the concentration of 120 and 60 μM



M14K 5000 events/ well	Q1 (7AAD+)	Q2 (7AAD+AnnexinV+)	Q3 (7AAD- AnnexinV-)	Q4 (AnnexinV+ 7AAD-)
	%	%	%	%
Negative control (NC)	1.1	2.8	94.6	1.5
Cecropin A 120 μM	2.4	6.6	73.6	17.4
Cecropin B 120 μM	2.2	4.1	81.1	12.6
β - Defensin - 1, human 120 μM	1.9	6.9	72	18.2
Dermaseptin 120 μM	2.8	15.2	63.6	18.4

Table 2
EVALUATION OF CELLULAR
APOPTOSIS BY FLOW
CYTOMETRY TECHNIQUE
WITH ANEXIN V AND 7-AAD

Table 3
ANALYSIS OF NORMAL HEK CELL EVOLUTION BY MTT ASSAY AFTER 72 h OF INCUBATION WITH THE FOUR PEPTIDES STUDIED

HEK tumor cell line exposed to HP-1 defensin for 72 h			
	Concentration (μM)	Absorbance average (570/620 nm)	% of cytostasis = $(1 - A_{\text{cells peptide}} / A_{\text{negative control}}) \times 100$
NC	-	0.899	0
Cells (HEK) with saline solution	-	0.897	2.78
β - Defensin - 1, human	120	0.842	10.32
Dermaseptin	120	0.808	10.11
Cecropin A	120	0.826	11.00
Cecropin B	120	0.813	9.56

M14K (human mesothelioma) tumor cells incubated with physiological saline (150 μL) showed a 2.78% cytostasis. This obtained value falls within normal (physiological) limits because cell cytostasis is considered to be normal up to a value of 10%. In conclusion, we can say that the physiological serum did not influence the viability and proliferation of the tumor cells studied.

The results obtained in these experimental studies on the biological effect of cytotoxic peptides (cecropin A, cecropin B, β - defensin - 1 and dermaseptin) on normal cells (HEK) demonstrated that cell viability and proliferation was not significantly influenced by these. The biological effect of natural peptides can be tracked either on tumor or normal cell lines and experimental animal model [5,13-15].

The mechanism by which cytotoxic peptides are functional depends on a number of their physicochemical properties such as: primary sequence, secondary structure, net charge, amphipathy, hydrophobicity as well as their biomembrane concentration and membrane cell composition [16]. The presently accepted model by which antimicrobial peptides manifest their functionality involves the existence of several successive steps such as: peptide association on membrane surface, assembly of peptides in secondary structures defined at membrane surfaces, insertion of peptides into biomembranes, and finally formation of transmembrane pores [17,18]. Changes in cell membrane have major implications for the progression of infections because they play a key role in the cell's response to its environment [19].

The study of interactions between cytotoxic peptides and lipid membranes could highlight both the conditions that make membranes responsive to the action of these peptides and the possibilities to improve their selectivity [20]. The influence factors are determined on the one hand by the characteristics of the biological environment in which these interactions are manifested, and on the other hand the characteristics of the intramembrane environment, namely the type of lipid molecules entering the membrane structure that directly influence the electrical profile of the membrane and its fluidity. Understanding these factors is essential for studies that relate to the synthesis of peptides with better selectivity and specificity [21]. To date, two distinct types of ion channels formed by the aggregation of cytotoxic peptides have been described, namely: Classical pores whose interior is formed only by the polar parts of the peptides and the toroidal pores for which the internal structure of the inner structure also contributes to the polar ends of the lipids in the composition of the cell membrane [22-24]. The addition and progressive addition of new monomers (cytotoxic peptides) can lead to increased pore size eventually leading to the dispersion or infiltration of a cell's

content and thus its death [23, 24]. These cytotoxic peptides, by forming ion channels, permeabilize the cell membrane by inducing cellular efflux of K^+ and other cellular components with cell evolution towards apoptosis [25]. M14K tumor cells (mesothelioma) have been shown to be vulnerable to the action of the cytotoxic peptides used.

Conclusions

In this in vitro experimental study, we found that the cytotoxic effect of the four cytotoxic peptides used depended on their concentration in the tumor cell culture medium, being significant at concentrations of 120 μM and maintained at concentrations of 60 μM . At 30 μM concentrations these tumoricidal effects were insignificant.

According to the literature as well as our study, the cytotoxic potential depends on the type of peptide, the toxicity being similar for the two cecropins at the same concentration for the same tumor line. Of all the studied peptides, dermaseptin has the strongest effect and β -defensin-1 the weakest. For this reason we plan to continue researching dermaseptin on other tumor cell lines. We can demonstrate in this way whether the tumoricidal effect also depends on the type of tumor cell line. In conclusion, both the concentration of the cytotoxic peptide with which we incubate a tumor cell line is important, but the type of peptide is also important. These peptides are water-soluble and non-hemolytic at their effective antimicrobial concentrations. At low concentrations it inhibits the growth of numerous species of bacteria, fungi and induces osmotic lysis in protozoa [25]. Therefore, the amino acid composition, amphipathicity, cationic charge, and size allow cytotoxic peptides to attach to and insert into the phospholipid bilayer of the cell membrane with the formation of transmembrane pore that will modify the permeability of the cell membrane and will determine the evolution of that cell toward apoptosis, limiting the degree of multiplication of tumor cells and causing tumor regression.

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