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Doctoral Thesis Summary

*“Cytotoxic effects induced to some
tumor cell lines by some peptides”*

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Cytotoxic peptides characterization

From specific literature data it was shown that most of the natural and synthetic peptides have a broad spectrum antimicrobial antiviral and some of them have a potential anticancer activity.

The present thesis has as purpose the evaluation of the antitumor effect of some natural peptides (magainin II, defensin HNP-1, cecropin A and cecropin B) on several cellular lines: MDA-MB231 (mammary adenocarcinoma), HT29 (colorectal adenocarcinoma), M14K (human mesothelioma), A549 (human alveolar carcinoma) and some cell lines of normal human epidermal keratinocytes (HEK).

Magainin II is part of a cytotoxic peptide class that was isolated from frog epithelium (*Xenopus Laevis*). Magainins show antibiotic activity towards Gram-positive, Gram-negative bacteria, and towards some fungi and protozoa [1]. The primary structure of this peptide is given by the following amino acid sequence:

NH₂-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-CONH₂

The amphiphilic structure of α -helix type allows this peptide molecule to target the cell membranes. At this level it can form permeable ion channels which can lead to membrane depolarization and finally to the death of the cell [2, 3].

The **cecropins A** and **B** belong to a polypeptide class that has bioactive properties. They consist in a chain of 34-39 amino acid residues. They were first isolated from the haemolymph of silk butterfly (*Hyalophora cecropia*) [4, 5, 6]. The primary structure of Cecropin A:

H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Gln-Asn-Ile-Arg-Asp-Gly-Ile-Ile-Lys-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr-Gln-Ile-Ala-Lys-NH₂

Cecropin B has the following amino acid sequence:

H-Lys-Trp-Lys-Val-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asn-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Ala-Val-Leu-Gly-Glu-Ala-Lys-Ala-Leu-NH₂

Both cecropins have a α -type secondary structure, the NH₂ terminal end intensely amphipathic while the COOH end from the α -helix is hydrophobic [6].

Many studies proved that cecropin A and cecropin B molecules have antitumor activity against mammalian leukemia, against lymphoma and colon carcinoma tumor cell lines [7, 8], lung cancer and gastric cancer. Cecropin A and B show activity against Gram-positive and Gram-negative bacteria in micromolar concentrations [9, 10].

Defensin HNP-1 (Human Defensin Neutrophil Peptide-1)

Defensins are cationic, amphipathic peptides consisting in 29-47 amino acid residues. The sequence in the case of *defensin HNP-1*:

Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

Most defensins are amphipathic peptides that have a sequence of hydrophobic and hydrophilic amino acids placed on one side and the other of the α -helix type structure. This allows them to interact with the microbial cell membrane [11].

In vitro, the defensins proved to have a wide range of antimicrobial activities against Gram-positive and Gram-negative bacteria, against fungi viruses and parasites [12, 13] but they are also cytotoxic for different types of mammalian cells [14, 15].

Materials, methods and researching techniques

The viability test using MTT [3-(4,5-dimethylthiasolyl-2-yl)-2,5-diphenyltetrazolium bromide]. The test was first described in 1983 by Mosamann and it analyses the cell metabolism and it is based on the capacity of the mitochondrial succinate dehydrogenase from living cells to reduce the soluble tetrazolium salts from MTT- (yellow) and to form insoluble crystals of formazan (purple) (fig. 1).

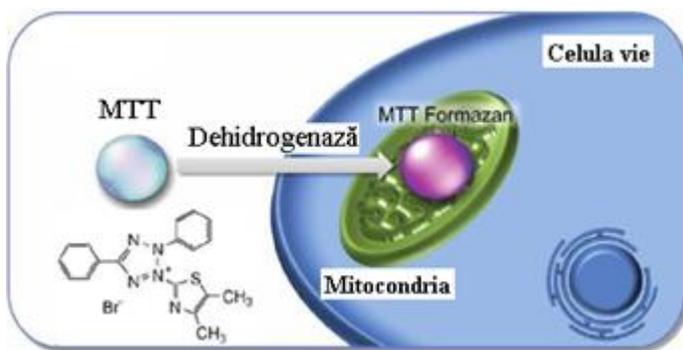


Fig. 1. The principle of the method (adapted from *www*).

The formazan is not soluble in water but it can be solubilized using isopropanol, dimethylsulfoxide (DMSO) or other organic solvent. The extinction was read at a spectrophotometer with culture plates with 96 wells (Tecan SunriseTM) at 570/620 nm wave length [16].

Cell apoptosis evaluation using flow cytometry technique

The cell apoptosis can be observed using the flow cytometry technique. For this purpose we used annexin V (BD Pharmingen) coupled to phycoerythrin fluorophore. The annexin V has an affinity for phosphatidylserine with which it bonds in the presence of calcium

ions in the external layer of the lipid bilayer of a cell in apoptosis (fig. 2) [17, 18, 19].

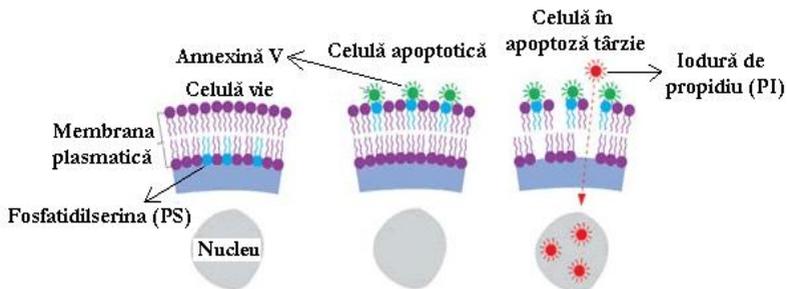


Fig. 2. The representation of apoptotic cells decantation using annexin V [20].

If the cell membrane is injured then the second fluorophore used, the 7-AAD (7-amino-actinomycin D) will enter the cell and will insert at nuclear level having an increased affinity for the nitrogenous bases guanine-cytosine. Thereby the normal living cells do not exhibit fluorescence being double negative for both annexin V and 7-AAD.

The cells that are in their early apoptosis keep an intact cell membrane so that they will exhibit fluorescence only for annexin V, unlike the cells in late apoptosis that have a damaged cell membrane and exhibit fluorescence for annexin V and 7-AAD [21].

The evaluation of cell proliferation using the RTCA DP technique (**Real-Time Cell Analyzer Dual Plate**).

The RTCA DP instrument consists in:

- double plate real time cell analyzer- RTCA DP.
- control unit RTCA 1.1 with preinstalled 1.2 RTCA software.
- E-Plate 16 or CIM plate 16 (Fig. 3).

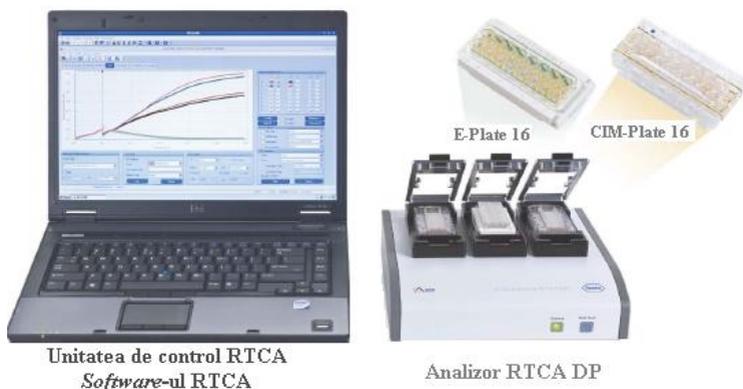


Fig. 3. *The RTCA DP instrument with the main components [22].*

The biological samples are cultivated on culture plates formed of 16 wells provided with a network of golden microelectrodes. Each well of the plate for the cell analyze contains some sensor electrodes placed “in network” that allow cell monitoring in the well [22].

The cell indices are parameters used to measure the relative modification of the electric impedance represented by the cell position. The impedance values show quantitatively the biological status of the cells, including the cell number, their viability and their morphology. The RTCA DP instrument is extremely precise regarding accuracy, precision and easiness in using [22, 23].

Cytotoxicity evaluation of magainin II and cecropin A and B on tumor cell lines

The evaluation of cell viability using the viability test with MTT

For this experiment it had been used a culture plate with 96 wells. The cells were incubated in 200 μL complete media/well containing magainin II, cecropin A and cecropin B in different final concentrations (60 μM , 30 μM , 15 μM , 7,5 μM , 3,75 μM , 1,87 μM and 0,9 μM) at 37 $^{\circ}\text{C}$ for 72 hours. The negative control was made of cells placed in wells without cytotoxic peptides.

Positive control was represented by the presence of doxorubicine hydrochloride (sindroxocin) 60 μM . After 72 h incubation, to each well there were added 20 μL MTT (Sigma, 5 mg/mL). The cells were incubated for 4 h at 37 $^{\circ}\text{C}$, afterwards the culture media was eliminated and replaced with 100 μL DMSO in order to dissolve the formed formazane crystals by the reduction of MTT. The extinction was read at spectrophotometer and the experiment was made three times.

From the obtained data on colorectal adenocarcinoma cells it could be said that the three types of peptides did not act on cells, the evolution to apoptosis being obvious in the case of doxorubicin (cytostasis 50 %).

After 72 h of incubation of tumor cells of mammary adenocarcinoma the three types of peptides it was shown that they do not exhibit a cytotoxic effect on this type of cell. The positive control showed a significant percent of cytostasis (90 %).

The human alveolar carcinoma cells incubated using magainin II, cecropin A and cecropin B do not exhibit any changes in contact with the peptides. The cytostasis percent in positive control is about 70 %. In the case of human mesothelioma cells incubation for 72 h with magainin II, cecropin A and cecropin B it could not be showed

a cytotoxic effect on cells. In the case of doxorubicine hydrochloride (60 μM) the cytostasis percent was about 80 % [24, 25].

Apoptosis evaluation using flow cytometry technique

After 72 h incubation the culture media of each well that contains dead cells was collected in polystyrene tubes of 12x75 mm that labeled for the flow cytometry analysis. The wells were washed with PBS and the adhering cells were detached using tripsine EDTA after 3-10 minutes of incubation depending on the cell line.

The tripsine was inactivated by adding complete media that was collected after washing the well and adding the corresponding tube for FACS. After two washings with cold PBS, centrifugation at 300 g for 5 minutes, the sediment was again suspended in 100 μL annexine V diluted and incubated for 15 minutes in darkness with 5 μL PE annexine V and 5 μL 7-AAD.

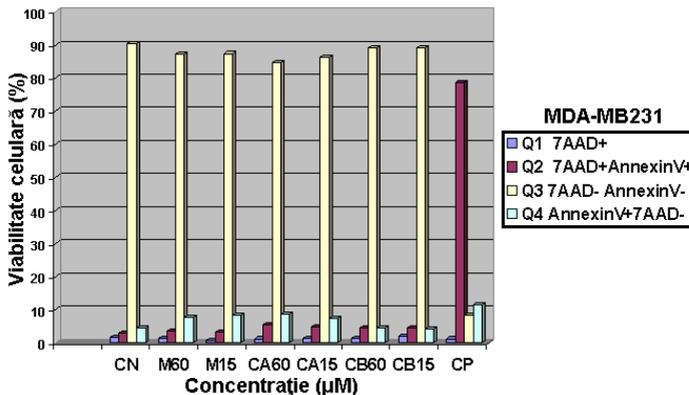


Fig. 4. MDA-MB231 cell viability analysis using flow cytometry, evaluation made after 72 h of incubation with magainin II, cecropin A and cecropin B, in comparison with a positive and a negative control.

The analysis using flow cytometry showed cell viability for the line MDA-MB231 after 72 h incubation with magainin II, cecropin A and cecropin B of 60 μM and 15 μM concentrations similar to that of negative control (more than 80 %).

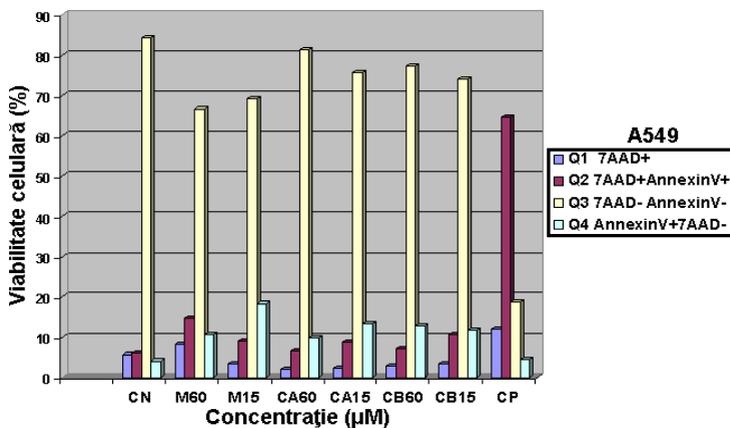


Fig. 5. A549 cell viability analysis using flow cytometry, evaluation made after 72 h of incubation with magainin II, cecropin A and cecropin B, in comparison with a positive and a negative control.

The evaluation of A549 cell viability after 72 h incubation with 60 μM and 15 μM concentrations of magainin II, cecropin A and cecropin B lead to the conclusion that the used peptides did not have a significant biological effect on the cells.

The positive control (doxorubicine hydrochloride 60 μM) proved significant reduced cell viability. In the case of A549 and MDA-MB231 cells the apoptosis rate that was induced was about 15 %.

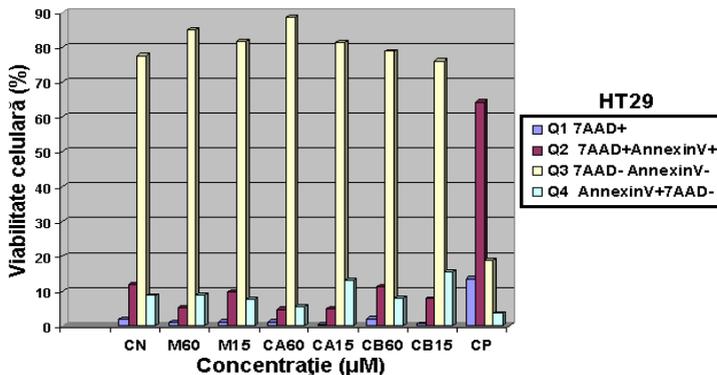


Fig. 6. HT29 cell viability analysis using flow cytometry, evaluation made after 72 h of incubation with magainin II, cecropin A and cecropin B, in comparison with a positive and a negative control.

After HT29 cell incubation with 60 μM and 15 μM magainin II, cecropin A and cecropin B it was observed that cell viability was not affected by the peptides. Only in the case of positive control the HT29 cells begin the apoptosis.

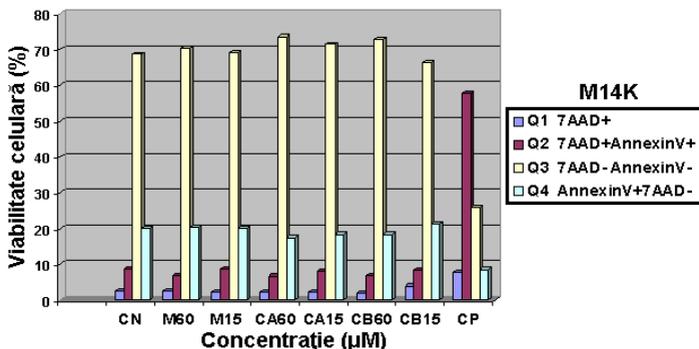


Fig. 7. M14K cell viability analysis using flow cytometry, evaluation made after 72 h of incubation with magainin II, cecropin A and cecropin B, in comparison with a positive and a negative control.

The M14K cell evolution at their impact with the three types of cytotoxic peptides is similar to that of the HT29 cells.

The cell proliferation evaluation using the RTCA DP technique (*Real Time Cell Analyzer – Dual Plate*)

Another technique used for the biologic effect evaluation of these peptides on the tumor cell lines was the RTCA DP method. This system xCELLigence RTCA for real time cell analysis-it measures the electric impedance of the signals given by the cells that adhere to the surface of the golden microelectrodes, thus quantifying in real time cells proliferation and the viability.

The cells were cultivated in duplicate, 5000 cells/well/200 μ L in E-Plate 16 (Roche), 2 stripes of 8 wells. The effect of the three peptides (magainin II, cecropin A and cecropin B) on the HT-29 cells was observed for 72 h after two peptide concentration (60 μ M and 15 μ M) incubation.

The peptide free incubated cells showed negative control while the wells having cells with doxorubicine hydrochloride 60 μ M added proved positive control. The cell indices are arbitrary units used in order to express the electric impedance.

From the graphic representation (Fig. 8) it can be observed the growth of the cell proliferation in the case of HT-29 tumor cells in the presence of the three types of peptides comparing to those of the free peptide cultivated tumor cells (negative control).

HT-29 tumor cells 60 μ M doxorubicine hydrochloride incubated (positive control) had a similar evolution to those of negative control the results were obtained in the first two hours of incubation.

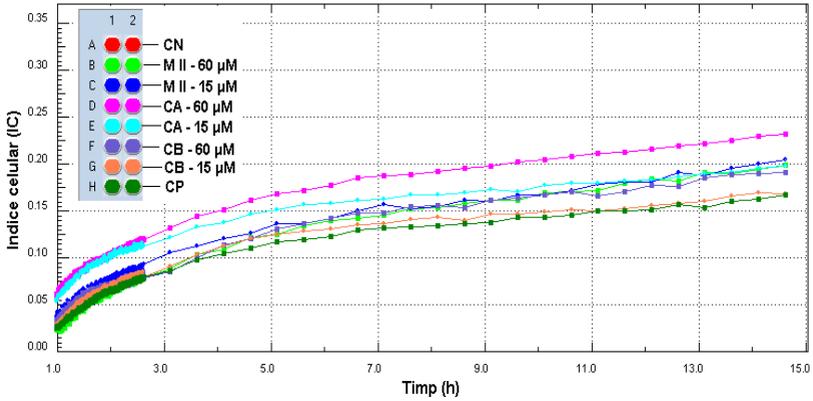


Fig. 8. HT-29 cell culture observation for 15 h from incubation in the case peptide free cells (negative control-red line) and of the peptide added cells of 15 μM and 60 μM concentration, comparing to doxorubicine hydrochloride added cells (positive control- green line).

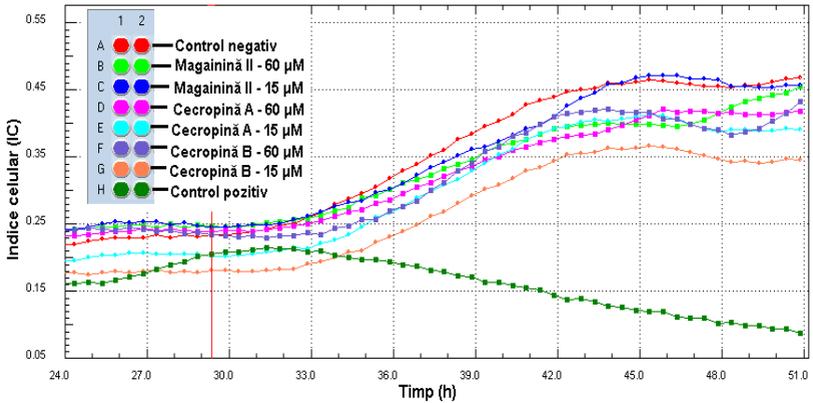


Fig. 9. HT-29 cell culture observation for 51 h from incubation with the three types of peptide (15 μM and 60 μM) comparing to positive control- green line and negative control- red line.

The experiment was made in duplicate. The experimental results obtained using the two peptide concentrations (15 μM and 60 μM) for the HT29 tumor line, proved a significant decreasing of the cell viability in time (51 h), comparing to that of negative control (peptide free). In the case of HT29 tumor cells treated with 60 μM doxorubicine hydrochloride (green line) if initially it was observed a slight cell proliferation, than it was observed an important cytostasis.

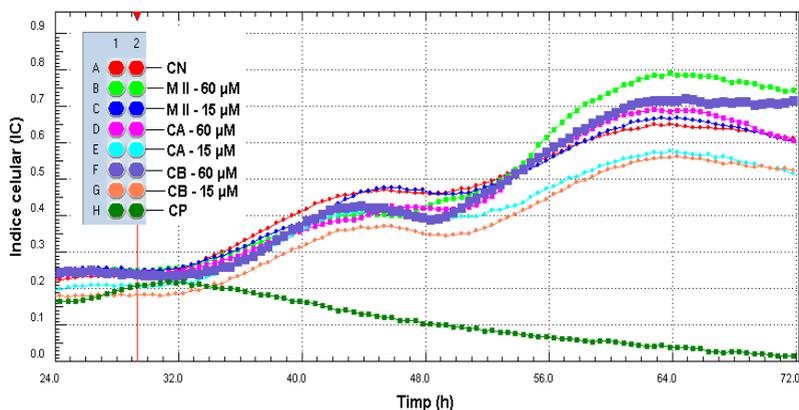


Fig. 10. HT-29 cell culture observation for 72 h from incubation in the case peptide free cells (negative control-red line) and of the peptide added cells of 15 μM and 60 μM concentration, comparing to doxorubicine hydrochloride added cells (positive control-green line).

From this graphic representation it can be observed that after longer incubation most of the HT29 peptide incubated tumor cells present a increased proliferation comparing to that of the negative control, exception the cecropin A and cecropin B that showed a decreasing of cell proliferation at 15 μM concentration comparing to the peptide free incubated cells (negative control).

Defensin HNP-1 cytotoxicity evaluation on normal and tumor cells

In vitro, defensins are substances that show many antimicrobial activities against Gram-positive and Gram-negative bacteria, against fungi, viruses and parasites [12, 13]. In the specific literature there are data that prove the cytotoxic effects for different types of mammalian cells [14, 15].

The obtained results after the treatment of the tumor cell lines with different defensin concentrations were compared to those of the results obtained for a normal adult cell line HEK human epidermal keratinocytes. For the defensin induced cell cytotoxicity evaluation on tumor cells it was used the MTT method.

The cells were incubated in 200 μ L RPMI complete media/well containing HNP-1 defensin of different final concentrations (14,52 μ M, 7,26 μ M, 3,63 μ M, 1,81 μ M, 0,9 μ M, 0,45 μ M, 0,22 μ M, 0,11 μ M și 0,05 μ M) at 37 °C for 72 h.

The negative control was made of tumor cells of the same type, meaning the equally distributed cells in the wells in which no cytotoxic peptide was added.

In order to verify if the cells are injured by the solution (saline) used to dissolve the cytotoxic peptides it was used a well containing: 150 μ L saline with 10⁵ tumor cells and 50 μ L culture media.

The positive control was represented by the doxorubicine hydrochloride. From the analysis made on colorectal cancer cells it is observed that in the case of the cells incubated with defensin HNP-1 the percentage of cytostasis is significant at the defensin concentration (14.52 μ M). A significant cytostasis percentage (86.38 %) was obtained in the case of doxorubicine hydrochloride of 15 μ M concentration incubated cells (fig. 11).

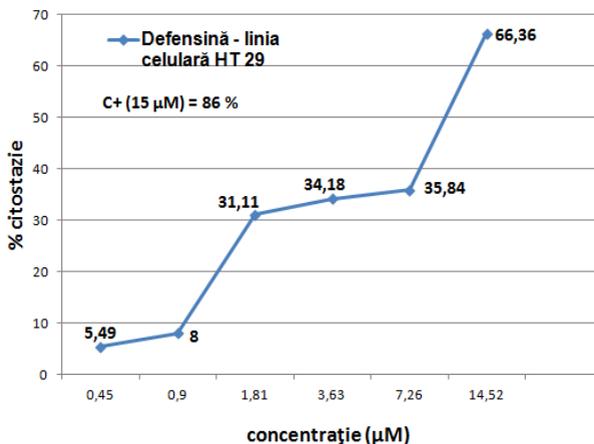


Fig. 11. The graphic representation of the dependence cytostasis percentage-peptide concentration (defensin HNP-1) for the HT29 cell line.

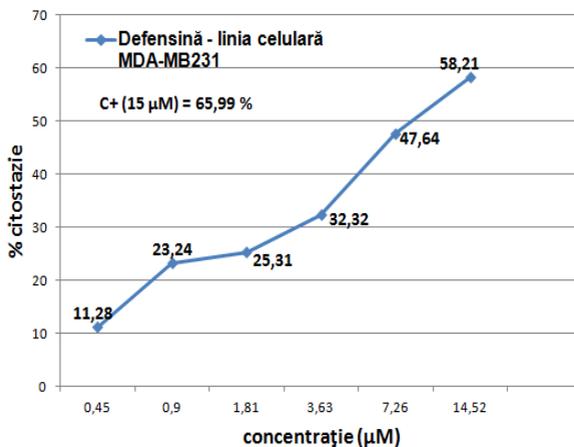


Fig. 12. The graphic representation of the dependence cytostasis percentage-peptide concentration (defensin HNP-1) for the MDA-MB231 cell line.

In the case of the mammary cancer tumor cells incubation with defensin of different successive concentrations beginning with 14.52 μM it was observed a cytostasis percentage of 58.21 %.

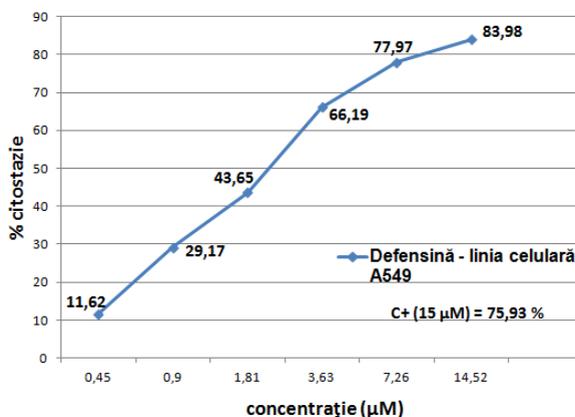


Fig. 13. The graphic representation of the dependence cytostasis percentage-peptide concentration (defensin HNP-1) for the A549 cell line.

Alveolar carcinoma tumor cells incubated using saline (150 μL) showed a cytostasis percentage of 2.78 %. The value obtained is within normal (physiological) limits because the cytostasis of the cells is considered normal till a value of 10 %.

For the human mesothelioma tumor cell line a cytostasis percentage of 81.61 % was obtained at a defensin (HNP-1) concentration of 14.52 μM .

In the case of M14K tumor cell incubation for 72 h with doxorubicine hydrochloride the cytostasis percentage was of 87.86 %. In the case of these tumor cells incubated with saline the cytostasis percentage is of 2.76 % (Fig. 14).

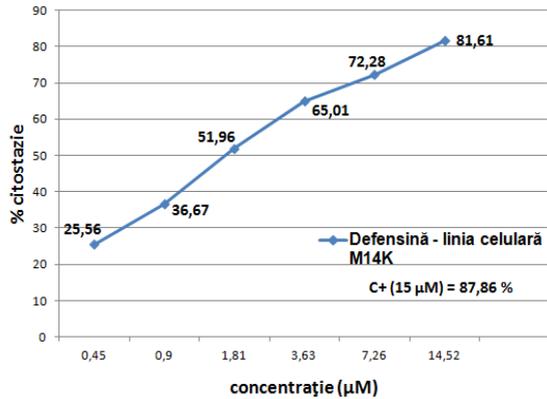


Fig. 14. The graphic representation of the dependence cytostasis percentage-peptide concentration (defensin HNP-1) for the M14K cell line.

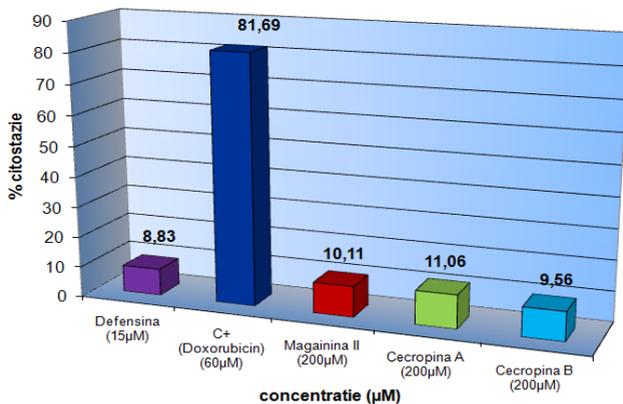


Fig. 15. The graphic representation of the dependence cytostasis percentage-peptide concentration (defensin HNP-1) for the HEK cell line.

In the analysis made on the HEK (*Human Epidermal Keratinocyte*) cell line for 72 h at the same defensin concentrations it

could be said that cytostasis percentage was different comparing to that of studied cell lines.

Also, the results obtained in these experimental studies, regarding the biological effect of magainin II, cecropin A and cecropin B on normal cells (HEK) proved that the cell viability and proliferation was not significantly influenced by the peptides.

Conclusions

This study had as purpose the evaluation of the cytotoxic effects of some natural known peptides (magainin II, cecropin A and cecropin B) on tumor cell lines (MDA-MB231 mammary adenocarcinoma, HT-29- colorectal adenocarcinoma, M14K- human mesothelioma, A549- human alveolar carcinoma) and some cell lines of normal human epidermal keratinocytes HEK.

After conducting these experiments we conclude:

- The optimal concentration necessary for the evaluation of the cytotoxic effect of these peptides was 10^5 cells/200 μ L media/well
- The incubation of the cells using 60 μ M magainin II, cecropin A and cecropin B had a biological effect only in the case of A549 and MDA-MB231 cells, the induced apoptosis rates being up to 15 %.
- Despite the fact that apoptosis was observed for A549 and MDA-MB231 cells, the viability of the studied cell lines was not significantly affected.
- The cells incubates using saline (150 μ L) showed a cytostasis percentage of 2.78 %. The value obtained is within normal (physiological) limits because the cytostasis of the cells is considered normal till a value of 10 %. In conclusion it can be said that saline did not have a influence on the viability and proliferation of the studied tumor cells.
- Defensin is a cytotoxic peptide that can have a significant influence on the viability and proliferation of tumor cells at a concentration of 14.52 μ M. Defensine did not have a significant influence on the surviving and proliferation of the normal cells (HEK) used in these experiments comparing to the evolution of tumor cells.

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