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INVESTIGATING THE ANTICANCER ACTIVITY OF SOME CATIONIC ANTIMICROBIAL PEPTIDES IN EPITHELIAL TUMOR CELLS

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Abstract. Cationic antimicrobial peptides (AMPs) became a promising alternative to the conventional cancer drugs. We investigated the effects of 5 AMPs (melittin, galanin-mastoparan, cecropins A, B and P1) on the viability of HT-29 and AR-42J carcinoma cells. The viability of the cells was evaluated and used to calculate the AMPs therapeutic index. Also, cellular apoptosis and necrosis were analyzed. Melittin and galanin-mastoparan inhibited HT-29 cells only by necrosis. Melittin, galanin-mastoparan and cecropin A and B inhibited AR-42J cells by both apoptosis and necrosis. These data support the working hypothesis of the specific action of some cationic AMPs on cancer cells.

Key words: antitumor peptides, HT-29 cells, AR-42J, melittin, cecropins, mastoparan.

1. INTRODUCTION

Although efficient in the early stages of cancers, classic treatments (radio- and chemotherapy) proved to be nonspecific for cancer cells affecting healthy cells in a similar manner [1]. Moreover, due to the drug resistance that tumor cells acquire during treatment, these therapies are unable to remove all cancer cells, which is considered to be the main cause of cancer relapse [2]. Therefore, the development of new and more efficient anticancer agents has become imperative.

AMPs represent an ancient innate defense system of the living organism against a wide variety of pathogens [3]. Surprisingly, cationic AMPs have displayed high specificity for cancer cells [4].

Their specificity towards tumor cells is dictated in the first instance by the differences between the membrane of normal and cancer cells. Compared with the neutrally charged membranes of normal cells, tumor cell membranes are usually negatively charged due to an increase, in the outer leaflet of the membrane, of

anionic molecules like phosphatidylserine [1]. Therefore, the electrostatic interaction facilitates the binding of the cationic peptides to the tumor cell membranes, followed by membrane disruption and cell death [5]. One of the mechanisms through which cationic peptides interact with the membranes leads to membrane lysis, while others can trigger specific pathways which can induce cellular apoptosis. It was proved that cationic peptides like magainins disrupt the mitochondrial membrane triggering apoptosis in cancer cells [1].

In this study, we explored the tumoricidal potential of five cationic AMPs against two carcinoma cell lines: HT-29, a human colorectal adenocarcinoma cell line with epithelial morphology and AR-42J, a rat pancreatic tumor cell line which is a model for acinar cells, the most predominant cells found in the exocrine pancreas (around 80%) [6]. Therefore, the toxic effects against tumor cell lines were tested and discussed based on the therapeutic index (TI). Furthermore, the mechanism of AMP action was investigated, emphasizing the role of apoptosis in the antitumoral effect.

2. MATERIALS AND METHODES

2.1. MATERIALS

Lyophilized melittin (Mel – GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), cecropin A (CA – KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2), cecropin B (CB – KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH_2), cecropin P1 (CP1 – SWLSKTAKKLENSAKKRISEGIAIAIQGGPR-OH) and galanin-mastoparan (GM – GWTLNSAGYLLGPINLKALAALAKKIL-NH_2) were purchased from Bachem AG (Budendorf, Switzerland) and reconstituted in a saline solution prior to application on the cells. Drabkin reagent, standard hemoglobin, Acridin-orange (AO), Ethidium homodimer–1 (EthD-1) and Triton X-100 were purchased from Sigma. All cell cultivation media and reagents were purchased from Biochrom AG (Berlin, Germany).

2.2. CELL CULTURE AND CULTURE CONDITIONS

HT-29 and AR-42J cells (ATCC, USA), were grown in appropriate media and conditions according to the provider instructions. For viability experiments, the cells were seeded into 96-well tissue culture plates at a density of 5000 cells/well for HT-29 cells and 15000 cells/well for AR-42J cells. For apoptosis experiments cells were seeded into 12-well tissue culture plates at a density of 40000 cells/well for HT-29 cells and 120000 cells/well for AR-42J cells. After 24h, the cells were incubated for another 24h with different peptide concentration.

2.3. *IN VITRO* ANTICANCER ASSAY

Cells viability was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, USA) following the manufacturer's instruction. The cell viability was calculated as a percent value relative to the control. The IC50 value represents the concentration of the tested peptides causing 50% cell death and was obtained by fitting the experimental data with a sigmoid function.

2.4. MEASUREMENT OF THE HEMOLYTIC ACTIVITY

The hemolytic activity of AMPs was determined using an adapted protocol of the ASTM F 756 – 00 standard [7] based on hemoglobin release from human red blood cells (hRBCs) resulting after cell lysis. Briefly, the blood was collected from volunteers, diluted with PBS to a concentration of hemoglobin ~10 mg/mL and then incubated with various concentrations of AMPs for 4 h at 37 ^{o}C. After 4 h the cells were centrifuged, the supernatant was collected, transferred into 96-well tissue culture plates and mixed with Drabkin reagent which converts hemoglobin to cyanmethemoglobin (absorption peak at 540 nm). After 15 minutes the absorbance of the samples was read at 570 nm using a plate reader. The data obtained were corrected for the background, dilution factors and in the end used to calculate the hemolytic index as percentage of the hemoglobin released after AMP treatment relative to control samples. hRBCs in PBS and distilled water were used as negative and positive controls.

2.5. APOPTOSIS AND NECROSIS EVALUATION BY IMMUNOFLUORESCENCE MICROSCOPY

Cellular apoptosis and necrosis were investigated by morphological characterization of the cells after staining with both AO and EthD-1. Untreated cells were used as negative control (C-). Cells treated with Bleomycin (60 μg/mL, 1 h) were used as positive controls (C+) [8]. The cells were treated for 24 h with two different concentrations for each of all five peptides: the smallest and the highest concentrations used in the MTS assay. After 24 h of incubation with AMPs, the cells were detached and washed with phosphate buffer saline (PBS) and then stained according to the protocol described earlier [9]. For each experimental condition at least 500 cells were analyzed.

2.6. STATISTICAL ANALYSIS

The data were presented as mean ± standard deviation (SD). The statistical differences were determined using One-Way ANOVA with the Dunnett post hoc

test (GraphPad Prism 5 Software, La Jolla, CA), in which the means of all treatment conditions were compared with the control condition (untreated cells). The differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. CYTOTOXICITY OF PEPTIDES AGAINST TUMOR CELL LINES

The viability of the cancer cells we investigated shows very different responses with respect to the AMPs used (Fig. 1). Two distinct trends are observed: peptides showing a clear toxic activity towards the cells and peptides which do not significantly affect the cells in the range of concentration we investigated. The IC50 factor was determined and is presented in Table 1 (the highest concentration was reported where IC50 was out of the evaluated concentration range).

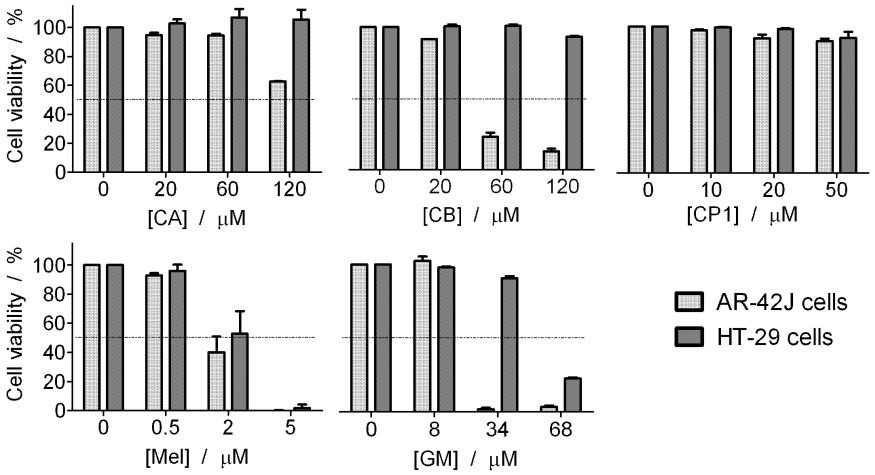

Fig. 1 – Cytotoxic effect of AMPs against AR-42J and HT-29 cells: the cell viability measurements.

For both cancer cell lines Mel was the most efficient, killing almost all cells at a concentration of 5 μM. IC50 value was 1.81 μM for AR-42J cells and 1.99 μM for HT-29 cells. A strong cytotoxic effect was observed for GM in both cell lines, though for AR-42J cells the IC50 was 21.3 μM, while for HT-29 cells was 52 μM. CB was effective only against AR-42J cells (IC50 = 43 μM), while against HT-29 cells, at 120 μM, only 9.5% of the cells were killed. Even though for CA the IC50 cannot be determined, the viability of AR-42J cells is reduced by 35% at the highest concentration tested (120 μM), while no effects were observed at the same

concentration for HT-29 cells. CP1 induced at 50 μM only a small decrease of cell viability, by 12% for AR-42J cells and 8 % for HT-29 cells. Comparing the two cell lines, pancreatic tumor cells AR-42J are more sensitive to peptide treatment compared with human colorectal adenocarcinoma cells HT-29.

3.2. CYTOTOXIC EFFECTS OF PEPTIDES AGAINST NORMAL CELLS – THE THERAPEUTIC INDEX

The therapeutic index (TI) was calculated as the ratio of the minimal peptide concentration that produces hemolysis (MHC, the concentration at which peptides induce a 5% hemolysis [17]) over anticancer activity (IC50). Thus, a larger value is indicative of a greater specificity of the APMs for cancer cells [10]. Table 1 shows the minimal hemolytic concentration (MHC), when no hemolytic activity was detected at the highest concentration tested, an MHC twice higher was used to calculate the therapeutic index [21]. Except for GM and CB, the MHC values were previously reported [11]. Mel and GM induced hemolysis in a dose-dependent manner (MHC was 1.82 μM for Mel and 9.09 μM for GM). In contrast, all cecropin family members did not induce hemolysis at the tested concentrations.

Table 1

Antitumor and hemolytic activity of the tested AMPs

Peptides (net charge, hydrophobic moment)	**IC_{50}/ μM**		**MHC[a] / μM hRBCs**	**TI**	
	AR-42J	**HT-29**		**AR-42J**	**HT-29**
Mel (6, 0.27)	1.81 ± 0.20	1.99 ± 0.28	1.82 ± 0.35	1	0.91
GM (3, 0.72)	22.45 ± 2.03	53.60 ± 0.71	9.09 ± 1.08	0.40	0.16
CA (7, –0.07)	> 120	> 120	> 120	–[b]	–
CB (8, –0.07)	35.68 ± 0.61	> 120	> 150	> 8.4	–
CP1 (5, –0.56)	> 50	> 50	> 120	–	–

[a] The minimal hemolytic concentration (MHC) was determined according to ASTM F 756 – 00.
[b] TI could not be calculated.

The TI calculated for AR-42J and HT-29 cells are reported in Table 1. With very similar TI values for both of the investigated cell lines, Mel shows no selectivity between cancer cells lines. Moreover, TI being very close to unit, Mel kills cancer cells at the same concentration at which hRBCs hemolysis was observed, proving no specificity for cancer cells. The TI values of GM are the lowest, which is consistent with the lack of specificity for cancer cells. However, the IC50 for HT-29 is more than twice compared to the one for AR-42J, suggesting a difference in the sensitivity of cell lines to the action of this peptide. CB exhibits the highest TI (> 8.4) and, similar to CA, kills only the AR-42J cancer cells. CP1

did not show any significant cytotoxicity against the studied cancer cells in the tested concentration range.

3.3. AMPS INDUCE APOPTOSIS AND NECROSIS TO AR-42J CELLS

After 24 h treatment with AMPs, the cells showed morphological alterations when compared with untreated cells as can be observed in Fig. 2 (see the Fig. 2 legend for details concerning the morphological features of cells in different states).

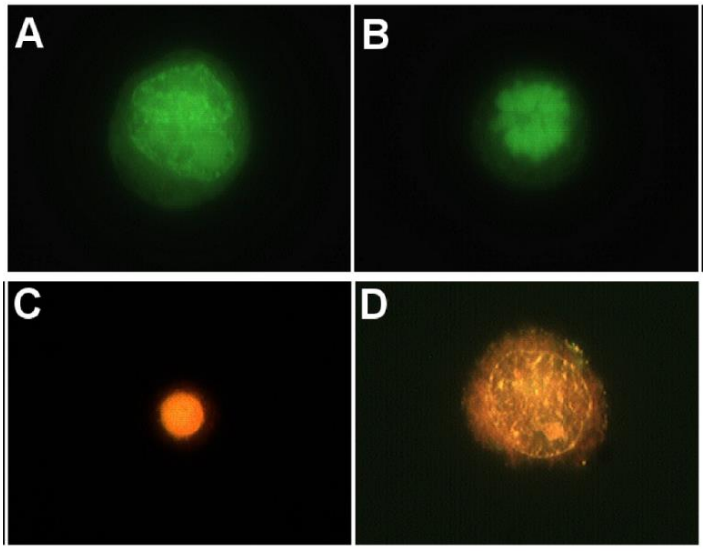

Fig. 2 – Representative images that show morphological changes of the cancer cells detected using dual staining of AO/EthD-1 (exemplified for AR-42J cells). Viable cells (A) stained only by AO show the healthy cells, intact with unaffected membranes; early apoptotic cells (B) stained by AO exhibit a pyknotic nucleus: late apoptotic cells (C) are stained by both AO and Eth-1have pyknosis nuclei and a small volume and necrotic cells (D) stained by both AO and Eth-1 have a larger volume and lose the integrity of the plasma membrane. Magnification of all images was 100×.

The results for AR-42J cells are presented in Fig. 3A. Compared with untreated cells Bleomycin (BLM) treatment decreases the percentage of viable cells from 93% to 71%. BLM treatment induces the death of the cells by necrosis as 22% were necrotic cells. Among the peptides, Mel was the most effective peptide. At a concentration of 5 μM 85.6 % were apoptotic cells (most of them, 64.8 %, were found in the late stage of apoptosis) and 8.8 % were necrotic cells. GM and CB at their highest concentrations (68 μM and 120 μM respectively) also induced a decrease of the viable cells but mainly through necrosis. For GM 85% of the cells are killed by necrosis, while for CB ~ 70% of the cells were necrotic. CP1,

at 50 μM, reduced the number of viable cells to 57%, but the main mechanism was through apoptosis, as 39% of the cells were in either the early (~ 19%) or late (~ 19%) stage of apoptosis and ~ 4% were necrotic cells. CA had the weakest toxic effect against the cells: at 120 μM 81.6% are viable cells; around 10% were apoptotic cells and 9% necrotic cells.

3.4. AMPS INDUCE NECROSIS TO HT-29 CELLS

The untreated HT-29 cells have around 93% of viable cells (Fig. 3B), which decrease after BLM treatment to 68%. BLM treatment induces cell death by both apoptosis (around 21%) and necrosis (10%) in these cells. Mel proves to be less effective against HT-29 cells compared to AR-42J at 5 μM, where the percentage of viable cells is only 21.7%, while apoptotic cells are 34.7 % (23.9 % in the late apoptosis stage) and the majority of the cells (43.4 %) are necrotic. GM kills the HT-29 cells through necrosis, (around 38%). CP1 induces HT-29 cell death mostly by necrosis, (17% were apoptotic cells and 26% were necrotic cells at 50 μM). CA and CB do not induce any effects with the increase of concentrations. The results show that HT-29 cells are more resistant to peptides action than AR-42 J cells and are mostly killed through necrosis.

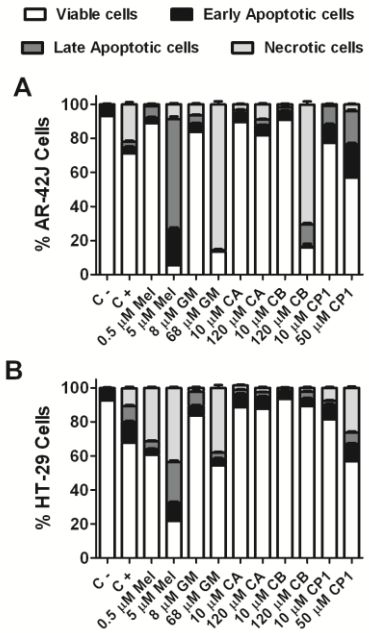

Fig. 3 – Percentage of AR-42J and HT-29 cells (viable, apoptotic and necrotic) observed after treatment with different concentrations of peptides. The results represent the mean ± SD from at least two independent experiments.

4. DISCUSSIONS

Current cancer drugs are known for their effectiveness, but also for the severe side effects leading to small therapeutic indexes. Therefore, intensive work has been carried out to find new medical products with reduced side effects. Very good candidates have proved to be found within the large family of AMPs, both of natural origin and synthesized [1, 12]. The reason lies in their features: simple structure, low molecular mass, positive net charge and amphipathic character [1, 5]. Recent studies suggest that AMPs may kill cancer cells in a selective manner with respect to healthy cells [1, 4] and are also active against multidrug resistant cancer cells [12], with reduced risk of inducing drug resistance or other side-effects.

To assess the utility of an AMP as tumoricidal agent we need to consider both the effects against tumor cells as well the effects induced against healthy cells (like human erythrocytes and lymphocytes). Based on this assumption, two distinct groups of cationic peptides could be described: (i) peptides with no specificity for bacteria, cancer or normal cells (*i.e.* melittin, defensins) which are normally expected to induce important side effects, and (ii) peptides with specificity for bacteria and cancer cells, but not for normal cells (*i.e.* cecropins and magainins) which are expected to have less side effects [1]. For our study we selected five cationic peptides: two from the first group (melittin and galanin-mastoparan) and three which belong to the second group (cecropin A, B and P1).

We focused our work on two cancer cell lines less studied with respect to AMP antitumoral effect. Very few reports were published regarding AMP effectiveness on HT-29 cells. To our knowledge, there are no studies performed on AR-42J cells using antimicrobials peptides.

Melittin can form channels in lipid bilayers and proved to be toxic at small concentrations for bacterial cells, cancer cells, but also healthy cells, including erythrocytes, by multiple mechanisms (reviewed in [14]). Several studies have demonstrated that bee venom and/or melittin have anti-cancer effects including prostate [15], liver [16], breast, cervical, renal [17], ovarian [18] and other cancer cells [14]. A previous study showed that Mel inhibits cells viability of HT-29 cells at 1.2 μM and Caco-2 cells at 1.8 μM [13]. Our IC50 values (~ 1.8 μM) are in agreement with the above cited report on HT-29 and Caco-2 cells also confirming other data from literature where the toxic effects of Mel are highlighted at concentrations higher than 2 μM independent of the cell type [19]. The lack of Mel specificity for a cell type can also be observed from the TI values which for both cell lines are close to 1. According to our data, Mel induces cell death through necrosis in HT-29 cells. Even though for AR-42J cells we found an IC50 similar to the one of HT-29 cells, in this case the peptide promotes both apoptosis and necrosis in pancreatic cancer cells. These findings are in agreement with many

reports where Mel had been proved to exert both apoptotic and necrotic effects on cancer cells (reviewed in [14]).

Galanin-Mastoparan, is a hybrid of Galanin (a neuro-peptide expressed in the nervous and endocrine system) and Mastoparan (a wasp venom peptide with a high antimicrobial potential [20]). To our knowledge, there are no reports about the cytotoxicity of GP on mammalian cells. GM kills AR-42J pancreatic cancer cells at smaller concentrations as compared with the colorectal adenocarcinoma HT-29 cells proving a higher affinity of GM for AR-42J. The sensitivity of AR-42J cells can be correlated with the proved presence of galanin receptors in acinar cells [21]. Its TI values smaller than 1 show high potency in inducing side effects. Even though GM shows higher affinity for AR-42J cells, it induces death in both cancer cell lines through the same mechanism: necrosis. Such a result is supported by the proved ability of GM to rapidly penetrate the plasma-membrane and invade the cytosolic membrane system [22].

Cecropin family is a class of peptides secreted generally by insects, like the giant silk moth Hyalophora Cecropia, with the exception of cecropin P1 found in mammals. CA and CB proved to be toxic for a set of four bladder cancer cells at concentrations at least three times smaller than those inducing toxic effect to normal fibroblasts [23]. Interestingly, the authors report significantly higher efficiency of CB compared to CA. Our results also prove this difference between CA and CB. Moreover, their CB IC50 (approx. 40 μM) is very close to our findings for CB on AR-42J cells. At least in the case of CB, a strong lytic activity on cancer cells membrane was proved by patch clamp measurements on stomach carcinoma cell line Ags [24]. Application of CB in the range of 10-60 μM induces bursts of outward currents proving the formation of pores through the membrane, this result strongly supporting a potent anticancer effect of CB. To our knowledge, there is only one paper reporting CA and CB effects against HT-29 cells [25]. Compared with our experimental conditions here, the cells were treated for 48 h. According to this report, CA had almost no effect on HT-29 viability, similarly to our findings. On the other hand, CB had a more pronounced effect compared to our data, a difference that is probably due to the longer time of exposure. According to our data, CB has the best TI among cecorpins, found against AR-42J cells. CP1, the last member of the cecropin family we tested, kills cancer cells in a dose-dependent manner, but the concentration range we tested is not large enough to determine an IC50 value.

5. CONCLUSIONS

To summarize, we asses AMP tumoricidal capacity against two epithelial tumor cell lines less studied with respect to their interaction with AMPs, but

representative for the colorectal and pancreatic cancers. We showed that peptides exhibit significantly different cytotoxic effects against the two cell lines, AR-42J cells being more susceptible to AMP treatment compared with HT-29 cells with CB having the highest TI value. The differences we observed in the particular mechanism whereby each AMP can kill one or more type of cancer cells, strongly suggest that a translation to *in vivo* studies needs a deeper understanding of the AMP killing mechanism for each particular type of cancer cells.

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