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Cationic Antimicrobial Peptides Cytotoxicity on Mammalian Cells: An Analysis Using Therapeutic Index Integrative Concept

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Abstract Antimicrobial peptides (AMPs) represent a class of molecules synthesized by different organisms as an ancient innate defense mechanism against different pathogens like bacteria, fungi, viruses. Their characteristics make them good candidates to fight against bacteria together with or as an alternative to antibiotics. To decide on AMPs suitability for use in mammalian systems we redefined a 'therapeutic index' using the concentrations for which AMP is active against pathogens without inducing cytotoxic damage to the mammalian cells. Here we analyzed the toxic effects of eleven, highly active cationic AMPs towards human cells. The AMPs cytotoxicity was determined using common standardized assays measuring their effect on red blood cells (hemolytic index) and on lymphocytes (cell viability). The therapeutic index was calculated for all the AMPs tested. The highest therapeutic index was found for cecropins followed by magainins and the smallest for Melittin. For two peptides, Cecropin A which presents the highest therapeutic index and Melittin with the smallest therapeutic index we characterized in detail the cell death process distinguishing between apoptosis and necrosis. The toxic effects produced by Cecropin A and Melittin are induced mostly by means of apoptosis suggesting that the definition of therapeutic index has to consider the apoptotic effects of AMPs. Thus we provided here a unitary way to characterize the side effects of AMPs. The analysis of in vitro cytotoxic effects of AMPs using the global concept of therapeutic index can be a powerful way to decide which peptide can be taken for further testing in preclinical trials.

Keywords Antimicrobial peptides · Apoptosis · Cytotoxicity · Hemolysis · Therapeutic index

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Introduction

Due to the alarming rate at which the bacterial strains gain antibiotic resistance (Radu et al. 2011; Watkins et al. 2013), there is an increasing interest in finding and developing novel antimicrobial compounds as an alternative to existing drugs. Antimicrobial peptides (AMPs), a group of small peptides (10 kDa) with high therapeutic potential, proved that they can become an alternative weapon in the fight against pathogens: bacteria, fungi, viruses (Izadpanah and Gallo 2005).

AMPs are molecules synthesized by a wide range of organisms, from vertebrates to insects, plants, even bacteria, and represent an ancient innate defense mechanism against microbial infections (Cederlund et al. 2011). To date, more than 500 natural peptides are described in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). AMPs are characterized by a highly diverse amino acid composition and structure. The specificity of AMPs interaction with cellular membranes is dictated by characteristics like peptide size conformation and structure, amino acid sequence, net charge, and amphipathicity (Brogden 2005; Yeaman and Yount 2003). The largest class of AMPs comprises cationic peptides, characterized by a positive net charge and a hydrophobic region which

allows them to insert into the lipid membranes and contributes to the peptide partition in the membrane bilayer (Hancock et al. 1995). AMPs are more prone to interact with bacteria than with mammalian cells due to particular biophysical and biochemical properties of the bacterial membrane. Thus, the bacterial cell membrane is negatively charged compared with mammalian cell membranes, increasing the affinity of cationic peptides. The presence of cholesterol inside the mammalian cell membrane is another factor that leads to a weaker hydrophobic interaction between the peptides and the membrane. Moreover, the transmembrane potential in bacteria is significantly more negative than in the mammalian cells favoring the interaction with AMPs (Yeaman and Yount 2003). Consequently, cationic AMPs possess a high selectivity for the bacterial cells.

The ability of AMPs to interact preferentially with bacteria is an essential property, which supports their usage as therapeutic agents. For therapeutic purpose AMPs should ideally have a strong interaction with bacteria and no interaction with mammalian cells. In practice, the differential interaction of AMPs with bacterial versus mammalian cell membranes has a degree of variability for each particular AMP.

In order to assess the utility of AMPs as a new generation of antibiotics, the side effects induced by their interaction with healthy mammalian cells needs to be thoroughly evaluated. All the cationic peptides have been reported to present a certain level of cytotoxicity towards mammalian cells (Hancock et al. 1995). While research in the last decades was focused on discovering new, more potent AMPs, their cytotoxicity against mammalian cells was not addressed in a standardized manner. Most data concerning AMPs cytotoxicity report on their hemolytic activity and, sometimes on the effect on mammalian cells viability typically determined on a fibroblast cell line. However, given that the procedures to assess the hemolytic index and the viability are very diverse in these reports, it is difficult to perform a consistent comparison of the results. Moreover, the mechanism of toxicity needs to be taken into account in order to compare the cytotoxic effects induced by AMPs in the host healthy cells. The most addressed mechanistic model attributes the AMP toxicity to its pore-formation capacity (Yeaman and Yount 2003). Although this may explain well the hemolytic effect on red blood cells, for other cell types such as the white blood cells, the cytotoxic effects can be induced by apoptosis and/or necrosis implicating more complicated mechanisms of action. The limitation of using the hemolytic effect as sole parameter to characterize AMPs cytotoxicity against healthy mammalian cells has been highlighted in the literature more than one decade ago (Yeaman and Yount 2003). Most studies that consider AMPs cytotoxic effects, besides their hemolytic action, analyze globally the cell culture viability. Only recently, in the case of yeasts, increased consideration has been given to the mechanisms implicated in the AMP induced cell death process with apoptosis being highlighted as "A new concept on mechanism of antimicrobial peptides" (Choi et al. 2013).

The therapeutic index (TI) was suggested as an integrative way to characterize the AMPs effect (Chen et al. 2005). According to Chen and his colleagues, TI is defined as the ratio of the hemolytic index (a measure of the minimal concentration inducing erythrocytes lysis) and minimal inhibitory concentration (MIC), the concentration at which the growth of bacteria is completely suppressed. Thus for therapeutic purposes the TI is a direct measure of AMPs specificity in killing bacteria or other pathogens without affecting the mammalian cells. Given that this definition of the TI only considers hemolysis as a secondary cytotoxic effect of AMPs it can only provide a limited characterization of AMPs.

The aim of our work was to investigate in a unitary way a set of representative cationic AMPs and to assess their side effects on mammalian cells. For this purpose the hemolytic activity and the effects on lymphocytes viability were investigated using standardized methods. We re-defined the global parameter of therapeutic index to consider a wider range of cytotoxic effects and we demonstrate the suitability of this concept to characterize AMPs in a proof of concept work. Moreover, in order to better understand the role of apoptosis in AMPs cytotoxicity against mammalian cells, we have studied the cell death process distinguishing between apoptosis and necrosis.

Materials and Methods

Materials

The peptides used in this study were purchased from Bachem (Budendorf, Switzerland): Melittin (Mel), Cecropin A (CA), Cecropin B (CB), Cecropin P1 (CP1), Indolicidin (Ind), Polistes Mastoparan (PM), Tachyplesin I (TL I) and Dermaseptin (DS1) and from Sigma (Seelze, Germany): Magainin I (Mag I), Magainin II (Mag II), and Lactoferricin B11 (LF11). Drabkin reagent, standard hemoglobin, and Triton X-100 were purchased from Sigma and all reagents for cellular cultures were purchased from Biochrom AG (Berlin, Germany).

MTS Cytotoxicity Assay

Fresh blood was collected from healthy human volunteers and the peripheral blood mononuclear cells (PBMCs) have been separated by a common protocol using centrifugation

on density gradient (Petcu et al. 2006). PBMCs were seeded into 96-well tissue culture plates at a density of 10^6 cells/well and incubated with various concentrations of AMPs for 24 h at 37 °C in a humidified atmosphere with 5 % CO_2. The cytotoxic effect of the AMPs was assessed using Cell Titer 96Aqueous One Solution Cell Proliferation Assay kit, Promega (Madison, WI, USA) according to the manufacturer protocol. The percentage of viable cells was calculated using untreated PBMCs as negative control.

Hemolytic Activity

The hemolytic activity of AMPs was assessed using an adapted protocol of the ASTM F 756—00 standard (ASTM 2000) based on hemoglobin release from human red blood cells (hRBCs) resulting after cell lysis. Briefly, the blood was collected, diluted to a concentration of hemoglobin ~10 mg/ml and then incubated with various concentrations of AMPs for 4 h at 37 °C.The hemolytic index was calculated as:

$$\%\ \text{Hemolysis} = 100\,\% \cdot \frac{A^{SB}}{A^{TB}}$$

where, A^{SB} is the corrected absorbance of the hemoglobin released in the supernatant after treatment with peptides and A^{TB} is the corrected absorbance of the total hemoglobin released after treatment with Triton X-100.

The untreated hRBCs or treated with Triton X-100 were used as negative and positive control, respectively.

Apoptosis

The apoptotic effect induced by two representative peptides, CA (for the highest TI) and Mel (for the lowest TI) was evaluated by a two colors (acridine orange and ethidium homodimer) assay based on the morphological changes suffered by the PBMCs nuclei (shape and color of nucleus, nucleus fragmentation), as previously described (Petcu et al. 2006).

Untreated cells were used as negative control, while for the positive control two conditions were used: (i) cells grown without FCS and (ii) cells irradiated with 100 mJ/cm^2 UV at 312 nm (Ozawa et al. 1999).

Statistics

At least three replicates were used for each experimental condition, and each experiment was performed at least twice. The results are presented as mean ± SD (standard deviation). The statistical significance of the results was analyzed using One-way Anova with Dunnett post-hoc test, in which all treatment conditions were compared with the control condition.

Results

Cytotoxicity of AMPs

To assess the cytotoxic effect induced by the 11 AMPs in eukaryotic cells, we first determined the PBMCs viability. Viability curves were obtained for each peptide tested for a given concentration range. Where possible, the half maximal viability concentration (IC_{50}) was calculated. Figure 1 shows the dose–response curves on PBMCs viability obtained for two different peptides (Mel and CA). The data of the IC_{50} values obtained for each peptide are shown in Table 1.

According to our results, the AMPs analyzed can be divided into three different classes: (i) AMPs which do not affect the viability of the cells for the tested concentrations, such as: TL I, CP1, Mag I and LB11; (ii) AMPs which induce a slight cytotoxic effect (for the tested concentrations a slow decrease of viability was observed, but it was not possible to determine the IC_{50}), such as: CA, CB, and Mag II and (iii) AMPs which exhibit a strong cytotoxic effect in PBMCs: Mel, PM, Ind and DS1.

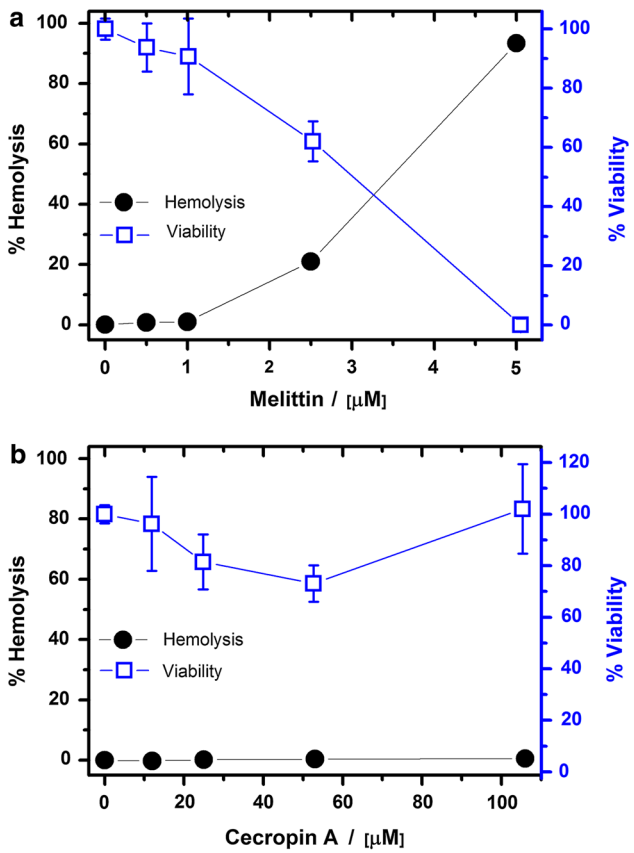

Fig. 1 Dose–response curves of the hemolytic and cytotoxic activity of **a** Mel and **b** CA

Table 1 Half viability concentration (IC_{50}), minimal hemolytic concentration (MHC), minimal inhibition concentration (MIC) and therapeutic index (TI) for the analyzed AMPs

AMPs (name, symbol and sequence)	IC_{50} (μM)	MHC (μM)	MIC[a] (μM)	TI	Geometric mean TI
Cecropin A (CA) KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2	NC (>110)	NH (>110)	0.19 (Schadich et al. 2010) 0.62 (Miskimins Mills 2010)	177.42–579	>27
Cecropin B (CB) KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH2	NC (>90)	NH (>65)	0.5 (Wang et al. 2011) 1.7–3.3 (Moore et al. 1996)	19.69–130	>12.23
Cecropin P1 (CP1) SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	NC (>90)	NH (>120)	0.8-1.6 (Paulsen et al. 2013) 3.3 (Moore et al. 1996)	27.27–112.5	>11.82
Dermaseptin 1 (DS1) ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	14.3 ± 0.42	NH (>10)	1.45 (Mor and Nicolas 1994) 12 (Savoia et al. 2008)	1.19–9.86	3.32
Indolicidin (Ind) ILPWKWPWWPWRR-NH2	50.39 ± 0.73	31.92 ± 7.89	3.04 (Ryge et al. 2009) 7.98 (Nan et al. 2009)	3.04-7.98	3.31
Lactoferricin B, fragment4-14 trifluoroacetate salt (LB11) RRWQWRMKKLG	NC (>150)	NH (>50)	12.48 (Ulvatne et al. 2002) 20.72 (Liu et al. 2011)	2.41–4.01	>2.53
Magainin I (MagI) GIGKFLHSAGKFGKAFVGEIMKS	NC (>165)	NH (>165)	0.41–14.5 (Zasloff et al. 1988) 31.13 (Maria-Neto et al. 2012)	5.30–402	>20.18
Magainin II (MagII) GIGKFLHSAKKFGKAFVGEIMNS	NC (>33)	NH (>33)	0.4–2.8 (Zasloff et al. 1988) 10.14 (Schadich et al. 2010)	3.25–82.5	>9.26
Melittin (Mel) GIGAVLKVLTTGLPALISWIKRKRQQ-NH2	2.48 ± 0.19	1.82 ± 0.35	1.4 (Andra et al. 2007) 4.56 (Sovadinova et al. 2011)	0.4–1.3	1.3
Polistes Mastoparan (PM) VDWKKIGQHILSVL-NH2	74.75 ± 1.06	60.85 ± 0.21	10.35[b] (Cerovsky et al. 2008)	5.87	5.87
Tachyplesin I (TL I) KWCFRVCYRGICYRRCR-NH_2	NC (>15)	12.06 ± 1.07	0.5 (Imura et al. 2007) 5.07 (Ramamoorthy et al. 2006)	2.37–24.12	5.14

[a] The MIC values for *E. coli* are taken from the literature (see the references in brackets)

[b] Due to reduced information regarding PM we used the MIC value obtained on peptides from the same family

Further assessments of AMPs toxicity were performed by studying the process of hRBCs lysis and hemoglobin release due to the presence of peptides. The minimal hemolytic concentration (MHC) was considered as being the concentration at which the peptide induced 5% of hemolysis (ASTM 2000). Based on the MHC data, the tested peptides can be divided in two distinct groups: (i) AMPs which can induce hemolysis: Ind, Mel, PM, and

TL-I and (ii) AMPs which don't induce hemolysis for the tested concentration range: CA, CB, CP1, Mag I, Mag II, LB11 and DS1.

The data obtained from the toxicity studies were summarized in Table 1. For peptides having a strong toxic effect the IC_{50} and MHC values are shown in the table, while for the others, which do not exhibit any effects in the concentration range tested, the highest concentration is reported in each case. Table 1 shows also the MIC values, as found in the literature, for the peptides applied against *E. coli*. Given that different strains of *E.coli* have been used in different studies, we used a range of MIC for characterizing the AMPs efficiency on *E. coli*. The range limits are given by the smallest and the highest MIC values found in the reports for a given AMP.

Using these data, the TI was calculated as the ratio between the minimal concentration at which cytotoxic effects are observed and the concentration at which the antimicrobial effects are observed (TI = min [MHC, IC_{50}]/MIC). For the case where a range of MIC values were found in the literature, a range of TI was computed and the geometric mean was used as a final value of TI. A higher TI value suggests that a peptide is effective against bacteria at small concentration, exhibiting low or no toxic effects for the mammalian cells.

Apoptosis Induced by AMPs in PBMCs

In order to address the mechanism of cell death we have analyzed in detail the type of cell death (by apoptosis or necrosis) for two peptides: CA with the highest TI, and Mel with the lowest TI. For CA the concentrations tested were 10 μM which does not significantly influence the viability as compared to the control, and 30 μM, concentration for which a small decrease of viability to ~85 % was observed (see Fig. 1b). For Mel we tested concentrations of 1 and 2 μM, one below and the other above the MIC value. The mechanisms of cell death were observed by morphological analysis, differentiating between viable, early apoptotic or late apoptotic and necrotic cells (Petcu et al. 2006). The results obtained after 24 h treatment are shown in Fig. 2.

In the negative control (untreated cells), less than 10 % are apoptotic cells, the majority being in early apoptotic stage; no necrotic cells are observed in this condition. For the positive control represented by cells grown without FCS, 20 % apoptotic cells (early or late) were observed, in the absence of necrotic cells. For the positive control represented by UV-irradiated cells the percentage of viable cells was reduced to 50 % with a large percentage of the dead cells (~40 %) being found in the late stage of apoptosis. The results obtained for the negative and

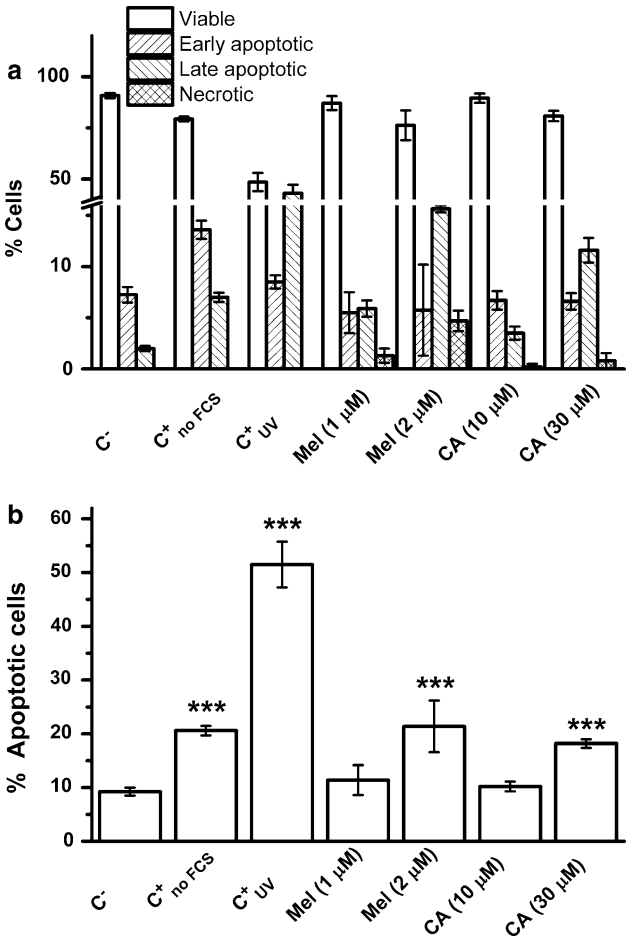

Fig. 2 **a** Apoptotic effects induced by Mel and CA on PBMCs. **b** Total percentage of apoptotic cells for all the conditions studied. C^{+}_{UV}, $C^{+}_{no\ FCS}$ and C^{-} are the apoptosis positive and negative controls, respectively. ***$p < 0.0001$ comparing with the negative control

positive controls are consistent with published data validating the assay used (Ozawa et al. 1999; Petcu et al. 2006).

For both peptides tested, the percentage of apoptotic cells increased as a function of AMP concentration (Fig. 2a). The fraction of cells in early apoptosis is not different (for both tested concentrations) as compared to the negative control. The distribution of apoptotic cells is almost the same for low CA concentration as compared to the negative control. Instead, the small concentration of 1 μM of Mel produces an increase in late apoptotic cells and a small percentage of necrotic cells (less that 2%) comparing to the control. The increase of peptides concentration induced a decrease in the viable cells fraction. The changes are in agreement with the global viability measured in the cytotoxicity assay, Fig. 1. A corresponding significant increase of late apoptotic fraction (~16 % for Mel and ~12 % for CA) accompanied by a small

percentage of necrotic cells (~5 % for Mel) is noticed (Fig. 2a).

In order to better capture the apoptotic effect of the AMPs tested, in Fig. 2b the percentage of early and late apoptotic cells was presented. Our results show that CA at 10 μM and Mel at 1 μM had no toxic effects, the percentage of apoptotic cells being not significantly different to the control. A highly significant increase to ~20 % of apoptotic cells was encountered for both CA and Mel at the high concentration, with a small excess of necrotic cells for Mel that proved to be more toxic. These data demonstrate that the cytotoxicity of CA and Mel in PBMCs is mainly due to induction of apoptotic cell death mechanisms.

Discussion

TI as an Integrative Parameter Characterizing the AMPs Cell Selectivity

Natural AMPs have been discovered and studied in the last 50 years along with many more artificially synthesized AMPs. The aim of this work is to propose a modality to identify compounds with high efficacy against pathogens, and low toxic effects on normal cells to inform on the AMPs suitability for testing in preclinical trials. AMPs efficiency on pathogens has been proven in vitro; however their toxic effects have not been fully investigated. Due to this concern and due to production costs only a small number of compounds went for preclinical testing and there is no AMPs based drug currently approved by Food and Drug Administration (Seo et al. 2012).

The reports on AMPs efficacy and secondary toxicity on mammalian cells contain primarily data on red blood cell hemolysis and effects on cell viability. However the assays are not designed in a standard manner and the reported results are difficult to compare. It was proposed that the selectivity of AMPs can be determined using the TI, that considers hemolysis as sole cytotoxic parameter according to Chen et al, 2005. Given the new insights into the mechanisms of AMPs cytotoxicity, particularly the prevalence of apoptosis, TI need to be re-considered to address directly the effects of AMPs on the viability of complex mammalian cells. Here we propose to add to the TI another index of cytotoxicity, the IC_{50} measured on PBMC by a common viability test.

Accordingly, we propose to redefine the TI as the ratio of min [MHC, IC_{50}]/MIC. This approach addresses the toxicity of AMPs on mammalian cells by two in vitro tests, hemolysis and lymphocytes viability, assessing the AMPs side effects more realistically. Both indexes (MHC and IC_{50}) are measured by standardized assays i.e. MHC acc ASTM F 756—00 and IC_{50} by a MTS proliferation test).

To test this working formula of TI we used a set of 11 AMPs, the only common feature of these being the cationic nature. In order to ensure that this approach can be applied to a wide range of AMPs we selected peptides with various secondary structures (alpha-helix, beta-sheet or random coil). The results summarized in Table 1 for the 11 AMPs tested reveal their different toxicity against mammalian cells and the fact that the newly defined TI integrates their ability to differentiate between bacteria and mammalian cells.

Besides its efficiency against bacteria in the micromolar range, Mel exhibits strong toxic effects against both hRBCs and PBMCs, at similar concentration (~2 μM) resulting in the smallest TI of the tested AMPs (MHC/MIC = 1.3). These findings are in line of other reports proving the high toxicity of Mel (Sovadinova et al. 2011). We found that Mel kills PBMCs in a dose dependent manner, mostly through apoptosis.

The studies performed on the intact lactoferricin and two Lactoferricin segments (with 6 and 10 amino acid residues, the last being LB11) showed that only lactoferricin and LB11 are active against human leukemia and carcinoma cancer cells (Mader et al. 2005), while on the gastric cancer cell line only Lactoferricin is efficient (Pan et al. 2013), proving that the active center alone is not sufficient for peptide efficiency. Liu and its colleagues tested the antimicrobial and hemolytic activity of Lactoferricin and several derived peptides, including LB11. They showed that LB11 MIC against *E.coli* is at 20.72 μM higher than Lactoferricin, but only a slight hemolytic activity above 41.44 μM (two times MIC), which is lower than the one observed for Lactoferricin (Liu et al. 2011). For the smallest sequence the efficiency against bacterial strains was abolished proving again that the active center alone is not sufficient for peptide efficacy. Our studies confirm these data showing that LB11 didn't exhibit either hemolytic effect up to concentrations two times higher than its MIC neither cytotoxic effects on PBMCs at concentrations at least seven times higher than its MIC.

The Ind concentration that induced the hemolysis is similar to the one reported by Subbalakshmi and colleagues (Subbalakshmi et al. 1996), but is lower than the one at which the cytotoxic effects were observed for PBMCs. Staubitz et al. found the MHC for Ind (defined as the minimum concentration required to cause complete hemolysis of the erythrocytes) at 15.6 μM (Staubitz et al. 2001), lower than the one we report here. Based on our results we can say that Ind is more toxic at concentrations lower than the ones tested for cecropin and magainin families, PM or LB11, while their antimicrobial activity is comparable.

Previous studies performed on Dermaseptin S1 and its derivatives showed that in the range of micromolar

concentration are active against bacteria, fungi, yeasts and protozoa, while no toxic effects were observed against red blood cells, even at a concentration of 50 μM (Mor and Nicolas 1994; Savoia et al. 2008). Unexpectedly, we found that DS1 kills PBMCs in a dose dependent manner (IC_{50} ~14 μM). No hemolysis was observed for the concentration range tested here. Considering these values, the TI of DS1 is smaller than that of other peptides, making it less suitable for further development of therapeutic strategies.

As reported earlier (Ramamoorthy et al. 2006), TL I caused hemolysis to hRBCs and 5 % of hemolysis was observed at 12.06 μM, a concentration at which only a slight cytotoxic effect was observed on PBMCs. Although TL I is active against bacteria at small concentrations, due to their hemolytic effect the TI is pretty small (around 5), which indicates that TL I show only a reduced selectivity towards the bacterial cells. Therefore this compound presents as well reduced potential for further testing in preclinical studies.

PM is a mast cell degranulating peptide found in wasp venom, able to bind to and inhibit calmodulin activity and to stimulate the activity of phospholipase A2 (Argiolas and Pisano 1983; Barnette et al. 1983). Although not many studies of antimicrobial or toxic effects were performed on PM, molecular simulation studies proved its high antimicrobial potential (Avram et al. 2011). It was reported that for a concentration of 15.3 μM no hemolytic activity was observed (Argiolas and Pisano 1983). Our results showed that the toxic effects against mammalian cells are observed at concentrations higher than the one tested before. The toxic effects were observed around 70 μM, a concentration at least seven times higher than the MIC reported in the literature for its family members (Nakao et al. 2011).

Magainins are a class of AMPs which exhibit antimicrobial activity at low concentration and do not produce any toxic effects against mammalian cells. We found that Mag I and II didn't show any toxic effects for the highest concentrations tested, 165 and 33 μM respectively. Based on these results we found that they have a really high therapeutic index, especially Mag I, which is higher than 20.

The Cecropin family is active against bacteria at low concentration, and didn't show toxic effects, even at concentrations more than 100 times higher than the ones reported for MIC (see Table 1). Beside its antibacterial activity, it was found to exhibit antitumor activity in a number of cancer cells. CP1 and CB are active against bacterial cells at similar concentration with no effects against mammalian cells, resulting in a similar therapeutic index, around 12. From the 3 peptides tested, CA proved to be the most effective one, having the highest TI (>27).

According to our data, the Cecropin family, especially CA exhibited a high TI compared with the other peptides tested. The results indicate that that CA can selectively damage bacterial cells and not cause any damage to PBMCs and hRBCs. Similarly, the family of Magainins, especially Mag I also gave a high therapeutic index.

Apoptosis is the Major Pathway of PBMCs Death Induced by AMPs

To support our view concerning the definition of the TI, we additionally analyzed the mechanisms by which two of the investigated AMPs, Mel (smallest TI value) and CA (highest TI values), induce a cytotoxic effect on PBMCs. Few reports in the literature address the issue of AMPs apoptotic capacity on mammalian cells, even if in the case of yeasts the apoptosis has been very recently considered as an important action mechanism of AMPs (Choi et al. 2013). On the other hand, the apoptotic cells death is often investigated in studies of AMPs action on cancer cells (Hoskin and Ramamoorthy 2008). Our data proves that Mel and CA induce the death of PMBCs mainly by apoptosis, the percentage of the apoptotic cells being several times higher than the necrotic one for the concentrations used in this study. Mel was proven to induce necrosis in Jurkat cells at concentrations higher than 2.5 μM (Janko et al. 2013) by its lytic capability, but at small concentration (less than 1 μM) Mel suppressed the proliferation of vascular smooth muscle cell by apoptosis (Son et al. 2006). The concentrations we used in our experiments are at the limit of these ranges, so it was expected that both death pathways would be activated with a significant increase of the necrotic contribution at higher concentrations. In the case of CA, no cytotoxicity has been proved on normal mammalian cells, but at 30 μM the apoptosis has been shown to occur by a caspase independent pathway in human promyelocytic leukemia cells (Ceron et al. 2010), which is in agreement with our data.

All these data sustain that apoptosis is one of main pathways activated by AMPs in mammalian cells at cytotoxic concentration. Consequently, the TI definition has to consider alternative parameters to MHC, a measure only of the AMPs lytic effect, associated mainly with necrotic cell death (Janko et al. 2013).

Conclusions

It is hard to predict whether the AMPs will be efficiently active in vivo based only on the results obtained in vitro. Our study argues in a proof of concept work that the rigorously determining a therapeutic index is the starting point to select the best potential candidates for a clinical trial. We suggest that a formula of TI that includes both the direct lytic effect measured on human erythrocytes, and an

index, of human lymphocytes viability would provide a more realistic way to globally characterize the cellular specificity of the AMPs by TI. Our data prove that apoptosis is one of the major cell death mechanisms triggered by AMPs in the range of concentrations where these are toxic for mammalian cells. In order to make appropriate comparisons among different reports on AMPs TI values, standardized assays have to be used in measuring the hemolytic index and the lymphocytes viability index.

Thus, we demonstrated the utility of testing in similar conditions the AMPs toxicity towards eukaryotic cells (PBMCs and hRBCs) and the power of the therapeutic index concept to compare different AMP candidates to clinical trials.

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Conflict of interest Mihaela Bacalum and Mihai Radu declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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