The influence of selected antimicrobial peptides on the physiology of the immune system

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ABSTRACT

Antimicrobial peptides (AMPs) are an essential part of the innate immune system that serves as a first line of defense against invading pathogens. Recently, immunomodulatory activities of AMPs have begun to be appreciated, implying the usefulness of AMPs in the treatment of infectious disease. The aim of this strategy is the modulation of host immune responses to enhance clearance of infectious agents and reduce tissue damage due to inflammation. Although AMPs could be used as therapeutic agents, a more detailed understanding of how they affect host cells is needed. Hence, several AMPs have been investigated for their potential as a new class of antimicrobial drugs in this study. Synthetic AMPs and AMPs of natural origin were tested on human leukocytes by flow cytometry. Dose- and time-dependent cytotoxic effects could be observed by propidium iodide staining. Different leukocyte subtypes seem to be susceptible to AMP treatment while others were not affected, even in high concentrations. In conclusion, AMPs have an impact on host immune cells. However, their role in stimulation of chemokine production and enhanced leukocyte recruitment remains a crucial aspect and further studies are needed.

Keywords: antimicrobial peptides, cytotoxicity, flow cytometry, leukocytes

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1. INTRODUCTION

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune system and still have their function in the defence against invading pathogens. They are ubiquitous among all eukaryotes, including mammals, amphibians, insects, plants and protozoa. The majority of AMPs fight pathogens by permeabilizing the membranes of microbial cells. However, some AMPs penetrate bacterial membranes. In the cell they interact with intracellular targets, disturbing physiological processes, e.g. synthesis of a specific membrane proteins or DNA [1-4]. These modes of action seem not to be affected by the resistance mechanisms developed by microbes towards conventional antibiotics [4-6]. As many bacteria developed resistance against common antibiotics, the working mechanism of AMPs yields a starting point for antimicrobial treatment in the future.

However, antimicrobial peptides are being considered to be universal multifunctional molecules because their functions extend the role of an antimicrobial agent by far. It was proved that they possessed many additional biological activities, such as anti-tumour, mitogenic, and anti-viral [4-7]. Moreover, it has been shown that endogenous peptides act on immune cells and hence alter functionality of the immune system by different mechanisms. They can act proinflammatory (chemoattraction of immune cells, induction of chemokines and differentiation responses, angiogenesis) but also immunosuppressive (blocking TLR signalling, inhibition of LPS-stimulated production of cytokines, hampering septic shock) [6].

Consequently, AMPs are good candidates as a new generation of antibiotics as well as innate immune modulators. Presently, only few AMPs have entered phase 3 clinical-efficacy trials. Suitable applications of AMPs have been found for curing or preventing impetigo and diabetic foot ulcers (Pexiganan), oral mucosaitis (iseganan), sepsis (Neuprex), catheter-associated infections (Omiganan) [6]. Although many AMPs indicated broad-spectrum antimicrobial and immunomodulatory activity *in vitro,* for *in vivo* use in clinical applications their working mechanisms, characteristics such as retention time and other factors must be examined in detail and taken into consideration for a possible therapeutic agent. Important are among others toxicology, pharmacokinetics, and preferably a limited half-life of the drug [8]. In particular, little is known about their cytotoxicity against normal eukaryotic cells [9]. However, it was proven that some AMPs cause cell death by DNA damage, disruption of the transmembrane potential in mitochondria, or membrane permeabilization [10-13]. Furthermore, differences in toxicity of AMPs against certain cell types, i.e. different subpopulations of immune cells such as mononuclear cells or granulocytes, activated or non-activated human lymphocytes, apoptotic, and normal cells were observed [12-17]. These properties imply usefulness of AMPs in new therapeutic strategies and applications.

The aim of the present study was to investigate the cytotoxic activity of different AMPs on human leukocytes. The cell viability of human leukocytes was tested after treatment with eleven peptides. AMPs of natural origin from different species and AMPs with modified structure or synthetic ones were included into the study. We used flow cytometric detection of the time course of cell killing by propidium iodide (PI) staining to assess cytotoxic effects of AMPs. Use of PI is recommended as it does not interfere with the killing action of cytotoxic agents [18].

2. METHODS

2.1. Synthesis of antimicrobial peptides (AMPs)

AMPs were synthesized manually by the solid-phase methodology on Polystyrene AM-RAM resin or 2-chlorotrityl chloride resin (Rapp Polymere, Tübingen, Germany) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [19].

After synthesis, the peptides were cleaved from the resin and simultaneously deprotected using trifluoroacetic acid (TFA) in the presence of scavengers (triisopropylsilane and water). The cleaved peptides were precipitated with diethyl ether and if required (AMPs containing cysteine) oxidized by 0.1 M iodine in methanol. Subsequently, peptides were purified by reverse phase high-performance liquid chromatography (HPLC) on a Knauer K501 two-pump system with a Kromasil C8 column (10 x 250 mm, 5 µm particle size) with several gradients of acetonitryle (ACN) in 0.1% TFA. The eluates were fractionated and analyzed by the analytical RP-HPLC. The purity of the peptides was checked on a Varian ProStar HPLC system controlled by Galaxie Chromatography Data System with Kromasil C8 column (4.6 × 250 mm, 5 µm particle size) with several linear gradients of ACN in 0.1% TFA. Fractions containing >98% peptide were pooled and lyophilized. All peptides were additionally analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The values of the molecular ions were as expected (table 1).

	antimicrobial peptide	molecular weight [Da]	MIC [µg/ml]	
			Gram$^+$	Gram$^-$
natural peptides	n-1	1480.77	16-32	128-256
	n-2	2434.89	8-32	128-256
	n-3	1615.96	8-16	128-256
	n-4	2294.7	4-16	32-128
	n-5	1397.77	8-64	512-1024
	n-6	1778.14	4-32	128-256
synthetic/modified peptides	s-1	1771.28	1-4	4-32
	s-2	1905.35	0.5-16	16-128
	s-3	2439.01	16-512	256-1024
	s-4	1780.15	0.5-16	16-128
	s-5	586.42	1-2	-

Table 1: List of used AMPs with corresponding molecular weight. AMPs were of natural origin or were synthetic or modified from the original structure. MIC: minimal inhibitory concentration against gram$^+$ and gram$^-$ bacteria

2.2. Isolation and preparation of leukocytes

EDTA blood was taken from healthy adult volunteers and immediately processed after collection. Leukocytes were isolated using Histopaque 1,077 g/ml density gradient medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The layer of leukocytes was collected, washed once in PBS and maintained in RPMI 1641 medium supplemented with 10% FCS (Sigma Aldrich Chemie GmbH). Cells were counted using an improved Neubauer hemocytometer. Cell suspension was diluted to a concentration of 250.000 cells per sample.

2.3. Cell viability assay

AMPs were dissolved in PBS at a stock concentration of 1mg/ml. AMPs were added to cell suspensions at a final concentrations of 1, 10, 100 µg/ml and incubated at 37°C. Different incubation times were used for each peptide (5, 30, 60 min). After incubation reaction was stopped by putting samples on ice. For negative control PBS was added to the cell suspension instead of AMP, for positive control H_2O_2 (final concentration: 0.2%). Cell viability was tested by PI staining (final concentration: 0.5 µg/ml) for 5 minutes on ice. Immediately after staining samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences) at high speed. PI was excited with the 488nm laser line and fluorescence was detected by using a 675 LP filter. A total of 10,000 events were measured per sample.

2.4. Data analysis

Flow cytometric data were analyzed using the FlowJo software (version 7.5; FlowJo, LLC, Ashland, OR). Leukocyte subsets (lymphocytes, monocytes, neutrophils) were identified by gating on the FSC vs. SSC dotplot (figure 1).

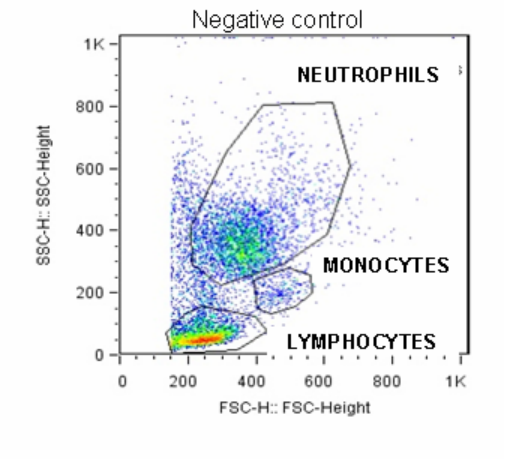

Figure 1: Identification of leukocyte subtypes (untreated sample). Lymphocytes, monocytes, and neutrophils were gated on FSC vs. SSC dotplot of negative control. Gates were applied for other samples.

In order to include cells into analysis that are affected by the AMP but are completely disrupted (and hence not measurable by flow cytometry), the percentage of live cells was calculated as follows:

$$CL = 100 * (1 - \frac{CC_S / t}{CC_{NC} / t})$$

$$RC = 100 - CL$$

$$rCC_{alive} = RC - RC * \frac{rCC_{dead}}{100}$$

CL: cell loss [%]

CC_S: cell count in sample

CC_{NC}: cell count in negative control

t: run-time of data acquisition on FACSCalibur [s]

RC: remaining cells in sample [%]

rCC_{alive}: relative count of live cells in sample [%]

rCC_{dead}: relative count of PI^+ cells in sample [%]

Cell viability of leukocyte subsets was determined by calculating the percentage of live cells within the samples using the same formulas as for the overall cell population.

2.5. Statistics

Results represent the median values of at least three independent experiments. Effect of AMPs on cell viability was determined by comparing the AMP treated sample with negative control by Mann-Whitney Rank Sum Test (SigmaStat, SPSS Inc., Chicago, IL). Level of significance was set to $p \leq 0.05$.

3. RESULTS

AMPs were added to freshly isolated human leukocytes in order to test their cytotoxic effects on immune cells. Cell viability was determined by PI exclusion test for lymphocytes, monocytes, and neutrophils with three different concentrations of AMPs and different incubation times.

We could not find a significant effect of any of the tested AMPs at concentrations of 1µg/ml and 10µg/ml. However, at 100µg/ml with some AMPs (n-5, s-1, s-5) the number of dead cells (PI^+) increased significantly compared to negative control. Moreover, exposure to these peptides caused decrease in cell concentration, i.e. a lower cell count than estimated was measured by flow cytometry indicating disruption of cells. The cell loss is a direct effect of the AMPs. As 'lost cells' cannot be determined by flow cytometry, the number of dead cells, assessed by PI fluorescence, must be corrected by the likewise dead cells that are not existent as a cell anymore. Hence, in order to correctly assess the effect of AMPs on cell viability the measured cell count was related to the initial cell count in each sample and leukocyte subpopulation. With the formulas we compiled for this purpose

(see methods), the overall effect of AMPs on cell viability could be determined. The loss of cells within the sample was in particular evident after 60 minutes exposure to AMP n-5 at a concentration of 100µg/ml (figure 2).

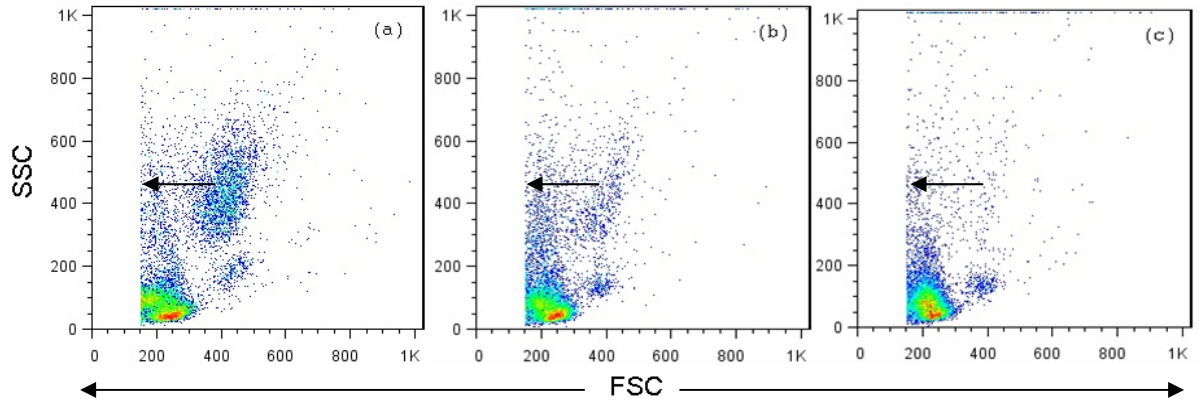

Figure 2: Leukocytes treated with AMP n-5 (100µg/ml). It can be seen from the FSC vs. SSC dotplot that the number of cells decreased with time of incubation (a: 5 minutes, b: 30 minutes, c: 60 minutes). Concurrently, more cell debris showed up at low FSC values presumably from fragmented cells (arrow).

As can be expected from the disruption of cells, some AMPs caused changes in cell size and morphology (n-5, s-1, s-3, s-4, s-5). An example for the morphological changes is shown in figure 3. This effect on cells is also visible in the altered cell distribution in the FSC vs. SSC dotplot (figure 4). However, most of the AMPs did not significantly influence the leukocytes and were not toxic at all tested concentrations and incubation times (table 2).

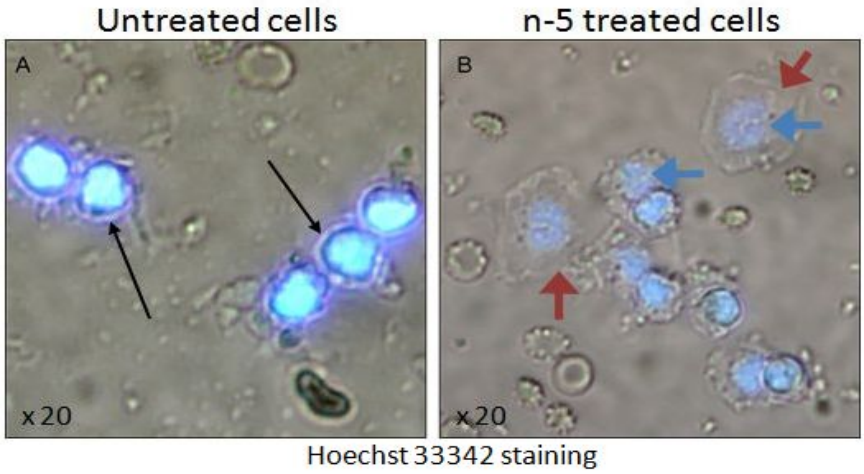

Figure 3: Effect AMP n-5 on isolated leukocytes. While untreated cells (A: negative control) are spherical with a bright (Hoechst 33342 stained) nucleus (black arrows), cells treated for 60 minutes with n-5 (B) are larger, flat (red bold arrows) and also the nucleus seems to be affected (blue bold arrows).

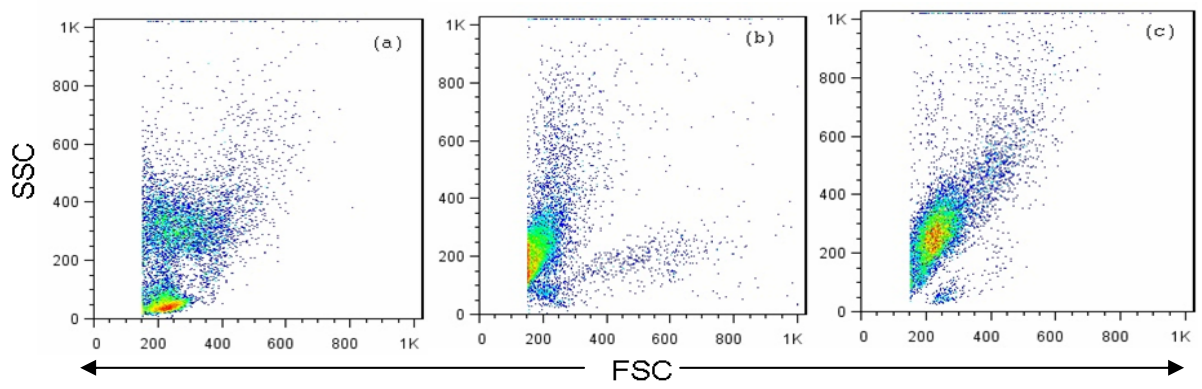

Figure 4: Scatter alterations caused by AMPs. Some AMPs had an effect on cell size and morphology (b: s-1, c: s-5) while others showed no such effect (a: n-2). Dotplots show leukocytes after 1-hour-exposure to AMPs (100µg/ml): n-1 (a), s-1 (b), s-5 (c). AMP n-2 has no effect on scatter whereas after treatment with s-1 or s-5 there the number of events with low FSC and SSC increased. This means that these AMPs have an effect on cells.

	incubation time		
AMP	5 min	30 min	60 min
n-1	n.s.	n.s.	n.s.
n-2	n.s.	n.s.	n.s.
n-3	n.s.	n.s.	n.s.
n-4	n.s.	n.s.	n.s.
n-5	n.s.	**41.5**	**20.0**
n-6	n.s.	n.s.	n.s.
s-1	n.s.	**18.1**	**8.9**
s-2	n.s.	n.s.	n.s.
s-3	n.s.	n.s.	n.s.
s-4	n.s.	n.s.	n.s.
s-5	**42.1**	**29.8**	**5.1**

Table 2: Cell viability of leukocytes. Table contains the percentage of live cells (all leukocytes) at different incubation times at a concentration of 100µg/ml of AMP. Results are the median values of three experiments (bold: statistical different from control, $p \leq 0.05$). Only n-5, s-1 and s-5 lead to significant cell killing.

3.1. AMP effect on leukocyte subtypes

Leukocytes were subdivided into lymphocytes, monocytes, and neutrophils by gating on the FSC vs. SSC dotplot. However, the changes in cell size caused by some AMPs made it difficult to identify the whole cell population. In order to get comparable results, gates were set on the negative control and adopted to the analysis of the other samples. The number of leukocyte subtypes was determined within the respective gate. Cell viability was corrected by the cell loss as described for the whole leukocyte population. A summary of the results is shown in table 3.

	lymphocytes			monocytes			neutrophils		
AMP	5 min	30 min	60 min	5 min	30 min	60 min	5 min	30 min	60 min
n-1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n-3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n-4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n-5	n.s.	n.s.	**39.2**	n.s.	**38.8**	**24.8**	n.s.	**29.3**	**6.7**
n-6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
s-1	n.s.	**27.6**	**6.9**	n.s.	**0.0**	**0.0**	n.s.	**4.9**	**0.8**
s-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
s-3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**26.6**	**23.4**
s-4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**5.7**
s-5	**7.8**	**6.8**	**6.1**	n.s.	n.s.	**7.9**	n.s.	n.s.	**1.3**

Table 3: Cell viability of leukocyte subtypes. Table contains the percentage of live cells (lymphocytes, monocytes, and neutrophils) after different incubation times with AMPs (100µg/ml). Results are the median values of three experiments (bold: statistically different from control, $p \leq 0.05$).

Assessment of cell viability on isolated leukocytes is influenced by the isolation procedure, sample preparation and the *ex vivo* situation itself. For this reason, viability results were always compared to negative control. Even within the negative control, after 60 minutes incubation in RPMI medium there were ~30% PI^+ monocytes and ~50% PI^+ neutrophils. Lymphocytes on the other hand were not significantly affected.

It could be observed that AMPs had a different cytotoxic effect on leukocyte subtypes. Some AMPs (e.g. s-1) were toxic to all subtypes while others (e.g. s-3) affected only neutrophils (figure 5). Moreover, also the incubation time seems to play an important role. In most cases cytotoxic effect was measurable after 30 minutes exposure to the AMP. However, AMP s-4 showed only after 60 minutes an effect on neutrophils. On the contrary, after 5 minutes exposure to AMP s-5 less than 10% of lymphocytes were still alive.

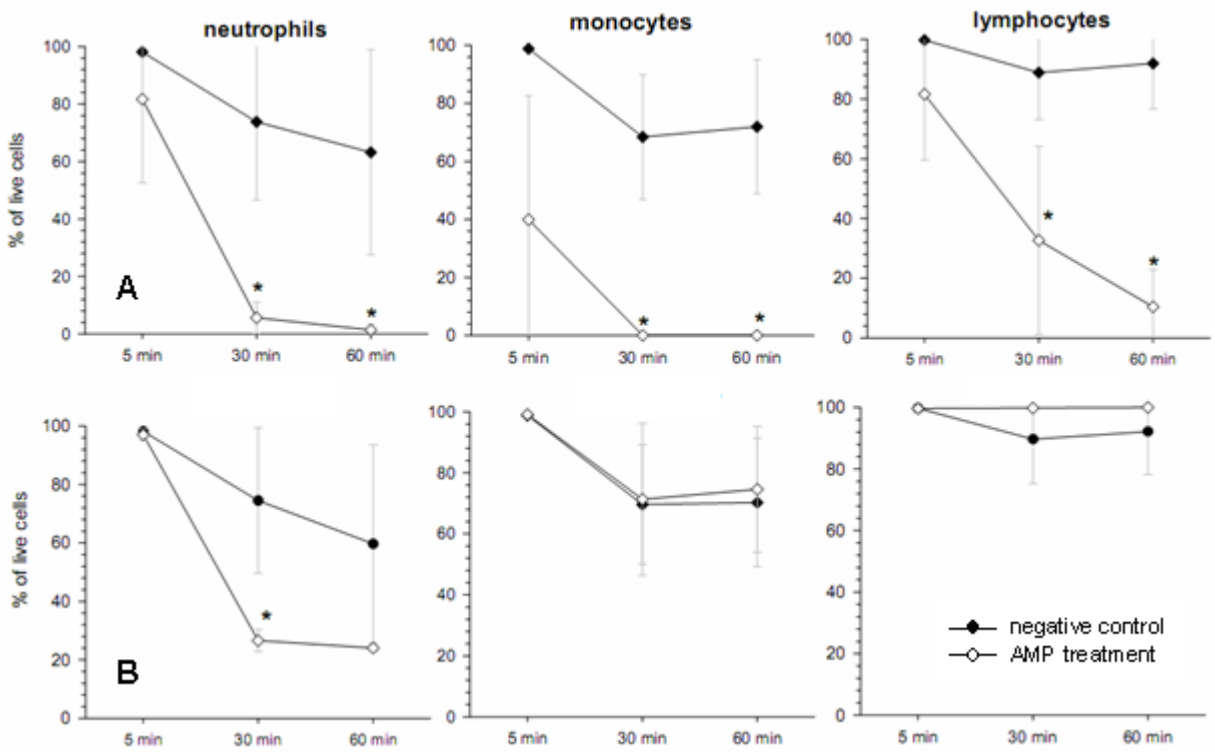

Figure 5: Leukocyte subtype specific cytotoxicity for two AMPs. It can be seen that not all leukocyte subtypes are affected the same way during AMP exposure. A: AMP s-1, B: AMP s-3, * significant differences to negative control.

4. DISCUSSION

AMPs are nowadays regarded as broad-spectrum molecules that possess many valuable properties. The fact that they act directly on bacteria can be considered to be better than pharmaceuticals that have an indirect approach [20]. Moreover, new potent applications of AMPs are now being considered as immunomodulatory compounds that can serve in anti-infective therapeutic strategies [6]. There is a need to further characterize already identified AMPs and to search for new similar compounds.

We studied eleven AMPs of different origin. They vary in sequence, structure, size and antimicrobial activity. Some of them have an effect on bacteria at very low concentrations, e.g. at a Minimal Inhibitory Concentration (MIC) against $Gram^+$ bacteria of 2-4µg/ml (AMP s-1). On the contrary, others need a concentration, which is two-hundred-times higher to show antimicrobial activity (e.g. APM n-2). In general, AMPs of natural origin seem to unfold their antimicrobial activity at higher concentrations than the synthetic ones. Moreover, the antimicrobial activity of the AMPs may also depend on the molecular weight. Short AMPs seem to have an effect on bacteria already at low concentrations considering the low MIC values and the cell viability results for the tested AMP with the lowest molecular weight (AMP s-5).

Cytotoxicity of AMPs on immune cells was assessed by flow cytometry. This technology is ideally suited for analyzing the effects of compounds on the immune system [21,22]. Immunophenotyping and flow cytometric analyses allows for the analysis of cell alterations [23] and the effect of drugs on different cell subsets. It was proved to be very useful in predicting the immunotoxicity of certain families of chemicals and to determine selective cytotoxicity [21]. Therefore, in this study immunophenotyping and flow cytometry were used to assess the influence of AMPs on the viability of leukocytes. Selective cytotoxicity and the range in which they kill immune cells could be determined. However, the majority of the AMPs tested by us did not render detectable cytotoxic activity. This does not exclude that they act in some other way on leukocytes or leukocyte subsets. This may include immunomodulation such as cell activation or silencing. Further high-content and high-throughput flow cytometry tests are needed to scrutinize such potential effects. This may include effects on signal transduction pathways by phosphoflow [24], cytokine production by polychromatic standardized protocols [25], macrophage function [26], among others. With the introduction of automated high-throughput image and flow cytometers [27,28] in combination with data standardization and automated data analysis [29,30] the assays can be easily scaled up to manage thousands of samples in a short amount of time.

We could demonstrate that all AMPs did not cause significant decrease of cell viability at concentrations of 1 to 10µg/ml. However, at 100µg/ml some of the tested AMPs revealed cytotoxic effect on leukocytes. This means, at concentrations at which AMPs possess an antimicrobial activity they show no cytotoxicity on immune cells and could be safely used for treatment.

Due to the increasing bacterial resistance to antibiotics, there is a great need to find alternatives in antimicrobial treatment. AMPs may be a solution. Several applications of AMPs are under development [5,6,18]. For example, there is the possibility that AMP-derived products will be used to kill bacteria in catheter-related infections [6]. However, testing AMPs (from natural sources or synthetic ones) is a crucial aspect in developing new applications. We could show that AMPs have a different effect on leukocyte subtypes. That implies the usefulness of AMPs in the treatment of e.g. immune disorders. But also other therapeutic and non-therapeutic applications should be considered where depletion of certain cell lines is needed. Nevertheless, further analyses should be performed.

Before AMPs may enter clinics as therapeutic agent, their effect on basic physical parameters of immune cells must be tested. Moreover, the selective cytotoxic activity of AMPs must be confirmed in animal studies.

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