

Antitumor activity of antimicrobial peptides against U937 histiocytic cell line

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We investigated cytotoxic activity of antimicrobial peptides of different origin (both naturally occurring and synthetic), structure and known mechanisms of action against human histiocytic lymphoma cell line U937. The strongest cytotoxic activity against U937 cell line was shown by Pexiganan MSI-78, followed by Citropin 1.1, Protegrin 1 and a synthetic lipopeptide, *N*- α -palmitoyl-L-lysyl-L-lysine amide (Pal-Lys-Lys-NH₂). The cytotoxic activity of the peptides was more dependent on the time of incubation than concentration. Only for the lipopeptide, whose mode of action was restricted to disruption of electric potential of the cell membrane, the correlation between cytotoxicity and concentration was almost linear. The high cytotoxicity of Pexiganan MSI-78, Protegrin 1 and the lipopeptide could be basically explained by their membranolytic activity leading to necrosis. However, in the case of Citropin 1.1, the cell membrane integrity was disrupted only slightly and independently of the peptide concentration. Therefore, some other mechanism of action might be responsible for its strong dose-dependent cytotoxic activity, e.g., membranolytic activity leading to apoptosis. Furthermore, TNF- α production due to LPS (lipopolysaccharide) stimulation was suppressed by the presence of Citropin 1.1, Pexiganan MSI-78 or Protegrin 1, but not by Buforin 2 or the lipopeptide. Our experiments have shown that cytotoxic activity is not limited to some specific molecular structure of a peptide, but rather to the length of the peptide chain as it is likely to affect the efficiency of the tumor cell membrane disruption and interaction with LPS.

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INTRODUCTION

Antimicrobial peptides (AMPs) constitute an evolutionarily conserved part of the innate immune defense mechanism found in a wide range of prokaryotic and eukaryotic organisms. They are active against bacteria, fungi, viruses and also take part in anti-tumor response making them promising candidates for therapeutic use (Lehrer & Ganz, 1999; Reddy *et al.*, 2004; Dubin *et al.*, 2005). Over 1000 AMPs have already been isolated and characterized at the level of their primary structure (Bulet *et al.*, 2004). Most of these naturally occurring peptides are generally 12- to 50-amino acid long, have cationic properties, meaning that they have an overall positive

net charge at physiological pH, and fold into a variety of different structures, including α -helices, β -sheets, extended helices, hairpins and loops. They can also adopt an amphipathic structure with a hydrophobic and a hydrophilic side (Bulet *et al.*, 2004).

Their general mode of action is disruption of the cell membrane, in which their tendency to form amphipathic structures is believed to be pivotal by facilitating their interaction and insertion into the membrane (Oren & Shai, 1998; Cudic, 2002). As they are generally cationic, they can be drawn electrostatically to the target membrane and accumulate on the surface. When a critical concentration of AMPs is reached, some conformational changes occur leading mainly to the formation of ion channels or aqueous pores and resulting in the death of the cell through hypoosmotic lysis (Shai, 1999). Although most of the AMPs seem to act mainly at the membrane level, their translocation into the cytoplasm is not uncommon and leads to intracellular changes including inhibition of protein or DNA synthesis (Boman *et al.*, 1993; Reddy *et al.*, 2004). Some AMPs, for instance Buforin 2, can even lack the ability to disrupt cell membrane and instead form non-permeabilizing pore-like structures that allow translocation of the peptide into the cytoplasm without cell lysis (Park *et al.*, 1996; Kobayashi *et al.*, 2004).

Most AMPs have some level of selectivity towards specific target cells, mainly towards prokaryotic ones. It is based on a complex balance of many different biophysical properties of AMPs such as secondary structure, overall charge and hydrophobicity as well as many different biophysical properties of the target cell membranes, such as, for instance, its phospholipid composition, curvature or the presence of cholesterol (Polozov *et al.*, 1997; Matsuzaki *et al.*, 1998; Shai, 1999; Wu *et al.*, 1999; Bradshaw, 2003). It also leads to enhanced sensitivity of tumor cells to the lytic action of AMPs, due to the differences in composition of the cell membrane between tumor and normal cells and the disturbed cell membrane asymmetry in the former. The outer leaflet of the cell membrane of tumor cells contains negatively charged phosphatidylserine (PS) (3–9% of the total membrane phospholipids) in contrast to normal cells in which PS is localized exclusively to the inner leaflet of the membrane (Utsugi *et al.*, 1991). The negative charge of the

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Abbreviations: AMPs, antimicrobial peptides; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; LPS, lipopolysaccharide; Pal-Lys-Lys-NH₂, *N*- α -palmitoyl-L-lysyl-L-lysine amide; TNF- α , tumor necrosis factor-alpha

tumor cell membrane is also increased by higher levels of O-glycosylated mucins (Carraway *et al.*, 2007). The resulting higher negative net charge of the cell surface of tumor cells compared to normal cells contributes to the selective lytic activity of antimicrobial peptides. For instance, cecropins and magainins kill neoplastic cells at concentrations lower than those required to lyse normal cells such as peripheral blood lymphocytes (Cruciani *et al.*, 1991; Moore *et al.*, 1994). AMPs can also function as mediators of inflammation as they can affect epithelial and immune cells modulating, e.g., proliferation, angiogenesis, cytokine release, and chemotaxis (Beisswenger & Bals, 2005) by binding to cellular receptors at low concentrations and activating signalling pathways (Koczulla & Bals, 2003).

In this publication, we present a study on the cytotoxic activity of a variety of antimicrobial peptides of different origin, structure and known mechanisms of action as shown in Table 1 against one tumor cell line — a human histiocytic lymphoma cell line U937. We also investigated the antitumor activity of a synthetic lipopeptide *N*- α -palmitoyl-L-lysyl-L-lysine amide (Pal-Lys-Lys-NH₂) (Kamysz *et al.*, 2007). As it carries only one fatty acid residue, it exhibits a reduced mitogenicity (Bessler *et al.*, 1985). Its direct activity against tumor cells through simple disruption of the membrane electric potential (Epanand *et al.*, 1999; Avrahami & Shai, 2004) was analyzed and used for comparison with a possibly more complicated mechanism of action of the other peptides analyzed. Such an approach should allow an analysis of the level of dependence between the cytotoxic activity of the antimicrobial peptides and their structure.

MATERIALS AND METHODS

Cell lines and peptides. The human myeloid leukemia cell line U937 cells were cultivated long-term as a suspension cell culture in RPMI1640 medium supplemented with 10% FCS (Gibco, Gaithersburg, MD, USA), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin. TNF- α -sensitive mouse A9 fibroblastic cell line (Wallach, 1984) cells were cultivated long-term in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. Both cell lines were cultivated at 37°C in a humidified atmosphere containing 5% CO₂ and split 1:4 every 5 days.

The peptides included in this study were synthesized manually in a microwave reactor by the solid-phase method using the 9-fluorenylmethoxycarbonyl chemistry (Fmoc) (Fields, 1990). The completeness of each coupling reaction was monitored by the chloranil test. The peptides were cleaved from the solid support with trifluoroacetic acid (TFA) in the presence of water (2.5%), and triisopropylsilane (2.5%) as scavengers. The cleaved peptides were precipitated with diethyl ether and the cysteine-containing ones were oxidized with 0.1 M iodine in methanol. The peptides were purified by high-performance liquid chromatography (HPLC). The crude lipopeptide was purified by solid-phase extraction by a previously described protocol (Kamysz *et al.*, 2002). The resulting fractions of purity greater than 95% were verified by HPLC or thin-layer chromatography (TLC) for the lipopeptide. The peptides were also analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

***In vitro* cytotoxicity assay.** U937 cells were cultured in 96-well plates at a density of 1×10^5 cells (for the 6-h assay) or 5×10^4 cells (for the 40-h assay) per well with serial dilutions of AMPs in 0.1 ml of complete RPMI1640 medium with 1% FBS and 1 mM pyruvate. Cell viability was measured with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The optical density of wells containing cells cultured without AMPs was assumed to represent 100% cell viability.

Determination of TNF- α concentration in cell culture supernatants. U937 cells were cultured in 24-well plates at a density of 5.2×10^5 cells per well with chosen dilutions of AMPs in 0.52 ml of complete RPMI1640 medium with 1% FBS and 1 mM pyruvate. When additional stimulation of TNF- α production was needed, PMA and LPS at final concentrations of 100 ng/ml each were added. Cells were incubated at 37°C in 5% CO₂ for 6 h. After incubation supernatants were collected and FBS was added to the final concentration of 10%.

The TNF- α concentration was measured by a standard cytotoxic assay coupled with the neutral red uptake assay using TNF- α -sensitive mouse A9 fibroblastic cell line (Wallach, 1984). A9 cells were incubated for 17 h with the analyzed cell culture supernatants at a dilution of 1:1 in culture medium with addition of cycloheximide (CHX) (0.25 mg/ml). The optical density of the wells containing cells not treated with the supernatant but treated with CHX was assumed to represent 100% cell viability.

Trypan blue uptake assay. Cell membrane integrity of U937 cells treated with AMPs obtained from the 6-h assay (as above) was analyzed using trypan blue. Pelleted cells were suspended in 100 μ l of RPMI medium and 20 μ l of cell suspension was mixed with equal volume of the trypan blue solution (0.4% in PBS; Sigma). After 5 min of incubation at room temperature, the cells were counted in a Neubauer Improved hemocytometer. The percentage of cells not stained blue, that is with an intact cell membrane, was calculated. As a control, cells not treated with AMPs were used.

Statistical analysis. Statistical analysis for the *in vitro* assays was performed using the Mann-Whitney U-test.

RESULTS

The effect of AMPs on tumor cell line U937 viability *in vitro*

The cytotoxic effect of the chosen AMPs was analyzed on the human histiocytic lymphoma cell line U937, a frequently used model in studies of TNF- α -mediated cytotoxicity (Sundstrom & Nilsson, 1976). All the peptides, with the exception of Buforin 2, were highly cytotoxic when incubated with tumor cells for 40 h. After such a long incubation period with the AMPs' at 20 μ g/ml, the cell viability was around 2% only, with no visible differences among the peptides. A decrease of the AMPs' concentration to 0.5 μ g/ml did not substantially increase the viability level in the 40-h assay. Only in the case of Omiganan MBI-226 did the viability increase to 15% (not shown). This suggests a stronger dependence of the antitumor effect on the duration of the assay than on the concentration of the peptides for the concentration range studied.

Table 1. AMPs used in experiments, their sequence, grouping according to characteristics of their molecular structure, and mechanism of action

Name and sequence	Grouping according to characteristics of molecular structure	Known mode of action
Buforin 2 TRSSRAGLQFPVGRVHRLLRK (Park <i>et al.</i> , 1996)	linear, α -helical peptide forming amphipathic helix distorted around Pro(11) with a flexible N-terminal region (Kobayashi <i>et al.</i> , 2004; Cho <i>et al.</i> , 2008)	forms and disintegrates non-permeabilizing pore-like structure to translocate without cell lysis then bind to DNA and RNA (Park <i>et al.</i> , 1998; Kobayashi <i>et al.</i> , 2004)
Citropin 1.1 GLFDVIKKVASVIGGL-NH ₂ (Wegener <i>et al.</i> , 1999; Wabnitz <i>et al.</i> , 1999)	linear, α -helical peptide without cysteines (Wegener <i>et al.</i> , 1999; Wabnitz <i>et al.</i> , 1999)	forms a 'carpet' on the membrane surface following 'carpet-like' model (Ambroggio <i>et al.</i> , 2005; Fernandez <i>et al.</i> , 2009)
Demegen P-113 AKRHHGYKRKFH-NH ₂ (Paquette <i>et al.</i> , 1997)	linear, α -helical peptide with a predominance of one or more amino acids — histidine-rich (Paquette <i>et al.</i> , 1997)	translocates into cytosol in <i>Candida albicans</i> (Jang <i>et al.</i> , 2008)
Omiganan MBI-226 ILRWPWWPWRK-NH ₂ (Isaacson, 2003)	linear, α -helical peptide with a predominance of one or more amino acids — tryptophane-rich (Isaacson, 2003)	induces membrane depolarization, inhibition of macromolecular synthesis and cell death (Melo <i>et al.</i> , 2007)
Pexiganan MSI-78 GIGKFLKAKKFKAFVKILKK-NH ₂ (Jacob & Zasloff, 1994)	linear, α -helical peptide without cysteines (Gottler & Ramamoorthy, 2009)	binds to the membrane surface, forms dimers that assemble toroidal pores, but not barrel-stave pores (Gottler & Ramamoorthy, 2009)
Protegrin 1 RGGRLCYRRRFVCVGR-NH ₂ (Koryakov <i>et al.</i> , 1993)	β -sheet structure stabilized by disulphide bridges (Gottler <i>et al.</i> , 2008; Mani <i>et al.</i> , 2006)	assembles β -barrel membrane pore structure forming the core of a toroidal pore (Mani, 2006; Gottler, 2008)
Temporin A FLPLIGRVLSGIL-NH ₂ (Simmaco <i>et al.</i> , 1996)	linear, α -helical peptide without cysteines, basic, highly hydrophobic (Simmaco <i>et al.</i> , 1996)	forms a transmembrane pore via a barrel-stave mechanism or forms a 'carpet' on the membrane surface via the 'carpet-like' model (Oren & Sahi, 1998), is also chemotactic to phagocytes (Chen <i>et al.</i> , 2004)

To find differences in the antitumor activity between individual peptides we had to shorten the incubation period to 6 h only. This enabled us to demonstrate higher antitumor efficiencies of Citropin 1.1, Pexiganan MSI-78 and Protegrin 1 in the U937 cell line model as only those peptides, together with the Pal-Lys-Lys-NH₂ lipopeptide, showed a strong cytotoxic effect in the shortened incubation time (Fig. 1). The lipopeptide, even with its strong ability to increase the permeability of the cell membrane (Avrahami & Shai, 2004), was less cytotoxic than the other three peptides. Those four peptides were chosen for a detailed analysis of the dose-dependence of their antitumor effect in the 6-h assay together with Buforin 2 to demonstrate its lack of cytotoxic effect against this tumor cell line (Fig. 2). Pexiganan MSI-78 had the strongest antitumor effect among the three peptides.

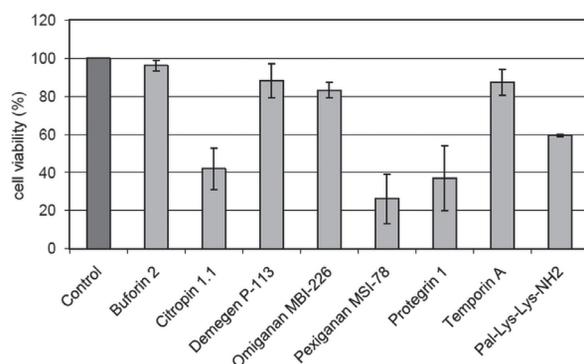


Figure 1. Cytotoxic effect of AMPs on U937 tumor cells AMPs at 20 µg/ml were added to cell culture for 6 h, followed by MTT cytotoxicity assay. Data points represent averages of 3 independent MTT tests. The standard deviations are marked.

The difference was statistically significant for the doses of 20 µg/ml ($P=0.01$) and 10 µg/ml ($P=0.05$). Although there was no difference in the cell culture viability at the dose of 20 µg/ml between Citropin 1.1 and Protegrin 1, the former showed a stronger antitumor efficiency at lower concentrations than did Protegrin 1 (statistically significant at 5 µg/ml; $P=0.05$). The cytotoxic activity of Pal-Lys-Lys-NH₂ at 20 µg/ml was similar to these of Citropin 1.1 and Protegrin 1. However, in contrast to the other peptides, the lipopeptide cytotoxicity had an almost linear dependence on its concentration indicating a much simpler relationship between the concentration of the agent and its influence on the tumor cell. We also observed a lack of a cytotoxic activity of Buforin 2, which is unable to permanently disrupt the cell membrane (Kobayashi *et al.*, 2004).

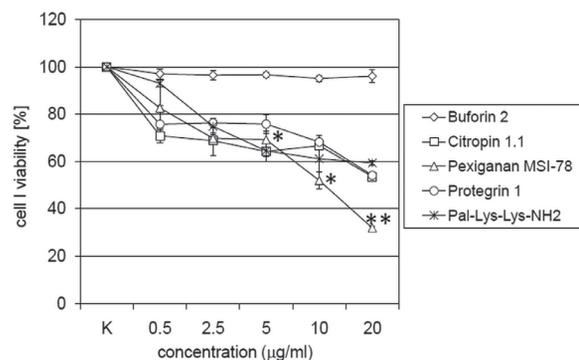


Figure 2. Dose-dependent cytotoxic effect of AMPs on U937 tumor cells AMPs were added to cell culture for 6 h, followed by MTT cytotoxicity assay. Data points represent averages of 6 independent MTT tests. The standard deviations are marked. * $P<0.05$; ** $P<0.01$.

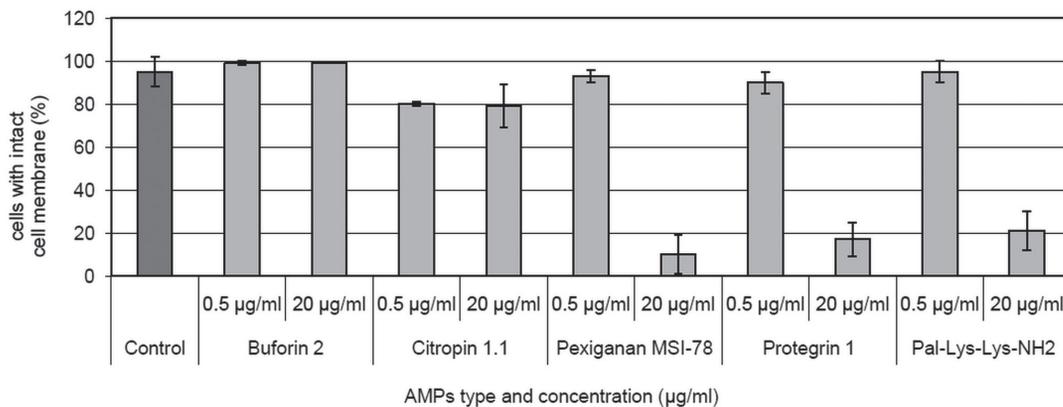


Figure 3. Dye uptake assay determining cell membrane integrity

Cell membrane integrity of U937 tumor cells after AMPs treatment for 6 h was checked using the trypan blue uptake assay. Low (0.5 µg/ml) and high (20 µg/ml) concentrations of AMPs were used. Data points represent averages of 3 independent tests. The standard deviations are marked.

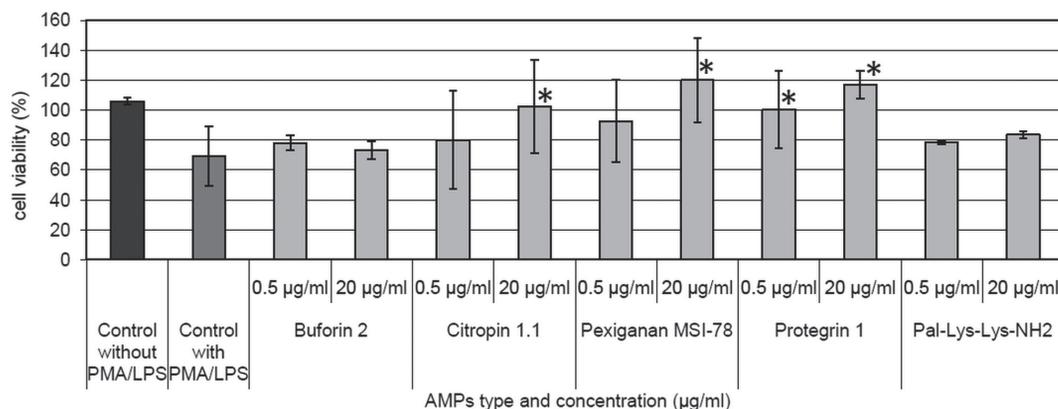


Figure 4. TNF-α concentration in supernatants of U937 cell culture treated with AMPs for 6 h in the presence of LPS and PMA.

Low (0.5 µg/ml) and high (20 µg/ml) concentrations of AMPs were used. TNF-α concentration in supernatants was measured as the cytotoxic activity against TNF-α-sensitive A9 cell line using a 17-h NR assay in the presence of CHX. Control supernatants were from cell cultures not treated with AMPs and treated/not treated with LPS and PMA. Data for A9 cells not treated with any supernatant were taken as representing 100% viability (not shown on graph). Data points represent averages of 4–6 independent NR tests. The standard deviations are marked. * $P < 0.05$.

Influence of chosen AMPs on cell membrane integrity of U937 cells *in vitro*

The best known mechanism of the action of AMPs is their membranolytic activity leading to necrosis. Microscopic observations have shown that cells seem to die of necrosis and apoptosis. We analyzed the mechanism of cell death using a dye uptake assay determining cell membrane integrity, which should be lost if cells die of necrosis. At the end of the 6-h cytotoxicity assay, the peptide-treated cells were stained with trypan blue — a dye that migrates into the cytoplasm of the cell only when the integrity of its membrane is compromised. This assay showed (Fig. 3) that the antitumor activity of Pal-Lys-Lys-NH₂, Pexiganan MSI-78 and Protegrin 1 could satisfactorily be explained by their membranolytic activity leading to necrosis. This effect was clearly dose-dependent. However, with Citropin 1.1, only a small percentage of the cells were dying of necrosis, and this level was similar both at high and low concentrations of the peptide. Consequently, in the case of Citropin 1.1., there should be an additional mechanism of action responsible for the antitumor activity of the peptide independent of its membranolytic activity and responsible for the dose-dependent increase in its cytotoxicity. This mechanism needs further clarification.

TNF-α release in supernatants of U937 cell line treated with AMPs

To elucidate further the mechanism of the AMPs antitumor activity we examined the complicated relationship among LPS, TNF-α and AMPs, as elevated levels of TNF-α are often induced by inflammatory response elicited by cancer (Esper & Harb, 2005), and some tumor cell lines, such as U937, can produce TNF-α upon stimulation with LPS and other immunomodulatory agents (Mander *et al.*, 1997).

First, we checked TNF-α production by the U937 cell line treated with AMPs at 0.5 or 20 µg/ml for 6 h. To determine of TNF-α concentrations in the cell culture supernatants we used the indicator cell line A9, mouse fibroblastic cells highly sensitive to the cytotoxic activity of TNF-α, especially in the presence of cycloheximide (CHX) — an inhibitor of protein synthesis. There was no sign of detectable TNF-α levels in the supernatants of the cells treated or not treated with AMPs (not shown). The AMPs alone did not affect the viability of the A9 cell line, either (not shown). We concluded that the AMPs had no stimulatory effect on TNF-α production by the U937 cell line without any additional stimulatory signal like that of LPS.

Next, we stimulated the U937 cells with LPS and PMA in the presence of the AMPs using an identical time frame and AMPs concentration. The U937 cells released detectable levels of TNF- α into cell culture supernatant under these conditions (Fig. 4, controls) and the TNF- α production was suppressed in the presence of Citropin 1.1, Pexiganan MSI-78 or Protegrin 1 (Fig. 4). This effect was small for the AMPs at 0.5 $\mu\text{g}/\text{ml}$, being statistically significant ($P=0.05$) for Protegrin 1 only. It became statistically significant for all three peptides at 20 $\mu\text{g}/\text{ml}$ ($P=0.05$). The highest activity in decreasing the level of TNF- α stimulated by LPS was shown by Protegrin 1, then by Pexiganan MSI-78, while the activity of Citropin 1.1 was substantially lower. The presence of Buforin 2 or Pal-Lys-Lys-NH₂ did not affect the capacity of LPS to stimulate production of TNF- α .

DISCUSSION

We examined the antitumor activity of a variety of antimicrobial peptides and its dependence on their structure and mechanism of action on one cell line, a human histiocytic lymphoma cell line U937, which is a frequently used model in studies of cytotoxicity (Sundstrom & Nilsson, 1976). This approach simplified the analysis as the peptide selectivity towards a specific target cell is not only dependent on a multitude of biophysical properties of the AMPs, but also on those of the target cell membranes (Reddy *et al.*, 2004).

It is thought that the amphipathicity of an AMP plays a pivotal role in its mechanism of action. The amphipathicity of a peptide depends, e.g., on its chain length, amino-acid sequence and the resultant net charge, and is enhanced upon formation of specific secondary structures such as α -helices, β -hairpins or extended polyproline-like helices and is, of course, modulated by their concentration, ability to oligomerize and form different structures in the cell membrane (Shai, 1999; Chen *et al.*, 2003; Papo & Shai, 2003). Our experiments showed that the antitumor activity was not determined by some specific molecular structure of the peptide, as the cytotoxicity levels of Citropin 1.1 and Temporin A — two linear, α -helical peptides of similar length (Simmaco *et al.*, 1996; Wegener *et al.*, 1999) were clearly different. Citropin 1.1 was highly cytotoxic to the U937 cell line, while Temporin A had almost no influence on its viability. Furthermore, Protegrin 1 containing a β -sheet as a dominant motif, showed similar cytotoxicity level as Citropin 1.1 at higher doses. This indicates that there is no simple link between the structure and the antitumor effect of those peptides. However, the highest cytotoxic activity of Pexiganan MSI-78 could be explained by its longer length of 22 amino acids (Jacob & Zasloff, 1994). Such peptides, consisting of more than 20 amino-acid residues can form α -helices of sufficient length to individually span a lipid membrane, thus enabling formation of different transmembrane structures in the target cell membrane than those formed by the shorter peptides. The transmembrane peptides can oligomerize into either 'barrel-stave' or toroidal pores, which work as non-selective channels disrupting the cell homeostasis and leading to cell death (Epanand & Vogel, 1999; Shai, 1999; Yang *et al.*, 2001). Shorter peptides are more likely to work *via* formation of a 'carpet' on the membrane surface, in which they aggregate on the surface until a critical concentration is reached, at which point they solubilise or lyse the membrane in a detergent-like manner (Oren & Shai,

1998; Epanand & Vogel, 1999; Shai, 1999). However, the length of the peptide is not the only critical factor, as shown by the low cytotoxic activity of Buforin 2 with its 21 amino-acid residues. It has been proposed that other factors, e.g., a peptide-lipid hydrophobic mismatch (de Planque *et al.*, 2003) and the peptide concentration relative to threshold values (Aisenbrey *et al.*, 2008) can play a role in the peptide cytotoxicity.

The effect on concentration was rather complex (Fig. 2) in the case of Citropin 1.1, Pexiganan MSI-78 and Protegrin 1, as above 5 $\mu\text{g}/\text{ml}$ the cytotoxicity of these peptides clearly increased, whereas at lower concentrations it was constant. Only for the lipopeptide Pal-Lys-Lys-NH₂ was its cytotoxicity almost linearly related to its concentration. We believe this simple relationship is due to the shortening of the peptide backbone and the presence of the palmitoyl residue (Kamysz *et al.*, 2007). The cytotoxicity of all the peptides, with the exception of Buforin 2, was significantly increased by extending the incubation time in the cytotoxicity assay, even at a low concentration (0.5 $\mu\text{g}/\text{ml}$) (not shown). This suggests a stronger dependence of the antitumor effect on the duration of the attack of the antimicrobial peptides on the tumor cells than on their concentration. This time factor and its lack of influence on the activity of Buforin 2, the only peptide used in this work that does not permanently disrupt the cell membrane (Kobayashi *et al.*, 2004), suggests that the antitumor activity of the peptides is mainly correlated with their ability to form pores in the cell membrane.

Our preliminary results obtained with the HL60 cell line derived from a human acute promyelocytic leukemia showed a similar, if slightly weaker, effect of the analyzed antimicrobial peptides, with Citropin 1.1, Pexiganan MSI-78 and Protegrin 1 affecting this cell line the most in the cytotoxicity assays. This suggests a universal link between the structure and cytotoxic effects of these peptides on various tumor cell lines.

We found that all the peptides which were highly cytotoxic to the U937 cell line disrupted the integrity of the cell membrane (Fig. 3). The best known mechanism of action of AMPs is their membranolytic activity leading to necrosis (Shai, 1999) and this mechanism can satisfactorily explain the antitumor activity of Pexiganan MSI-78, Protegrin 1 and Pal-Lys-Lys-NH₂. In the case of Pexiganan MSI-78 and Protegrin 1, even though they adopt different secondary structures, they disrupt cell membrane through the same mechanism — formation of toroidal pores (Mani *et al.*, 2006; Gottler *et al.*, 2009), in contrast to the other peptides used in this work (Table 1). Pal-Lys-Lys-NH₂ as a lipopeptide acts directly through simple disruption of membrane electric potential (Epanand & Vogel, 1999; Avrahami & Shai, 2004). However, in the case of Citropin 1.1, the situation is not so clear as the cell membrane integrity was disrupted only to a small extent and independently of the peptide concentration. Consequently, some other mechanism should be responsible for the strong and dose-dependent cytotoxic activity of this peptide, either a non-membranolytic one or another based on membranolytic activity leading to apoptosis (Mader & Hoskin, 2006; Mader *et al.*, 2007). The nature of this mechanism still needs to be elucidated.

Some peptides can induce non-membranolytic effects on target cells through binding to cellular receptors at low concentrations, activation of intracellular signalling pathways and stimulation of a variety of cellular functions (Koczulla & Bals, 2003). For instance, some of the

peptides, such as the neutrophil-derived heparin-binding protein (HBP), can stimulate the production of TNF- α by isolated monocytes (Heinzelmann *et al.*, 1998) even without additional stimulation by, e.g., LPS. TNF- α is quite often induced in cancer patients because of the inflammatory response elicited by cancer leading to cachexia (Esper & Harb, 2005). For this reason, we also decided to learn how our antibacterial peptides with the highest antitumor activity (and Buforin 2 as the least active one) affected TNF- α production. It turned out that the AMPs analyzed had no direct stimulatory effect on TNF- α production by the U937 cell line.

Moreover, we wanted to learn if they could affect the production of TNF- α induced in U937 cells by LPS and PMA stimulation. Infections are major causes of morbidity and mortality in patients with cancer (Smiley *et al.*, 2005) and the presence of LPS can increase proliferation of some tumor cells, as well as increase their invasive potential (Harney *et al.*, 2002; Takabayashi *et al.*, 2002). The cell line we used, the human histiocytic lymphoma cell line U937, is a frequently used model in studies of TNF- α -mediated cytotoxicity and can generate TNF- α upon stimulation with LPS and PMA (Sundstrom & Nilsson, 1976; Mander *et al.*, 1997). Some AMPs can interact with LPS by strongly binding to LPS aggregates causing their dissociation, preventing LPS from binding to the carrier lipopolysaccharide-binding protein or to its CD14 receptor, thus reducing TNF- α production by the cells (Rosenfeld *et al.*, 2006). Some of our peptides indeed suppressed the level of TNF- α in cell culture supernatants of LPS-stimulated U937 cells, with a statistical significance for Protegrin 1 and a strong tendency for Citropin 1.1 and Pexiganan MSI-78 at low concentration (0.5 $\mu\text{g}/\text{ml}$), and in a statistically significant manner for all three at the higher concentration of 20 $\mu\text{g}/\text{ml}$. Pal-Lys-Lys-NH₂ did not affect the ability of LPS to stimulate production of TNF- α . This suggests that a longer peptide backbone is needed. A similar lack of effect also for Buforin 2 seems additionally to suggest that the ability of the peptides to form stable pores, and especially toroidal ones, like those of Protegrin 1 (Mani *et al.*, 2006; Gottler *et al.*, 2008), is in some way involved. One explanation could be the ability of such peptides to form strong aggregates with LPS, resulting in the reduction of TNF- α production due to the LPS stimulation. Further studies are needed to answer the question through what molecular mechanism the peptides influence TNF- α production due to LPS stimulation.

As AMPs begin showing their potential as highly selective antitumor drugs, even against diseases resistant to chemotherapy (Dubin *et al.*, 2005; Mader & Hoskin, 2006), this potential should be taken advantage of to solve the main problems in conventional anti-cancer chemotherapy: toxic side-effects, low response of slow-growing tumors and dormant cells (Naumov *et al.*, 2003) and the development of multi-drug resistance by tumor cells (Gottesman, 2002) even against anti-angiogenic drugs (Ferrara & Kerbel, 2005). Understanding the relationships between AMPs structure and antitumor activity would be helpful in the development of synthetic peptides with the highest antitumor activity and selectivity and low side-effects, e.g., haemolysis. Our work showed that the cytotoxic activity of the analyzed peptides was not determined by some specific molecular structure, but was rather dependent on the length of the peptide chain, which is likely to affect the efficiency of the tumor cell membrane disruption and interaction with LPS. Therefore, some relationships between the structure and anti-

tumor activity of antimicrobial peptides are likely to exist and deserve further elucidation.

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