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Review

Novel properties of antimicrobial peptides

Wojciech Kamysz[1]✉, Marcin Okrój[2] and Jerzy Łukasiak[1]

[1]*Department of Physical Chemistry, Faculty of Pharmacy, Medical University of Gdańsk, Gdańsk, Poland,* [2]*Division of Cell Biology, Department of Histology and Immunology, Intercollegiate Faculty of Biotechnology, Medical University of Gdańsk, Gdańsk, Poland*

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Endogenous peptide antibiotics are known as evolutionarily old components of innate immunity. Due to interaction with cell membrane these peptides cause permeabilization of the membrane and lysis of invading microbes. However, some studies proved that antimicrobial peptides are universal multifunctional molecules and their functions extend far beyond simple antibiotics. In this review we present an overview of the general mechanism of action of antimicrobial peptides and discuss some of their additional properties, like antitumour activity, mitogenic activity, role in signal transduction pathways and adaptive immune response.

The innate immune system is the first barrier which invading microbes have to pass when entering the organism. This barrier consists of elements like epithelial continuity, mucous gland secretion, cilia movement, low pH in the gastrointestinal tract and skin surface, the presence of phagocytes, non-specific humoral factors, alternatively activated complement cascade, etc. Antimicrobial peptides are one of the most important elements of the innate immune system. These peptides are ubiquitous among all eukaryotes, including mammals, amphibians, insects, plants and protozoa (Gabay, 1994). Consisting no more than a dozen amino acids, rapidly produced and diffusable they seem ideal for fast and efficient defense against microbes (Nissen-Meyer & Nes, 1997). Their usefulness is also evident from their persistence throughout evolution. In mammals, epithelial cells in re-

✉Corresponding author: tel.: (48 58) 349 3152, fax: (48 58) 349 3206, e-mail: kamysz@amg.gda.pl

Abbreviations: EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; HNP, human neutrophil protein (defensin); HSV, human simplex virus; huPBL-SCID, human peripheral blood lymphocyte, severe combined immunodeficiency; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; TNF, tumour necrosis factor.

spiratory, gastrointestinal and genitourinary tract massively produce antimicrobial peptides, as do phagocytes and specialised cells in mucous glands (Lehrer & Ganz, 1999). So far, over 800 antimicrobial peptides have been isolated and described (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). The origin of a particular peptide is not a good criterion for classification because peptides derived from evolutionarily distant animals appear to share certain chemical features. Presently, the accepted classification distinguishes peptides with a high content of a particular amino acid, often Pro, Trp, Arg, His (prophenins, indolicidins, histatins), peptides with a predominantly β-sheet structure and intramolecular disulphide bonds (including defensins and protegrins), and α-helical peptides with an amphiphilic region (magainins, cecropins) (Lehrer & Ganz, 1999). Other classes of peptides were distinguished by Gallo (Gallo & Huttner, 1998), namely, loop peptides with a single disulphide bond, fragment peptides, and anionic, surfactant associated peptides. The primary structures of selected, naturally occurring antimicrobial peptides are presented in Table 1.

Table 1. The amino-acid sequences of some natural antimicrobial peptides

Peptide	Sequence of amino acids
Buforin 2	TRSSRAGLQFPVGRVHRLLRK
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
Defensin HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
Histatin 5	DSHAKRHHGYKRKFHEKHSHRGY
Indolicidin	ILPWKWPWWPWRR-NH_2
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
Protegrin PG-1	RGGRLCYCRRRFCVCVGGR-NH_2
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP

Almost all antimicrobial peptides are cationic or amphiphilic and this feature determines the mode of their antimicrobial action. Cationic parts of the peptides are capable of interacting with negatively charged structures of the microbial membrane and finally lead to its permeabilization. Four main mechanisms of membrane permeabilization have been described: barrel-stave model, wormhole model, carpet-like model and selective ion channel formation (Ganz & Lehrer, 1999; Chmiel, 2001). Cationic peptides exhibit a high affinity for lipopolysaccharides (LPS), which is the main component of the outer membrane of Gram-negative bacteria. Such peptides completely displace LPS-bound divalent cations stabilising the structure of the membrane (Hancock & Chapple, 1999). LPS-binding capacity of antimicrobial peptides is of great clinical advantage compared to classical antibiotics, because it prevents endotoxemia (Gough *et al.*, 1996). Uncontrolled systemic LPS release during antibiotic induced massive bacteria lysis induces proinflammatory cytokine production and finally leads to septic shock, often with fatal consequences. Selectivity of antimicrobial peptides stems from differences in membrane composition of higher eukaryotes and microbes. The outer membrane of higher eukaryotes is made of electrically neutral phospholipids like phosphatidylcholine and sphingomyelin, whereas bacterial membranes have exposed negatively charged phosphatidylglycerol and cardiolipin (Matsuzaki, 1999). Another difference is the lack of cholesterol in bacterial membranes. These parame-

ters were found to be important for selectivity of antimicrobial peptides (Andreu & Rivas, 1998). Some peptides exert their final effect, i.e., killing bacteria, without permeabilization of the membrane. They penetrate cells and interact with some physiological processes. For example, attacin inhibits the synthesis of a specific membrane protein (Carlsson *et al.*, 1998). PR-39 arrests DNA synthesis (Boman *et al.,* 1993) and defensins are able to break single strand DNA (Bateman *et al.*, 1991). Antimicrobial peptides also counteract fungal infections, especially those caused by *Candida* sp. (Nibbering *et al.,* 2002). Defensins and histatins kill fungi by nonlytic release of cellular ATP which subsequently binds to putative purinoergic receptor and activates cytotoxic pathways (Edgerton *et al.*, 2000). Defensins and synthetic analogues of magainin were shown to inhibit infection capability of some viruses (Daher *et al.*, 1986; Aboudy *et al.*, 1994). This effect is caused by the binding of peptides to the viral envelope since analogous treatment of nonenveloped viruses did not affect their infective potential. The properties of the antimicrobial peptides mentioned above show, that they are evolutionarily conserved, ubiquitous, simple and effective factors acting within the innate immune system. Moreover, because of their specificity and safety they seem to be suitable for medical use. Several peptides and their derivatives have already passed clinical trials successfully (Hancock & Chapple, 1999; Levy, 2000) and several others are considered to be potential therapeutics (van't Hoff *et al.,* 2001). Unexpectedly, antimicrobial peptides turned out to possess additional biological activities than those affecting bacteria, fungi or viruses.

ANTITUMOUR ACTIVITY

As noted above, the fundamental activity of antimicrobial peptides stems from their ability to interact with negatively charged molecules in a target membrane. Tumour cells can differ in membrane composition from nontransformed cells. In 1991 Utsugi *et al.* reported 3–7-fold higher phosphatidylserine content in the membrane of melanoma and carcinoma cells in comparison to that in normal human keratinocytes. Such differences can result in higher susceptibility of tumour cells to membrane-permeabilizing peptides. Magainins represent a group of cationic peptides isolated from the skin of amphibians which are able to lyse many types of tumour cells at a concentration 5–10-fold lower than that for normal cell lysis (Jacob & Zasloff, 1994). Baker *et al.* (1993) showed that magainin 2 and magainins' synthetic analogues exerted their antitumour activity towards murine ascites tumours, leukaemia, and spontaneous ovarian tumour cells, both *in vitro* and *in vivo*. All D-amino acid analogues of magainin were found to be as effective as doxorubicin towards ovarian tumour. Cecropins insect derived peptides are also able to lyse tumour cells. *In vitro* experiments proved the effectiveness of even in multidrug-resistant cell lines, where they destroyed within one hour tumour cells (Moore *et al.*, 1994). Defensin-peptides common for human granulocytes, induce tumour cell lysis in the concentration dependent manner, the effect being observed after 3 h, with a plateau between 8–14 h. *In vitro* incubation of murine teratocarcinoma cell swith defensins reduced their oncogeneticy *in vivo* (Lichtenstein *et al.*, 1986). Lytic activity of defensins was inhibited by serum. Peptide neutralisation and degradation in extracellular compartments can pose a real problem in their clinical use. There are several ways of coping with degradation. Replacement of amino acids by their D isomers in peptide sequence or modification of peptide terminal, e.g., by amidation, can prevent degradation and increase the compound's tumoricidal activity (Moore *et al.*, 1994). Loss of activity may be avoided by introduction of the gene coding for antimicrobial peptide directly into the tumour cell. Overexpression of cecropin and metillin

DNA decreased cell tumourgenicity. Tumour growth was either reduced or totally inhibited when these transformed cells were injected into nude mice (Winder *et al.*, 1998). Expression of antimicrobial peptides has been reported in several tumour cell lines where depending on their concentrations, they exert mitogenic or necrotic activity (Muller *et al.*, 2002).

ANTIMICROBIAL PEPTIDES AS MITOGENS

Initial evidence that antimicrobial peptides could have mitogenic properties was provided by Murphy *et al.* (1993). In concentration range required for antimicrobial activity, defensins stimulated growth of fibroblasts and epithelial cells *in vitro*. In this case effective concentration of defensins was comparable to the concentration expected during wound healing *in vivo*. These data suggest that defensins may perform a double role in skin injuries. Besides preventing microbial infection, they can also induce a wound healing process. Similar results were obtained by Aarbiou *et al.* (2002) for lung epithelial cells. Defensins at a concentration not exceeding 10 μg/ml enhanced proliferation of A549 lung epithelial cells. The stimulatory signal was found to be mediated by the MAP kinase pathway and independent of the EGF-receptor. The presence and expression of antimicrobial peptides did not seem to be strictly restricted to certain cells or structures, as exemplified by granulocytes and epithelial cells in mammals. The presence of human α-defensins was reported in normal kidney, where they were probably released by neutrophils. Moreover, biopsies of renal carcinoma tissue revealed the intracellular expression of these defensins and renal carcinoma cell lines studied *in vitro* demonstrated that defensins were present at a concentration not exceeding 25 μg/ml stimulated DNA synthesis (Muller *et al.*, 2002). This may suggest a possible role of α-defensins in proliferation of either normal or tumour cells in kidney.

ANTIMICROBIAL PEPTIDES AS SIGNALLING MOLECULES

Another aspect of antimicrobial peptides activity is their possible role in modulation of signal transduction. At least four peptides were reported to influence the activity of kinases. Human defensins (HNP-1, HNP-2, HNP-3) are potent inhibitors of protein kinase C (PKC), whereas they have little or no effect on the activity of myosin light chain kinase and protein kinase A (Charp *et al.*, 1988). The specificity of kinase inhibition can give rise to the theory that defensins are involved in particular signalling pathways. PKC activity can be upregulated by another neutrophil granule-derived peptide, CAP37 (Pereira *et al.*, 1996). Potentiated PKC activity was observed in endothelial cells and such a result can correspond to the *in vivo* situation, when neutrophils adhere to endothelium during the inflammatory process. PR-39 is a proline and arginine-rich peptide which can bind to the adapter protein p130 (Cas) (Tanaka *et al.*, 2001), involved in various cellular processes including cell adhesion, migration and transformation (Kirsch *et al.*, 2002). Moreover, it binds a subunit of phosphoinositide 3-kinase (PI3-kinase) and inhibits kinase activity (Tanaka *et al.*, 2001). The *PR-39* gene transfected cells showed a reorganisation of actin structure and suppression of cell proliferation. These cells were also characterised by decreased activity of c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAP), and decreased expression of cyclin D. The molecule, which was first discovered and described as the EGF receptor kinase inhibitor appeared to have also antimicrobial properties towards different species of Gram-positive and Gram-negative bacteria (Pogrebnoy *et al.*, 1998). The epidermoid carcinoma-derived antimicrobial peptide (ECAP) is respon-

sible for inhibition of EGFR autophosphorylation and decreased activity of non-receptor kinases like Lyn and Syk (Hobta *et al.*, 2001). These properties of ECAP are achieved at a concentration at least 10-fold lower than needed for antimicrobial and cytotoxic action. Such results may suggest that protein kinases are the physiological targets for antimicrobial peptides. Another fact suggesting a possible role of antimicrobial peptides in the modulation of signalling pathways is the identification of defensins in lymphocyte nuclei (Blomqvist *et al.*, 1999). The possibility of defensins being involved in regulation of transcription machinery as the last step of signal transduction should be investigated.

ANTIMICROBIAL PEPTIDES LINK INNATE TO ADAPTIVE IMMUNITY

Antimicrobial peptides, due to their membrane-permeabilizing properties towards a wide spectrum of microbes, are classified as effector molecules of innate immunity. Besides direct antimicrobial properties, these peptides are able to modulate other components of innate immunity. Defensins and PR-39 act as chemoattractants for monocytes and neutrophils, respectively (Territo *et al.*, 1989; Huang *et al.*, 1997). Since neutrophils and monocytes are phagocytes, such action enhances the innate immunity. Moreover, defensins influence the production of several cytokines. In monocytes, TNF and IL-1 expression is stimulated by defensins (Chaly *et al.*, 2000), while in lung epithelial cells defensins enhance the expression of IL-8 (van Wetering *et al.*, 1989). In 1996 Chertov *et al.* observed that subcutaneous injection of defensins into chimeric huPBL-SCID mice causes infiltration of CD3+ cells. This indicated that defensins could exert chemotaxic activity not only towards nonspecific phagocytes but also towards cells engaged in adaptive immune response. The role of defensins in adaptive immunity was elucidated by Yang and coworkers. First, they proved that defensins can bind to CCR6 chemokine receptor and displace other CCR6 ligands in a competitive manner. The cells transfected with human CCR6 exhibited a chemotactic response to defensins (Yang *et al.*, 1999). Later, defensins were shown to induce migration of human naive T cells and immature dendritic cells (Yang *et al.*, 2000). Chemotactic activity of defensins is generally observed at concentrations that are not bactericidal. This provides evidence of an additional function of these peptides, like recruiting immune cells to the sites of microbial infection. Defensins also act as adjuvants (Tani *et al.*, 2000). Mice, when immunized and treated with defensins, increase the production of antigen-specific IgG antibodies. At a molecular level, production of IFN-γ and IL-4 was increased. Thus, defensins enhance the antigen-specific immune response by inducing the production of lymphokines, which promote the adaptive immune response.

SUMMARY

Initially antimicrobial peptides were described as potent agents able to permeabilize the membranes of microbes and, subsequently, to inhibit their growth. Nowadays, these peptides are known as multifunctional molecules. Besides their antimicrobial activity, several other functions have been proposed and investigated (Table 2), i.e., influence on the expression of adhesion molecules (Chaly *et al.*, 2000), adrenocorticoids production (Fuse *et al.* 1993), chloride ions secretion (Lencer *et al.*, 1997), angiogenesis and wound repair (Gennaro *et al.*, 2002), and a role in fertilization (Garcia *et al.*, 2001). Since some of these functions are of great importance one can wonder, as to the primary role of antimicrobial peptides. The type of biological activity of antimicrobial peptides often depends on their concentration, e.g., at a lower

Table 2. Overview of the additional activity of antimicrobial peptides

Kind of activity	Peptide / references
inhibition of membrane protein synthesis	attacin (Carlsson *et al.*, 1998)
inhibition of DNA synthesis	PR-39 (Boman *et al.*, 1993)
	magainins (Jacob & Zasloff, 1994)
antitumour activity	defensins (Lichtenstein *et al.*, 1986)
	cecropins (Moore *et al.*, 1994)
stimulation of cell proliferation	defensins (Murphy *et al.*, 1993)
interference with signalling pathways	defensins (Charp *et al.*, 1988)
	CAP 37 (Pereira *et al.*, 1996)
	PR-39 (Tanaka *et al.*, 2001)
	ECAP (Hobta *et al.*, 2001)
chemoattractant for immune cells	defensins (Territo *et al.*, 1989)
	PR-39 (Huang *et al.*, 1997)
stimulation of cytokine expression	defensins (Chaly *et al.*, 2000)
	(van Wetering *et al.*, 1989)
stimulation of adhesion molecule expression	defensins (Chaly *et al.*, 2000)
angiogenesis	proline-rich peptides (Gennaro *et al.*, 2002)
chloride secretion	cryptidins (Lencer *et al.*, 1997)

concentration they may influence signal transduction or proliferation and at a higher one cause cell lysis (Fig. 1). Perhaps this universal character of the peptides contributed to their conservation throughout evolution. Some antimicrobial peptides are potential candidates for clinical applications and therefore the recognition of every aspect of their biological activity can be of extreme importance.

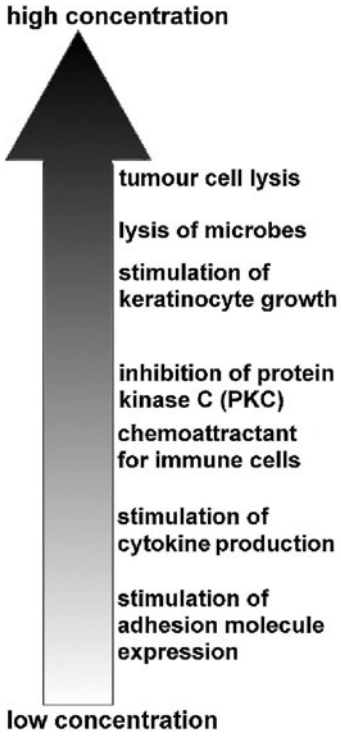

Figure 1. The spectrum of biological activity of defensins.

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