Lecture

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Are antimicrobial peptides an alternative for conventional antibiotics?

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Abstract

Antimicrobial peptides are widespread in living organisms and constitute an important component of innate immunity to microbial infections. By the early 1980s, more than 800 different antimicrobial peptides had been isolated from mammals, amphibians, fish, insects, plants and bacterial species. In humans, they are produced by granulocytes, macrophages and most epithelial and endothelial cells. Newly discovered antibiotics have antibacterial, antifungal, antiviral and even antiprotozoal activity. Occasionally, a single antibiotic may have a very wide spectrum of activity and may show activity towards various kinds of microorganisms. Although antimicrobial activity is the most typical function of peptides, they are also characterized by numerous other properties. They stimulate the immune system, have anti-neoplastic properties and participate in cell signalling and proliferation regulation. As antimicrobial peptides from higher eukaryotes differ structurally from conventional antibiotics produced by bacteria and fungi, they offer novel templates for pharmaceutical compounds, which could be used effectively against the increasing number of resistant microbes.

Key words: antimicrobial peptides, peptide antibiotics

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Introduction

One of the major difficulties modern medicine has to overcome is controlling microorganisms resistant to conventional antibiotics and dealing with the increasing number of new infections [1]. It should also be mentioned that diseases caused by microorganisms are the most significant etiologic factor to cause death worldwide (after cardiovascular disorders). Nowadays, a dangerous recurrence of infectious diseases can be observed in a number of countries, and the World Health Organization (WHO) has classified these diseases as the main menace to human beings. Therefore, an urgent need for new substances with antimicrobial properties still exists.

Antimicrobial peptides are ancient and essentially small cationic molecules of the host defence system. They are found in a great variety of species [2]. By the early 1980's, more than 800 different antimicrobial peptides were isolated from mammals, amphibians, insects and plants [3]. Since numerous antibiotic peptides possess a strong in vitro activity against microorganisms, which are resistant to conventional antibiotics, they provide attractive templates for the design of new antimicrobial agents for specific application. Various new substances are undergoing clinical trials. In the future, they may replace the drugs which have been used in medicine for many years.

Peptide antibiotics in medicine

Peptide antibiotics are widespread in nature and belong to the most significant elements of the immune system of *Prokaryota* and *Eukaryota*. Conventional antibiotics currently used in medicine are produced non-ribosomally in microbes by multienzymatic cellular systems or within various extraribosomal processes. Synthesized peptides gain their microbiological activity within the post-translatory treatment.

Antimicrobial peptides constitute a large group of known chemotherapeutics. However, because of their high toxicity and high cost of production only a small quantity of them has been used in medicine. These drugs constitute a diverse group of chemical substances. The peptide chain is mainly composed of L-amino

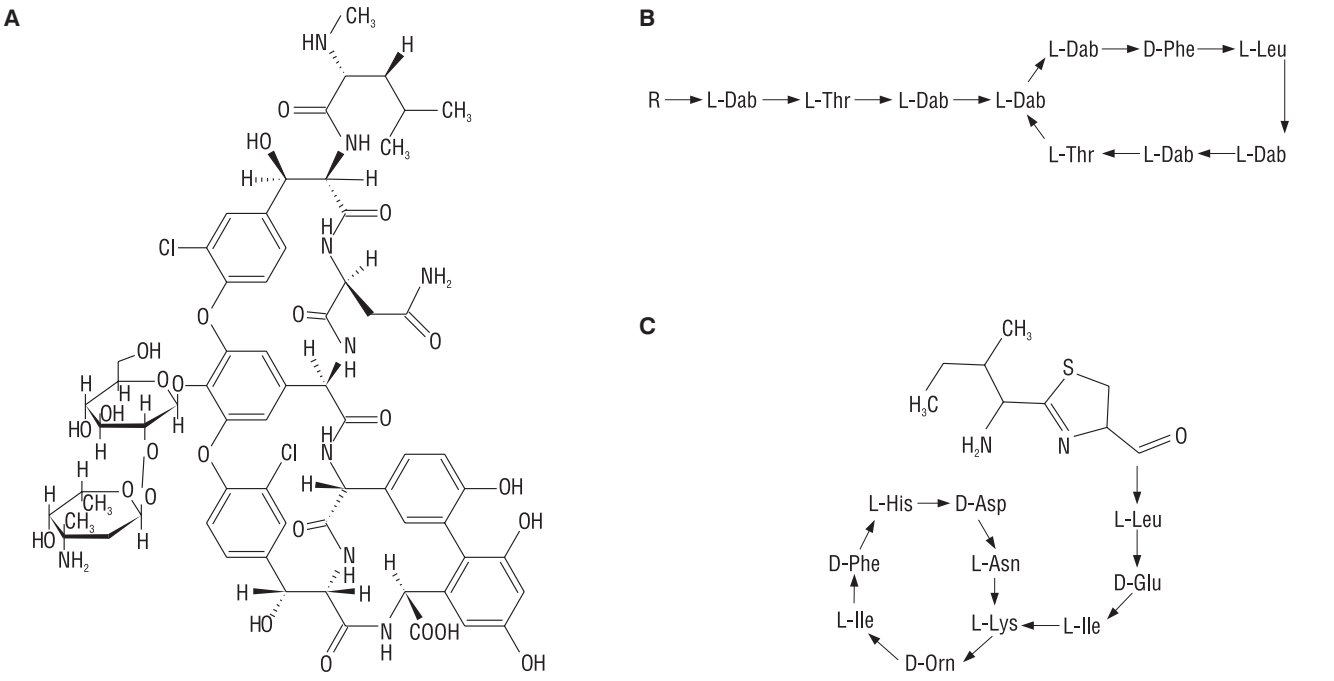

Figure 1. Structures of conventional peptide antibiotics: **A.** — vancomycin; **B** — polymyxin B, Dab — α, γ — diaminobutyric acid **C.** — bacitracin A.

acids or D-amino acids. Apart from the basic peptide skeleton, these antimicrobials contain nonprotein parts such as sugar fragments and fatty acid residues (Figure 1).

Bacitracin, vancomycin, and the polymyxins are relatively toxic drugs and have only a limited use in chemotherapy. Their modes of action differ: bacitracin and vancomycin affect cell wall synthesis whereas the polymyxins affect the cell membrane. Bacitracin and vancomycin are used for the treatment of infections caused by gram-positive bacteria; the polymyxins are used for treating gram-negative infections and are active against *Pseudomonas aeruginosa*.

The continuously rising resistance of microorganisms to the majority of drugs (including conventional peptide antibiotics) is the main cause of the constant search for new, more effective antimicrobial substances.

Bacteriocins — antimicrobial peptides from microorganisms

Bacteriocins are bacterial products and they show antimicrobial activity against other microorganisms [4]. They are usually produced by Gram-positive bacteria. Bacteriocins are peptides secreted by cells to inhibit or kill closely related species. They are divided into two basic types [5]. The first group comprises peptides which have been subjected to post-translatory treatment (modified bacteriocins - lantibiotics). The second group includes unmodified bacteriocins. Furthermore, bacteriocins comprise colicins and microcins, i.e. peptides produced by Gram-negative bacteria (e.g. *Escherichia coli*) [6].

Lantibiotics constitute the most popular group of bacteriocins. Their name refers to the occurrence of the unnatural amino acids of lanthionine or methyl lanthionine. Apart from this dehydroamino acid and other unnatural fragments, thioether bonds are present in the structures of these antimicrobial peptides. Lanthionine and methyl lanthionine residues have strong electrophilic centres, which can react with nucleophilic groups present in bacterial DNA or can inhibit the activity of certain enzymatic systems. These compounds are extremely important due to their potential biotechnological application. They may be used as biopreservatives of food or antibiotics. The most popular and often characterized is nisin, which is used as a biopreservative for dairy products (Figure 2) [7]. Nisin is bactericidal against gram-positive bacteria such as Clostridium. Moreover, it inhibits endospore germination and it has recently been proven to kill gram-negative bacteria Salmonella. The major obstacle in the use of lantibiotics is the high cost of production. Final purification is particularly troublesome.

Endogenous peptide antibiotics

Over the last 25 years, numerous antimicrobial peptides isolated from all living nature have been described (Figure 3) [3]. These new antibiotics are gene encoded peptides and they play a significant role in the innate immunity of all organisms. So far, over 800 different antimicrobial peptides have been isolated and described. More information concerning these substances is available on the websites [8, 9].

Historical background

The first reports concerning natural antimicrobials produced by higher organisms appeared 40 years ago. In the 1960s, 24-peptide bombinin was isolated [10]. This substance with hemolytic and antibiotic peptides was purified from the secretion of the skin

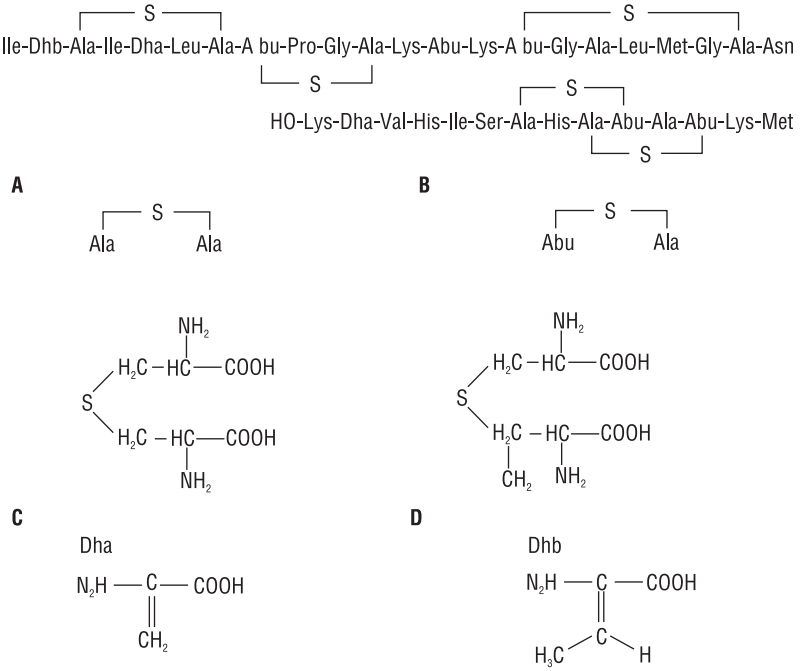

Figure 2. Structures of nisin and modified (unnatural) amino acid residues: **A.** — lanthionine; **B.** — methyl-lanthionine; **C.** — didehydroalanine; **D.** — didehydrobutyrine. Abu — aminobutyric acid.

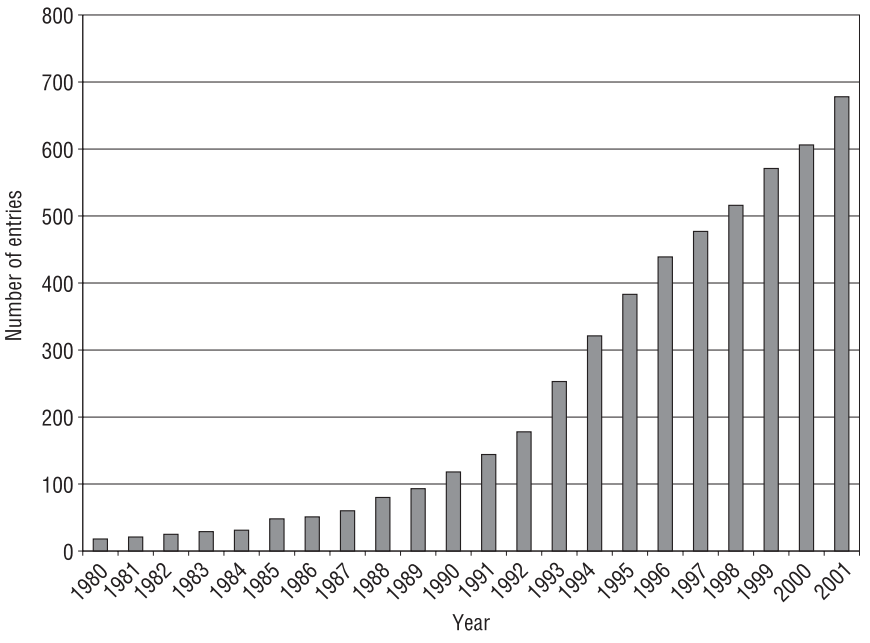

Figure 3. Number of antimicrobial peptides sequences inserted per year to AMSDb database [3].

of the frog *Bombina variegata* [10]. Unfortunately, hemolytic activity limited the scope of research on this antibiotic.

Ten years later, Habermann isolated melittin from bee venom [11]. This substance is currently being investigated by scientists who are looking for effective antimicrobials [12]. As in the case of bombinin, hemolytic activities limit the application of this peptide.

The research of Hans Boman was a milestone in the search for new antimicrobial peptides. In 1981, he isolated cecropins from the haemolymph of pupae of the cecropia month [13]. Cecropins are a family of 3–4 kDa linear amphipathic peptides. They constitute a main part of cell-free immunity of insects.

The research of Michael Zasloff is also of great importance. He isolated magainins, two linear peptides with a wide spectrum of activity, from the skin of *Xenopus laevis* [14]. Magainins are two

closely related peptides. Both of them are 23 amino acids and they differ only by two substitutions. At low concentration, they inhibit the growth of various species of bacteria and fungi and induce osmotic lysis of protozoa. A great the number of new publications is still rising.

Sources of antimicrobial peptides

The last 25 years turned out to be extremely fruitful as regards antimicrobial peptides and their function in living organisms. Antimicrobial peptides have been isolated from insects [16], amphibians [17], birds [18], fish [19] and mammals [20] and they constitute a significant part of the immune system of these creatures. Peptides are secreted by bone marrow derivatives (macrophages, granulocytes), most epithelial cells (keratinocites), Paneth cells of the small intestine, vaginal epithelium, airway epithelium, oral cavity epithelium and dermal glands in frog.

Insect peptides are one of the largest groups of known antibiotics. A single insect produces approximately 10–15 peptide antibiotics, each peptide exhibiting a completely different spectrum of activity [21]. Antimicrobial peptides can be detected in insect haemolymph as early as 2–4 after a septic injury [22]. The peptides are secreted directly to haemolymph (functional equivalent of blood) and are fast and effective protection against invading microorganisms.

Amphibian peptides are a large group of substances, mainly of linear and uncomplicated structure [17]. The majority of these substances is hydrophobic, cationic and forms an amphipathic α-helix in nature. These molecules are produced and stored in dermal structures called granular glands, which release their content onto the skin of a frog, upon adrenergic stimulation or injury. Other cationic peptides are expressed in the cells of gastric mucosa and in the intestinal tract. The best-known peptides isolated from frogs are brevinins, esculentins, magainins, ranatuerins and temporins [17].

The major classes of mammalian antimicrobial peptides are defensins and cathelicidins. Defensins are arginine-rich, amphiphilic b-sheet peptides containing 29–43 amino acid residues [23]. Six amino acids in the structure of defensins are cysteins linked by intracellular disulfide bonds. Depending on the concentration, defensins show antimicrobial activity against the most popular microorganisms and cause tumour cell lysis. At low concentration, they stimulate keratinocyte growth, cytokine production and adhesion molecule expression [24]. Defensins found in mammals are grouped into two main classes: a-defensins and b-defensins. Alpha-defensins are found in azurophil granules of neutrophils [25], macrophages and Paneth cells of the intestine [26]. Beta-defensins are found in neutrophils [27], respiratory tracks of cattle [28] and leukocytes of chickens [29].

Cathelicidins are a diverse group of antimicrobials, differing greatly in sequence, structure and the number of residues [30]. They are cationic and amphipathic molecules, which inhibit microbial function by targeting microbial membranes. In addition, cathelicidins interact with host pattern recognition receptors to stimulate cellular immune defence. Cathelicidins are expressed in various specific types of cells, including different epithelial surfaces. The peptides also appear to have a wide range of antimicrobial activity although they may be under-expressed in cystic fibrosis airways [31]. The development of topically administered antimicrobial peptides may have a significant role in the treatment of cystic fibrosis in the future. Cathelicidins have a wide spectrum of antimicrobial properties. Some of them exhibit endotoxin binding activity [32]. The most popular peptides belonging to this group are protegrins [33], bactenecins [34] and indolicidin [35].

Biosynthesis

Endogenous antimicrobial peptides are encoded in the genome as prepropeptides, with a classical N-terminal signal peptide targeting intracellular storage or extracellular release [36]. Because of their cationic structure (residues of Lys and Arg), antimicrobial peptides are toxic for intracellular organelles. The anionically charged prosegment neutralizes the cationicity (inhibiting the activity of the mature peptide) and may be responsible for intracellular trafficking and correct folding of the C-terminus as well. The fully functional antimicrobial compound is released by elastase-mediated cleavage [37].

Classes of antimicrobial peptides

In the past, the origin of antimicrobial peptides was the basis for their classification. This type of classification helped to make connections between the functions of the antimicrobial peptides originating from a similar group of animals and aspects of living conditions of the animals. However, the later discovery of a large number of peptides from many different animal species and the possession of a group of antimicrobial peptides, such as cecropins, by distantly related animal groups, undermined this type of classification. Today the grouping approach, based on the chemical and biochemical characteristics of peptides, is preferred. The solution structures of many peptides have recently been solved by NMR (Figure 4).

The present grouping combines sequence homologies, three-dimensional structures and functional similarities.

According to this classification, antimicrobial peptides can be divided into 5 main classes (Table 1):

1. Linear, mostly α-helical peptides without cysteine residue, with or without hinge region (bombinins, cecropins, magainins).
2. Antimicrobial peptides with one disulfide bond that form a loop structure with a tail (bactenecins, esculentins).
3. Antimicrobial peptides with two or more disulfide bonds giving mainly or only β-sheet structure (defensins, protegrins).
4. Linear peptides without cysteine residue and with an unusual composition of regular amino acids (histatins, indolicidin, temporins).
5. Antimicrobial peptides derived from larger peptides or proteins with other known functions (lactoferricins, MUC7).

Despite differences in structure, all the peptides studied display a similar motif: an amphiphilic structure, with one surface being highly positive and the other hydrophobic.

Mechanism of action

The precise mechanism of the action of antimicrobial peptides is yet to be explained. Generally, antimicrobial peptides disrupt the membranes of a target cell, causing lysis of the cell [45]. The knowledge of how it occurs and of the factors determining the activity and selectivity of these peptides is very limited. To

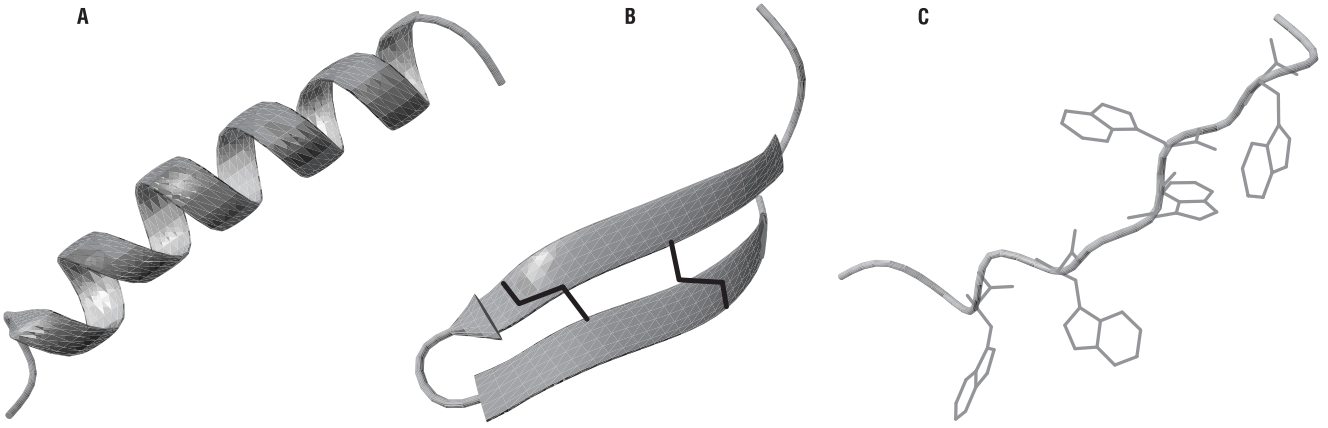

Figure 4. Structural features of some antimicrobial peptides: **A** — Magainin 2; **B.** — Protegrin 1; **C.** — Indolicidin. Figure prepared with MOLMOL [38].

Table 1. Major antimicrobial peptide classes and their representatives (G+ — gram-positive bacteria, G– — gram-negative bacteria)

Peptide	Sequence	Source of isolation	Spectrum of activity	References
α-helical peptides				
Bombinin	GIGALSAKGALKGLAKGLAEHFAN	Yellow-bellied toad	G+, G–, mammalian cells	[10]
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	Pig	G+, G–	[13]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	African clawed frog	G+, G–, fungi, cancer cells	[14]
Antimicrobial peptides with one disulfide bond				
Bactenecin	RLCRIVVIRVCR	Bovine neutrophils	G+, G–	[34]
Esculentin 2A	GILSLVKGVAKLAGKGLAKEGGKFGLELIACKIAKQC	Edible frog	G+, G–, mammalian cells	[39]
β-sheet peptides				
Defensin HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	Human	G+, G–, viruses, fungi	[40]
Protegrin 1	RGGRLCYCRRRFCVCVGR-NH_2	Pig	G+	[33]
Peptides with unusual composition				
Histatin 3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN	Human	G+, G–, fungi	[41]
Indolicidin	ILPWKWPWWPWRR-NH_2	Bos taurus	G+, G–, viruses, fungi	[35]
Temporin A	FLPLIGRVLSGIL-NH_2	European common frog	G+	[42]
Antimicrobial peptides derived from larger peptides or protein				
Lactoferricin B	FKCRRWQWRMKKLGAPSITCVRRAF	Bovine Cancer cells	G+, G–, viruses, fungi,	[43]
MUC7	LAHQKPFIRKSYKCLHKRCR	Human	G+, G–, fungi	[44]

understand the mechanism of action of these peptides a number of models have been proposed [46].

The majority of these substances are of cationic nature (the presence of lysine and arginine residues). This property enables them to interact with negatively charged fragments of biological membranes in particular lipopolysaccharide (LPS), which is the component of the outer membrane of gram-negative bacteria [47]. The incorporation of peptides into a membrane leads to the pore formation or destabilization of its structure and consequently to the lysis of the bacteria cell. This is the most common mechanism of action of peptide antibiotics. However, some natural peptides exhibit other mechanisms. For instance, buforins inhibit the cellular function by binding to DNA and RNA [48], attacins block the synthesis of integral membrane proteins [49] and PR-39 inhibits DNA synthesis [50]. A different mechanism is proposed for gram-positive bacteria. It is connected with the binding of bacteria to lipoteichoic acid (LTA) [51].

LPS-binding capacity of antimicrobial peptides is a great clinical advantage compared to classical antibiotics as it prevents endotoxemia. LPS stimulates lymphocytes B and macrophages (by binding to CD14, a surface receptor) to the production of inflammatory cytokines (TNF, IL-1, IL-6, IL-8) [52]. Uncontrolled and excessive production of these substances is considered to be the direct cause of death in the case of sepsis.

Table 2. Antimicrobial peptides in pharmaceutical development [80]

Peptide	Company	Mode of use	Application	Stage
D2A21	Demegen	Topical	Burn wound and skin infection	Phase I
Daptomycin	Cubist Pharmaceuticals	Systemic	Sepsis	Phase III
Demegen P-113 (Histatin analogue)	Demegen	Topical (oral)	Gingivitis	Phase II
Heliomycin	Entomed	Systemic	Antifungal	Preclinical
Iseganan IB-367 (protegin analogue)	Intrabiotics	Oral	Oral mucositis Lung infectious in patients with cystic fibrosis	Completed phase III, not approved by FDA Phase II
Lactoferricin B	AM Pharma	Systemic	Antifungal	Preclinical
MBI-594AN	Micrologix	Topical	Acne	Completed phase II
Neuprex (recombinant fragment of BPI)	Xoma Corp.	Systemic	Meningococcal miningitis	Completed phase III, not approved by FDA, in additional studies
Omiganan MBI-226	Micrologix	Topical	Catheter infection	Phase III
Pexiganan MSI-78 (magainin analogue)	Genaera	Topical	Infected diabetic ulcers	Phase III

Antimicrobial peptides also counteract fungal infections, especially those caused by *Candida* sp. [53]. Defensins and histatins kill fungi by nonlytic release of cellular ATP, which subsequently binds to putative purinergic receptors and activates cytotoxic pathways [54].

Apart from the antibacterial activity, antimicrobial peptides also possesses antiviral [55, 56] antiprotozoan [57] and antitumor activity [58].

Antimicrobial peptides are preferentially more selective towards the prokaryotic cell membrane. This might be caused by the fact that prokaryotic cell membranes are more anionic and that they do not have cholesterol [59]. Studies have shown that the presence of cholesterol in artificial membranes significantly reduced the lytic activity of antimicrobial peptides.

Additional activity of antimicrobial peptides

Apart from their titular role, antimicrobial peptides can possess a broad spectrum of additional activities [24]. They can prevent viral infections. Moreover, they can be cytotoxic for tumour cells [58]. However, killing is not the only function of common antimicrobial peptides. More sophisticated properties of these peptides have been proven. For example, defensins act as mitogens for epithelial cells and fibroblasts [60], suggesting their role in wound healing processes. Defensins are also potent inhibitors of protein kinase C [61]. Another peptide PR39 binds to p130 protein and phosphoinositole-3-kinase; both molecules of great importance in signalling pathways [62]. Some antimicrobial peptides act as chemoattractants for neutrofiles and monocytes [63]. Upregulation of proinflammatory cytokine production and competition for chemokine receptors are other properties reported in immune systems [64]. All the additional roles of antimicrobial peptides mentioned above are concentration-dependent and can have local character. Since these peptides are evolutionally conserved, they might have shown even more diverse activities in the past. Simple construction, rapid production and diffusability emphasize their advantages as useful and multifunctional molecules.

Applications

A survey of patent databases reveals a wide range of proposed applications, including the treatment of gastric ulcers [65], skin ulcers [66], oral cavity diseases [67, 68], ophthalmic diseases [69], sexually transmitted diseases [70] and sepsis [71]. Other applications are gene therapy [72], production of sterile coatings [73], use in cosmetics [74], use as food preservatives [75], production of transgenic plants and food animals [76] and production of new radiopharmaceuticals, which discriminate bacterial infections and sterile inflammations [77].

Although wide usage of new antibiotics may be difficult to achieve (due to high costs of production), their application in therapy requiring small quantities of them seems to be promising.

What hampers the introduction of new antibiotics to treatment is finding a suitable delivery system of a drug. Peptides are substances of low stability during storage time and intraintestinal usage. They are non-resistant to the photolytic enzymes of the gastrointestinal tract and large sizes (ca 2kDa) considerably limit their absorption into the digestive system [78]. Due to the above-mentioned properties, they are used mainly locally and are limited only to cases where a small quantity of a drug is required. The most popular substances under clinical test are Demegen P-113 [67], Iseganan IB-367 [68], Omiganan MBI-226 [79] and Pexiganan MSI-78 [66] (Table 2).

Peptide radiopharmaceuticals

In contrast to computerized tomography (CT), magnetic resonance imaging (MRI) and ultrasonography, which visualise anatomical changes, scintigraphy allows the localisation of functional changes in tissues and organs. Furthermore, this aim can be achieved in a non-invasive way.

Chemically-modified compounds like leukocytes [81], cytokines (IL-1, IL-2, IL-8) [82, 83], polyclonal or monoclonal immunoglobulins [84, 85], ciprofloxacin [86] and some types of peptides (chemotactic peptides (np. f-Met-Leu-Phe) [87], defensins [88]) are widely used in experimental and diagnostic approaches. Although several acknowledged radiopharmaceutics are well described and applied worldwide, it is still necessary to search for new ones. The majority of classical radiopharmaceutics act in a non-specific manner and they cannot distinguish between bacterial infections and sterile inflammation. Apart from specificity of action, an ideal radiopharmaceutic should be characterised by efficient accumulation and good retention in inflammatory foci, rapid clearance from the background, easy low-hazard preparation and wide availability at low cost [89].

All these requirements can be fulfilled by new peptide antibiotics labelled with short-lived radionuclides, such as technetium-99m (^{99m}Tc). The research of *Welling et* al. showed that ubiquicidin- and lactoferrin- based peptides labelled with ^{99m}Tc accumulated significantly in tissues infected with gram-positive and gram-negative bacteria as well as *C. albicans* [90–92]. These peptides could be accumulated only in sites of active infections, not sterile inflammation, while ^{99m}Tc-labeled ciprofloxacin was accumulated in both cases. Authors have also proved that these modified peptides were effective in monitoring the efficiency of antibacterial agents in infected mice.

Peptide radiopharmaceutics possess a variety of advantages. Compared to whole proteins, they have simple chemical structures, which is particularly important for costs of production. Peptides consisting of up to 50 amino acid residues can be automatically synthesized using solid-phase or liquid-phase synthesis [93]. It is also possible to produce them by genetic enginery methods. Problems concerning short plasma half-life (peptides are vulnerable to proteolytic enzymes) can by omitted by modifications such as substitution of D-amino acids instead of L-amino acids, incorporation of non-protein fragments or amidation and acetylation of terminal parts of the peptide chain. In an easy way, the whole panel of synthetic analogues with properties dedicated to certain *in vivo* effects can be produced.

As antimicrobial peptides become increasingly popular compounds as new pharmaceutics and are progressively applied in clinical research, it is to be expected that in the near future they will be applied not only in laboratories. However, increasing resistance to classical antibiotics is an emerging clinical problem. Natural antimicrobial peptides are a good alternative and we cannot exclude that they will substitute at least part of classical antibiotics soon.

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