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Antimicrobial Peptides: the Mode of Action and Perspectives of Practical Application

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Abstract—This review deals with antimicrobial peptides (AMPs) that demonstrate antibacterial, antiviral, and antifungal activity. It considers structure and mechanism of AMP interaction with lipid membrane and intracellular targets of pathogens. Special attention is paid to modern state and perspectives of AMP practical application and also to approaches that increase efficacy and reduce toxicity of AMP by chemical modification of their structure.

Key words: antimicrobial peptides, mode of action, practical application.

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INTRODUCTION

Endogenous antimicrobial peptides (AMPs) are[Ñ1] components of the most ancient defense system of organisms against pathogens [1]. They have been found in almost all living organisms from [Ñ2]bacteria to man [2]. During evolution multicellular organisms developed the whole AMP arsenal protecting them against various pathogens including Gram-positive and Gram-negative bacteria [2], DNA and RNA viruses [3, 4], fungi [5, 6], and protozoa [7, 8]. Antimicrobial peptides are characterized by a high rate of the bactericide effect [9, 10], which is usually attributed to AMP-induced pore formation in the bacterial membrane [11]. The increasing occurrence of bacterial resistance to existing antibiotics is driving a renewed interest on antimicrobial peptides. AMPs are now considered as novel perspective antimicrobial agents [2] due to the lack of microbial resistance development to AMPs [12]. However, despite of biological activity of numerous AMPs only some of them are employed clinically [13, 14]. Limited applicability is associated with the hemolytic effect to most AMPs, rapid degradation in vivo [1] and also rather high cost. At the same time some AMPs are free of these shortcomings [2] and therefore they may be used as the novel antimicrobial agents.

1. ANTIMICROBIAL PEPTIDES POSESSING [W3]ANTIBACTERIAL ACTIVITY

It is rather difficult to refer particular antibiotics to certain class due to existence of several classifications [15]. This problem also exists in the field of medico-biological science specialized on AMPs and sometimes AMPs are referred to lantibiotics [1] (bacterial polypeptides that contain polycyclic thioether amino acids such as lanthionine and methyl-lanthionine and/or unsaturated fatty acid residues) [16]). In this review we consider AMPs that consist of amino acids and lack nonpeptide fragments (e.g. carbohydrate, fatty acid residues, etc.).

Usually, AMPs exhibit low selectivity; they are toxic to both bacterial and eukaryotic cells (e.g. magainin [17] and indolicidin [18]). However, some AMPs [w4](e.g. pseudins [19]) in their antimicrobial concentrations do not possess the hemolytic effect. Understanding of mechanisms by which AMPs recognize pathogen cells is an important precondition for design of AMP-based drugs. Differences in composition of membranes of bacterial and eukaryotic cells are the main factor determining AMP selectivity. The external surface of eukaryotic cell membranes consists of zwitterionic phospholipids, while bacterial cell membranes contain high proportion of negatively charged phospholipids on both external and internal surfaces of the lipid bilayer [20]. Acidic features of the external surface of prokaryotic cells membrane determine its preferential interaction with positively charged AMPs. Lack of cholesterol in bacterial cell membranes is the second factor determining AMP selectivity [20]. It determines higher fluidity of the lipid bilayer of bacterial membranes and so pore formation by AMPs occurs more easily than in eukaryotic cell membranes. In addition, a high negative transmembrane potential across the inner membrane

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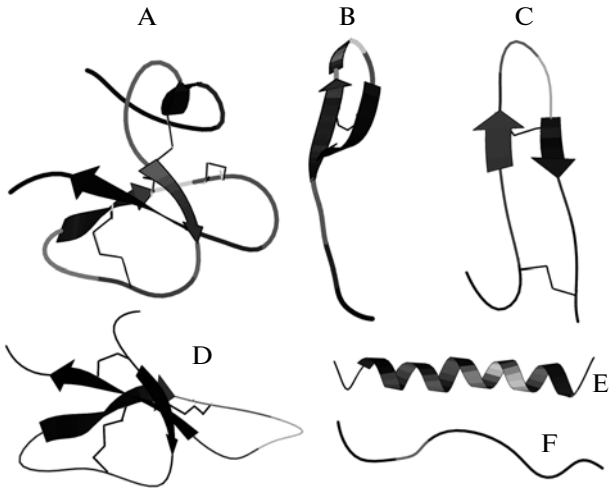

Fig. 1. Conformation of various antimicrobial peptides: (a)–β-defensin-2 (mixed conformation); (b)—tanatin (loop conformation), (c)–β-polyphemusin (β- hairpin), (d)—defensin-1 (mixed conformation), (e)—magainin (α-helix, (f)— indolicidin (extended conformation) (adapted from [24]).

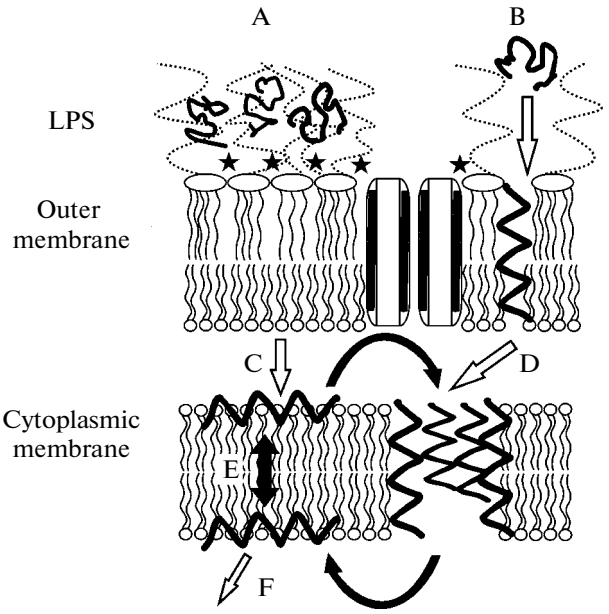

Fig. 2. Proposed mechanism of interaction of antimicrobial peptides with the cell wall of Gram-negative bacteria (adapted from [26]).

of bacterial cells also promotes pore formation by AMPs [21].

Usually, AMPs are amphiphilic compounds with well defined hydrophilic and hydrophobic parts [22]. Apart a few exceptions AMP molecules contain several lysine and/or arginine residues and at physiological pH values they are positively charged [22]. Structurally, AMPs may be subdivided into two large groups: cyclic peptides stabilized by -S-S- bonds and linear peptides (Fig. 1).

The liner peptides may be further subdivided into "typical" peptides forming α-helical secondary structure and linear peptides with atypical secondary structures. The latter group of peptides are usually enriched with Pro, Arg, or Trp residues. Most peptides have domains with various secondary structures and so when some peptide is referred to α-helical AMPs this implies predominance of α-helical domains in its structure [23].

In the lipid bilayer "typical" linear peptides may exist as α-helical and also as β-sheet structures. Both these structures demonstrate amphiphilic properties, and hydrophobic and hydrophilic parts are located on opposite sides of the polypeptide backbone. Amphiphilic features of AMPs promote such interaction with membranes, when polar peptide regions interact with polar head group of phospholipids while hydrophobic regions interact with membrane fatty acid residues via hydrophobic interactions [22].

Results of early studies suggested existence of direct interrelationship between formation of helical structures and lytic activity of AMPs towards microorganisms [24]. Hower, later it was demonstrated that the helical structure is more essential for cytotoxic activity towards eukaryotic cells. For example, cytotoxicity studies of pardaxin and melittin diastereomers have shown that loss of α-helical structure resulted in disappearance of the hemolytic activity, whereas the antibacterial activity against Gram-positive and Gram-negative bacteria remained the same [25]. Fluorescent studies on model membrane systems mimicking outer membranes of prokaryotic and eukaryotic cells confirmed results of the cytotoxic studies [25].

Positively charged regions are important characteristic features of antibacterial peptides. Using these regions AMPs find corresponding targets, microbial membranes. Peptides bind to negatively charged molecules of lipopolysaccharides (LPS), one of essential components of the outer membrane of Gram-negative bacteria [27]. Perturbations appeared in the LPS layer form co-called "vertical self-regulating channel", which facilitates peptide penetration to the plasma membrane [21, 28]. It is suggested that AMPs form "cracks" in the LPS layer (Fig. 2a) or bind to LPS sites (Fig. 2 b) responsible for interaction with divalent cations, Ca^{2+} and Mg^{2+}, required for stabilization of the cell surface and cross-binding the negative charges of LPS [29].

Passing through the outer membrane of the bacterial cell AMPs bind to the negatively charged surface of the cytoplasmic membrane. Transfer of peptides from the aqueous phase into a lipid bilayer environment is accompanied by changes in the peptide conformation [2, 30]. It remains unknown, at which moment the peptide adopts conformation typical for a

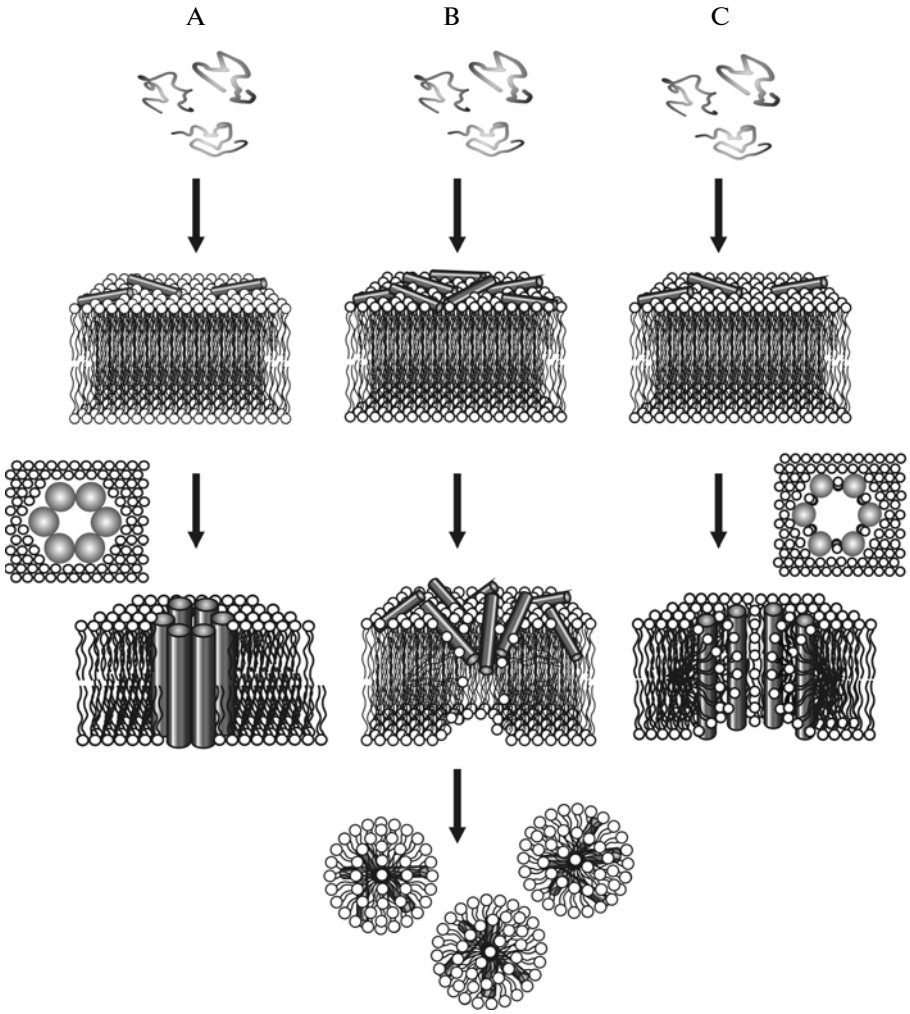

Fig. 3. Mechanisms of interaction of antimicrobial peptides with lipid membranes: (a)—formation of a cylindrical (barrel-stave) pore; (b)— carpet mechanism; (c)—formation of a toroidal pore (adapted from [23]).

particular lipid environment (during transfer through the outer[w5] membrane or during insertion into the cytoplasmic membrane). After insertion AMPs form various aggregates (Fig. 2d; hypothesis on the structure of these aggregates are considered below) or flip-flop exchange (Fig. 2e). After the flip-flop exchange AMP molecules may then dissociate into the internal medium of the bacterial cell and interact with such intracellular polyanions as DNA or RNA (Fig. 2f) [21].

Results of numerous studies indicate that after penetration into membranes of pathogenic microorganisms AMPs form aggregates [32, 33]. Interaction of peptide molecular with each other and with membrane lipids results in formation of complex structures; this represents a part of mechanism of action employed by many AMPs. The ability of peptides to form aggregate in the lipid membrane is determined by their amino acid sequences. For example, peptides containing hydrophobic and hydrophilic domains may "direct" orientation of their hydrophobic and hydrophilic surfaces towards corresponding membrane components and neighboring peptide molecules [23]. Peptide associates may form selective [34] or nonselective [35] pores or channels. For example, pore walls may be lined by hydrophilic regions of the lining peptide molecules, whereas hydrophobic regions will be oriented towards acyl chains of phospholipids and hydrophilic compounds will preferentially pass through such pore [34].

Accumulation of AMP molecules in the membrane surface of the lipid bilayer causes tangential tension[w6] increasing between two leaflets of the bilayer [23]. When concentration of AMP molecules on the outer surface of the lipid membrane reaches some threshold level, tension is compensated by various mechanisms (Fig. 3) including redistribution of peptide molecules between outer and inner membrane surfaces due to the increased flip-flop rate [36], formation of various pores [37, 38] or desintegra-

tion/rupture[w7] of the lipid membrane [39]. Below we consider these mechanisms in more details.

1.1. The Barrel-Stave Model (Fig. 3a)

In the case of domination of hydrophobic interactions peptide chains are inserted into the lipid bilayer and adopt orientation perpendicular to the bilayer surface. Subsequent recruitment of other polypeptide chains results in the increase of the transmembrane bundle of peptide molecules and formation of the barrel-stave type pore. The hydrophobic face of the peptide chain interacts with lipid acyl chains and the hydrophilic face forms the interior of the pore [27]. A minimal length of the peptide sequence required for realization of this model is about 22 residues for α-helical peptides and about 8 residues for peptides with the beta-pleated sheet structure [40]. The number of AMP molecules involved into pore formation may vary and depend on AMP concentration. For example, alamethicin forms pores that consist of 3–11 peptides with an average diameter of 4 nm [41, 42]. Such type of interaction is typical for small number of peptides (alamethicin, pardaxin) characterized by very low selectivity and also by toxicity to normal cells [40].

1.2. The Carpet Model (Fig. 3b)

This mechanism is realized in the case of very strong electrostatic interactions between positively charged regions of peptide molecules and negatively charged phospholipid polar head groups. Polypeptide chains are oriented parallel to the bilayer surface [44]. Accumulation of peptide above some critical threshold concentration on the lipid membrane causes its rupture[w8] followed by micelle formation [45, 46]. This is accompanied by formation of large (about 25 nm) toroidal pores (see more details about toroidal pores below) [35]. In this model pore formation is an intermediate step before the collapse of the membrane and such pore [w9]is not a stable structure [11].

1.3. The Toroidal Model (Fig. 3c)

According to this model, compensation of the membrane tension induced by insertion of peptide chain into the membrane occurs due to continuous bend of one membrane leaflet to the other one and association of membrane surfaces; this is accompanied by formation of toroidal pore [47]. Peptide molecules are inserted [w10]from the membrane surface into the hydrophobic part of the lipid bilayer. This results in formation of a pore structure, in which hydrophilic regions of peptide molecules and lipid head groups together form [w11]the pore wall (in contrast to the toroidal pore, the barrel-stave type pore is formed only by peptide molecules). Usually, toroidal pores have larger sizes than barrel-stave type pores [11]. For example, the toroidal pore formed by magainin (and consisting of 4—7 peptide molecules and about 90 phospholipid molecules) has inner and outer diameters of 3—5 and 7.0—8.4 nm, respectively [48].

Studies of mechanisms underlying AMP toxicity towards pathogens and host cells represent an important problem. It was long thought that AMPs killed microorganisms by forming numerous unrecoverable damages in their membranes. Indeed, AMPs may form pores in microbial membranes as described above. A microbe "attacked" by AMP molecules can die due to leak[w12]age of ions and metabolites and also due to membrane depolarization (followed by its dysfunction), inhibition of cell respiration and also biopolymer synthesis. However, recent data [49, 50] suggest that AMP-induced cell death may involve other mechanisms, for example, interaction of AMPs with intracellular targets. Thus, AMP-induced mechanisms of cell death require further studies and specification.

Below we consider processes occurring in the cell interacting with AMPs. The cytoplasmic membrane maintains normal functioning of microorganisms. It provides selective permeability, maintains the electrochemical gradient, electron transport, and oxidative phosphorylation (in the eukaryotic pathogens such as fungi, this process occurs on the inner mitochondrial membrane), synthesis and conjugation of peptidoglycan, chitin, and other biopolymers. This suggests that AMP-induced dysfunction of the outer and/or plasma membrane may cause impairments in one or a few these functions, which result (directly or indirectly) in cell death.

However, in many cases AMP-induced death of bacteria cannot be attributed only to membrane dysfunction [51]. For example, in the case of cytotoxicity of various peptides towards *S. aureus* there was no correlation between cytotoxicity and damage of bacterial membrane integrity. The other study [52] demonstrated that gramicidin S caused rapid depolarization of the cytoplasmic membrane of *Pseudomonas aeruginosa*. Nevertheless, this bacterium exhibited resistance to this peptide. On the other hand, toxic effects of polymixins B and E1 were associated with membrane depolarization of *Pseudomonas aeruginosa* cells. This suggests that AMP-induced damages of membrane integrity and bacterial cell death may represent independent events.

Inhibition of synthesis of peptidoglycan[C13]s, chitin, and other macromolecules also represents an important mechanism of AMP action. For example, normal functioning of the bacterial cell membrane is associated with peptidoglycan biosynthesis. Activated peptidoglycan precursors are transported across the cytoplasmic membrane and conjugate with each other in close proximity of this membrane. Cationic peptides cause membrane perturbations and thus cause impairments in the peptidoglycan biosynthesis cycle by direct or indirect inhibition of its synthesis and

translocation of peptidoglycan precursors and/or their conjugation. Considering [w14]high peptidoglycan content in the membrane of Gram-negative microorganisms it appears that they[w15] should be especially susceptible to the AMP effect employing this mechanism. For example, it was demonstrated that the AMP plasmin inhibits peptidoglycan synthesis in *E. coli* cells [53].

Although cell membrane damages are the key moment in the AMP-induced cell death, results of some studies indicate that peptide interaction with some intracellular targets also plays an important role in the mechanism of AMP cytotoxicity [54-56]. For example, in some cases there was rather prolonged period before death of microorganisms induced by AMPs; this suggests the cell death did not occur via the membrane lytic mechanism. The study of cytotoxicity of the antimicrobial peptide tPMP demonstrated [57] that *S. aureus* cells treated with this peptide remained viable [w16]for rather long period after damage of their membrane integrity. Subsequent death of these cells occurred due to direct inhibition of nucleic acid biosynthesis by tPMP.

Thus, AMPs demonstrating [Ñ17]antibacterial activity are abundant and most studied class of antimicrobial peptides. However, it should be noted that despite constant interest of the scientific community in this class of compounds only some of them are employed clinically. Increased attention to antibacterial AMPs is determined by the development of bacterial resistance to them and therefore design of new AMP-based antibiotics attracts researches.

2. ANTIMICROBIAL PEPTIDES POSSESSING ANTIVIRAL ACTIVITY

Besides antibacterial activity some AMPs also exhibit noticeable [w18]antiviral properties [24]. For example, it was demonstrated that the antiviral activity of numerous AMPs (e.g. lactoferrins) is determined by their binding to heparan sulfate, one of the most abundant components of the cell surface [58]. Heparan sulfate is a proteoglycan, with its protein component being covalently attached to one or a few sulfated glucosaminoglycan chains. Heparan sulfate is the most negatively charged component of the cell surface and this determines preferential binding of positively charged extracellular ligands [59] and also many pathogens including viruses [60]. Binding to heparan sulfate is the first step required for penetration of many viruses into the cell [61, 62]. There is evidence [63, 64] that blockade of cell surface heparan sulfate attenuated viral infection of these cells. It was also demonstrated that recombinant cells with decreased expression of heparan sulfate or chondroitin sulfate were less susceptible to herpes simplex virus (HSV) infection (by 80 and 60%, respectively) [65]. The increased content of cell-surface heparan sulfate is an important precondition for penetration of hepatitis C virus into the cell [66].

All these facts suggest that temporal blockade of cell-surface heparan sulfate may prevent development of viral infections in the organism[w19]. AMPs demonstrating antiviral properties have strong positive charge and therefore they easily bind to cell-surface heparan sulfate and prevent viral binding. For example, it was demonstrated that the antimicrobial peptide, melittin, prevented healthy cells against HSV infection [67]. According to isothermal titration calorimetry experiments, melittin exhibits increased affinity to heparan sulfate [68]. Lactoferrin and its analogues also exhibit increased affinity to heparan sulfate [69]. Cells treated with lactoferrin analogues were less susceptible to HSV than untreated cells, but treatment of the HSV suspension with these peptides did not inhibit viral activity [69]. The antiviral activity of the cathelicidin family of AMP is also attributed to peptide binding to heparan sulfate [70].

On the contrary, antiviral activity of some AMPs is determined by direct interaction with the viral particle. Usually, AMPs bind to viral envelope glycoproteins. For example, AMP defensins can bind to adenovirus particles. Defensins inhibit virus disassembly at the vertex region thereby restricting the release of an internal capsid protein, pVI, which is required for endosomal membrane penetration during cell entry. Thus, defensins prevent the release of adenovirus particles from endosomes, resulting in accumulation of virions in the lysosomal region where they are inactivated [71]. α-Defensin peptides can also inhibit adsorption of polyoma virus by binding to viral surface glycoproteins; they can also inhibit assembly of virus particles [72].

In addition, some AMPs may interact with the viral lipid envelope and cause its lysis, destabilization or pore formation [24]. For example, the AMP, indolicidin, inactivates HIV-1 by damaging the virion membrane [73]. Another peptide, dermaseptin, demonstrated similar activity [74]. This peptide did not influence directly the HSV membrane but inhibited virus adsorption on normal cells [75].

It should be noted that despite rather intensive studies of AMP antiviral activity the mechanism of selected AMP toxicity towards viruses still requires further investigation.

3. ANTIMICROBIAL PEPTIDES POSSESSING [W20]ANTIFUNGAL ACTIVITY

Many AMPs demonstrate significant antiviral activity against microscopic fungi [24] by inducing their lysis [5]. However, structure function relationship activity for antifungal AMPs is less studied compared with structure function relationship activity of antibacterial AMPs. For example, various peptides characterized by completely different structural motifs

demonstrate antibacterial activity. These include the antifungal peptide, containing 5 disulfide bridges, which was isolated from the *Eucommia ulmoides* plant [76], the peptide P18 possessing α-helical structure [77], the "extended" peptide indolicidin [78], and β-sheet pleated coleopteran isolated from *Acrocinus longimanus* [79].

The antifungal activity of AMPs is intensively studied in many laboratories [80-83]. Interestingly, interaction of AMPs with pathogenic fungi cannot be described by a universal mechanism (typical for AMP interaction with viruses and bacteria). For example, it was demonstrated [83] that peptide fragments from the lactoferrin sequence exhibit significant antifungal activity towards *Candida albicans*; they inhibit biosynthesis of outer membrane components and cause ATL leak from the fungal cells. However, pleurocidin, isolated from *Pseudopleuronectes americanus* acts by membrane lysis [w21][81]. Plant defensins bind to some outer membrane components of microscopic fungi and thus violate [w22]transmembrane transport and inhibit synthesis of their outer membrane components [80]. Piscidin-2 isolated from mast cells hybrid striped bass acts on the fungal plasma membrane by forming pores and causing cell death [82].

Fungicide activity of some AMPs represents a point of discussions. For example, it was suggested [84] that toxicity of the AMP, histatin 5, towards *C. albicans, C. neoformans* and some other fungi is determined by formation of reactive oxygen species (ROS) in the fungal cells induced by this peptide. Authors [84] proposed a hypothesis, by which after penetration inside cells of microscopic fungi histatin 5 enters mitochondria and inhibits coenzyme Q cycle; this results in accumulation of ROS in the fungal cell and cell death due to oxidation of intracellular substrates by the accumulated ROS. According to another viewpoint [85] histatin 5 does not cause the increase of ROS in the fungal cells and the fungicide effect of this peptide is determined by ATP leak[w23]age from fungal cells. These authors [85] assert [w24]that wrong conclusions made in [84] could be attributed to an unreliable method used for ROS detection in that study.

4. PERSPECTIVES OF PRACTICAL APPLICATION OF AMPS

High cost, sensitivity to proteolytic enzymes, and also the hemolytic effect typical for many AMPs are the main obstacles for AMP application in clinical practice [1]. Nevertheless, AMP-based drug preparations are developed by many pharmaceutical companies. Below we consider some examples of such preparations.

4.1. Omiganan

The active component is an analogue of the 13-residue antimicrobial peptide indolicidin. It is produced by Microbiologix Biotech. Phase III clinical trials of Omiganan as a drug decreasing colonization of vein catheters by microorganisms causing catheter-related bloodstream infections gave different results in various groups of patients; therefore its applicability in clinical practice is still questionable [86]. Repeated trials revealed that Omiganan 1% gel prevented catheter colonization by all known bacteria and microscopic fungi [87].

4.2. MX594AN

The active substance is an analogue of the 13-residue antimicrobial peptide indolicidin. It is produced by Microbiologix Biotech. MX594AN successfully passed through Phase IIb clinical trials as the drug for treatment of acne formation [w25]and now it is under Phase III clinical trials [86].

4.3 hIF1-11

This is a 11-residue peptide from the N-terminal part of human lactoferrin developed by AM-Pharma. Currently, it is under Phase II clinical trials as antifungal and antimicrobial drug [1, 88].

4.4. P113/P113D

The acting substance is a 12-residue peptide, modified histatin [89]. This drug produced by Demergen/Pacgen is active against oral candidoses. P113/P113D passed through Phase II clinical trials. Its inhalation drug dosage form has been prepared for Phase III clinical trial [1].

In addition to drugs considered in this secation there are some other AMP-based preparations , which are now passing earlier phases of clinical trials [1, 86].

CONCLUSIONS

Although AMPs exhibit high activity against various pathogens in vitro they are not widely used in clinical trials due to high cost, proteolytic susceptibility and hymolytic activity. This explains why design of modified AMPs, which would be free of these shortcomings, attracts much attention [90]. The main strategies used for optimization of AMP structures to obtain novel biocide agents include synthesis of cyclic AMP analogues [91], insertion of a fluorine atom or the trifluoromethyl group [92], synthesis of branched AMPs (dendrimers) [93, 94] and also synthesis of AMPs immobilized on various polymer matrices [95]. Thus, AMPs remain in the spotlight of many research groups, interested in scientific and practical aspects of their effects and application as novel antibiotics.

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