Antimicrobial peptides: from synthesis to clinical perspectives

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The increase in infections associated with microorganisms resistant to conventional antibiotics is a major concern for the clinical treatment of diseases and future public health problem. This is due to acquired resistance to antimicrobial agents by microorganisms associated directly to the indiscriminate use of antibiotics. Therefore, several studies have been conducted to discover substances that present effective antimicrobial activity. Thus, natural compounds represent a promising alternative for this purpose, in evidence, are the secondary antimicrobial peptides (AMP). The mechanisms of action developed by these peptides involve an interaction with the cell membrane which leads to destabilization and eventual cell death. The synthetic AMP production and development based on structural modifications are considered appropriate strategies to facilitate the application of these molecules a battle against microbial pathogens. However, recently, combining techniques from classic antibiotics and (AMP's) had a better effect in treatment against microbial biofilms, which reflects the importance of further study in this perspective. In the end, considering the high levels of infections correlated with biofilms and the slow progress in identifying new antimicrobial agents, new strategies to combat are invaluable clinical significance.

Keywords: antimicrobial peptides; antibiotic

1. Introduction

The discovery of Penicillin opened the doors for a new era in the treatment of infectious diseases. It hasn't seen just as a new drug or an isolated scientific event, but for the first time, there was a drastic reduction in mortality rates caused by infections and common bacterial diseases. These illnesses could be cured by so-called "miracle drugs" [1]. The emergence of the first antibiotic opened new ways to scientific investments and consequently detection of new active substances that would change the history of humanity.

Despite of the initial efficacy, the indiscriminate and extensive use of penicillin after second war tagged the appearing of the first non-susceptible bacteria strains. Years later, the same happened with antibiotics released in the market by the same reason [2]. The first case of penicillin-resistance bacteria was reported in 1948 and currently a large number of others microorganisms have been identified as strong to penicillin, giving to that kind of antibiotics a limited life [3]. In the same hand, this situation can be aggravated by constant genetic variability, moved by spontaneous mutations and recombination amongst genes, leading the microorganisms not to be affected by several antibiotics [4]. Apart from natural evolutionary phenomenon, the development of antimicrobial resistance is accelerated by poor hygiene in some countries, continuous travel flows and the delay in the diagnosis of emergent infections [5, 6].

Conversely to increase of the identification of microorganisms resistant to conventional antibiotics are the development of new antimicrobial agents, which has been reduced drastically over the past 30 years [3]. According to the WHO (World Health Organization), antimicrobial resistance has detained the scientific and social progress, apart from to be recognized as a global crisis of public health [7]. Therefore, research groups have considered the identification of new bioactive substances that may replace the standard antibiotics as a more suitable solution.

2. Antimicrobial peptides

The antimicrobial peptides (AMP) are components of the innate immune system of various multicellular organisms that were maintained during the course of evolution [8]. Is a group of molecules that enhance immune responses from the first line protection and modulate inflammatory responses induced by pathogens [9].

These molecules generally has low molecular weight (<100 amino acid residues), cationic structure with net positive charge at physiological pH (+2 to +9) and amphipathic character due to high amounts of hydrophobic amino acid residues [10]. These features from confer the ability these biomolecules to interact with structures in the cell suface in order to cause the eventual death of an external pathogen [11, 12].

In addition to the microbicidal activity against a broad spectrum of fungal, viral and bacterial species, the AMP has been mentioned as potential antitumor and anti-parasitic substances, as well [13]. However, the relevance of these

biomolecules as antimicrobial agents become themselves target of numerous studies from big research groups to explore their applicability in the prevention and treatment of infectious diseases [14].

The mechanism of action of these biomolecules occurs through the interaction between the peptides with bacterial components that could be intra or extracellular side. Peptides cause dysfunction of the plasmatic membrane leading to leakage of cytoplasmic material or alternatively, antimicrobial mechanisms have been characterized by target key cellular processes including DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis [11, 15, 16]. The form of nonspecific interactions of these molecules with lipids from biological membranes seems to be related to the low capacity of the micro-organism to develop resistance against antimicrobial peptides [16]. In contrast of action mechanism of most classical antibiotics, which are based on enzyme inhibition, and are relatively slow, the AMPs that act at the membrane level are quicker and effective [18,19].

2.1 Extracellular targets

The cell membrane, which consists of a lipid bilayer, operates in regulating the flow of metabolites between the external and the intracellular environment, functioning as a semipermeable barrier. This kind of surface assembles characteristics to become the main target for attack of many AMPs. Thus, the cationicity of the AMPs and a significant proportion of hydrophobic residues promotes selectivity for negatively charged microbial cytoplasmic membranes whereas the latter facilitates interactions with the fatty acyl chains. Since many linear AMPs are unstructured in aqueous solution and require a membranous environment to adopt a stable amphipathic conformation [20].

The Figure 1 shows mode of action usually involves lose of the integrity of the bacterial cytoplasmic membrane by several ways. In the classical models of membrane disruption, the peptides lying on the membrane reach a threshold concentration and insert themselves across the membrane. Initially, the peptides interact with the polyanionic LPS exterior and competitively dislocate the divalent cations that partially neutralize the LPS. This causes the disruption of the outer membrane, and the peptides are translocated. The target next is inner phospholipid membrane. The peptides bind to it, disrupt and disintegrate it causing eventually kill the cell [16]. Currently, four models describe the possible mechanisms of action of AMPs through the permeation membrane, they are: "barrel-stave" model, "carpet-like" model, "toroidal-pore" model and "disordered toroidal pore" model [21].

Barrel-stave" model
The model suggests the arrangement of the peptides in a transmembrane condition, organized in a format of "barrel". The hydrophobic regions aligned with the lipid core region of the bilayer whilst the hydrophilic peptide regions form the interior face of the pore.

"Carpet-like" model
In the ‘‘carpet-like’’ model the peptides cover the membrane like a carpet. When getting a threshold concentration, the AMPs penetrate the cell membrane and like "detergent action" it forms micelles containing membrane waste.

"Toroidal-pore" model
In this model, the hydrophilic region of the AMPs interacts with the polar portion of the phospholipid binding the lipidic monolayers to acquire a stable curvature and form toroidal pores. The peptides remain strongly bound to lipid polar groups throughout its extension.

"Disordered toroidal pore" model
This model is a recent modification to the model "toroidal pore". Less-rigid peptide conformations and orientations are formed and the inside pore is too lined by hydrophilic portion of the phospholipid.

In more specific cases, as related by Nguyen, peptide adsorption to the membrane can be enhanced by targeting them to oxidized phospholipids or other substance into the cell [22]. That occur when the peptide couple with small anions across the bilayer resulting in their efflux. [23] On the other hand, in some situations, the membrane potential can be dissipated without other any detectable damage or conversely, in the molecular electroporation model, the accumulation of peptide on the outer leaflet increases the membrane potential above a threshold that renders the membrane transiently permeable to various molecules including cytoplasmic constituents. [24] Other peptides can align parallel to the membrane surface can induce a quasi-interdigitated structure in the gel phase, while depending on the hydrophobic matching of the lipid bilayer core and the peptide membrane thinning or thickening can be observed in the fluid phase. [25] The clustering of anionic lipids to a region of the bacterial membrane would concentrate negative charge in a domain to which cationic peptides would congregate, possibly leading to the formation of a pore. [26] Finally, the induction of lipid polymorphism by antimicrobial peptides is involved in a series of events leading to the formation of pores and increases membrane permeability. [27]

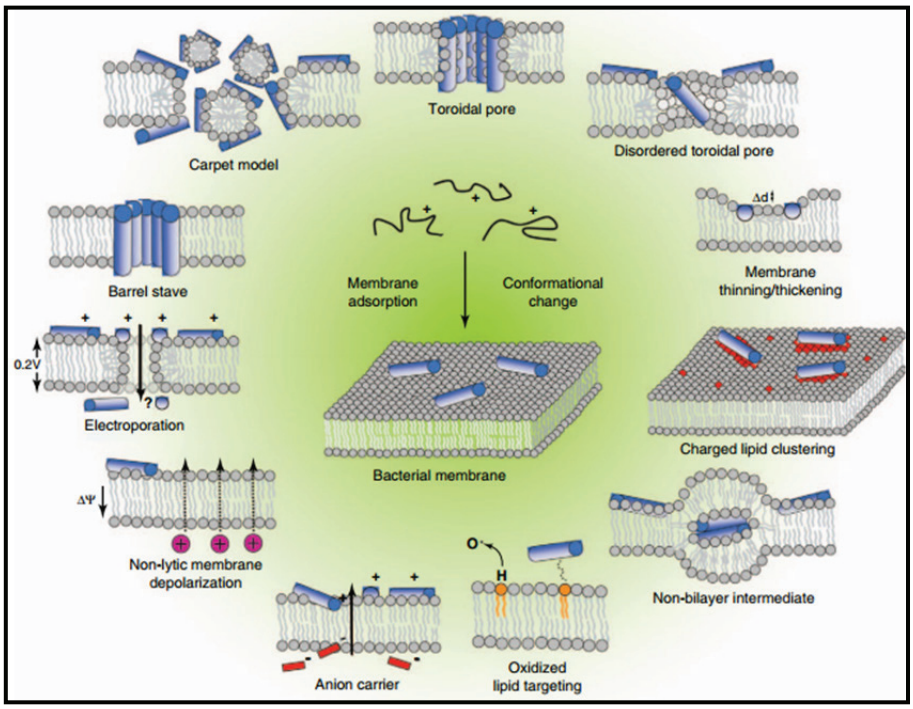

Fig. 1 Mechanisms proposed to explain the membrane permeabilization and cell death induced by AMPs. Image available at: *Nguyen LT, Haney EF and Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. Trends in Biotechnology September 2011, Vol. 29, No. 9*

2.2 Intracellular targets

Membrane interactions remain important even for intracellular-targeting peptides because they must have a means of translocation. As referred on Figure 2, AMPs can also act on multiple intracellular targets, modulating gene expression or inhibiting enzymatic processes important for cell viability maintenance [28, 29]. Some AMPs have the ability to interfere with the metabolism of nucleic acids, as is the case Microcidin B17 targets the DNA gyrase inhibiting DNA replication [30]. The peptide MccJ25 of bacterial origin may compromise RNA polymerase activity by preventing the transcription process [31]. While others peptides may directly act on proteins synthesis, as in the case of apidaecins and oncocins, causing a blockage in the formation of new protein products through binding to ribosomal proteins [32].

Changes in cell wall synthesis process can be triggered by peptides with antimicrobial intracellular targets. Mediated receptor binding was observed for Lactococcin G and Enterocin 1071, which binds to UPPP, an enzyme involved in cell wall synthesis. In the lantibiotic class of antimicrobial peptides produced by bacteria of different groups may serve important precursors for the synthesis of peptidoglycan, or by activation autolysins [33, 34].

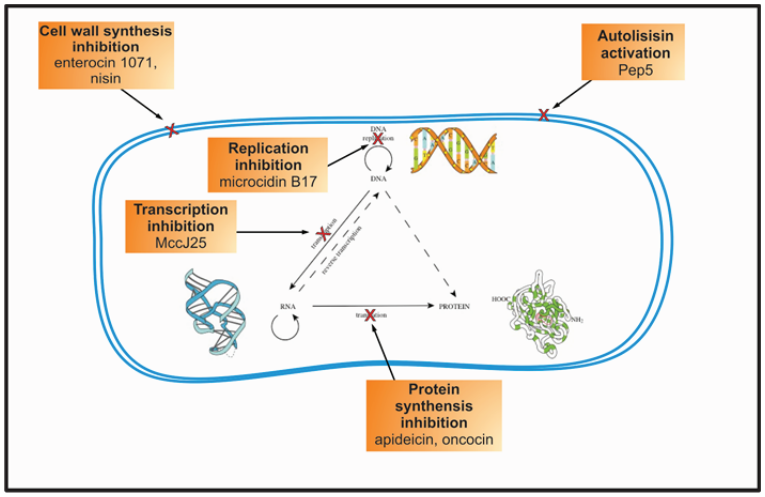

Fig. 2 Schematic illustration of the possible intracellular action mechanisms of AMPs.

3. Highlights of antimicrobial peptides

Currently the AMPs are recognized as ubiquitous biomolecules in nature, synthesized from simple organisms like bacteria to more complex as the human [39 - 63]. One of the first antimicrobial peptides identified has been isolated from the microorganism *Lactococcus lactis*. This peptide, known by Nisin, comes being used as antimicrobial agent in different process in food industry for decades [36, 37]. During the last years researches involving antimicrobial peptides have been intensified. Until December 2014, was registered a total of 2,493 to peptides *Antimicrobial Peptide Database* (APD), which are from different sources [38]. This database is an original construction that provides useful information about these relevant molecules, like discovery timeline, nomenclature, classification, glossary, calculation tools, and statistics. Then, this is a useful tool for both research and education. The antimicrobial peptides registered into APD cover the five kingdoms (bacteria, protists, fungi, plants, and animals) as well as the classification of the three domains of life (bacteria, archaea, and eukaryota).

Table 1 Antimicrobial Peptides from several sources in the five different kingdoms available at *Antimicrobial Peptide Database* (http://aps.unmc.edu/AP)

Peptide	No of residues	Source	Rerefence
		Monera	
Staphylococcin C55	59	*Staphylococcus aureus C55*	[39]
Mutacin	59	*Streptococcus mutans*	[40]
Microcin E492	59	*Klebsiella pneumoniae* RYC492	[41]
		Protista	
ASABF-alpha	59	*Ascaris suum*	[42]
Cecropin P1	59	*Ascaris suum*	[43]
		Fungi	
Antifungal protein	51	*Aspergillus giganteus*	[44]
PAF	55	*Penicillium chrysogenum*	[45]
PgAFP	58	*Penicillium chrysogenum RP42C*	[46]
		Plantae	
Ct-AMP1	49	*Clitoriaternatea*	[47]
Mj-AMP1	37	*Mirabilis jalapa*	[48]
Dm-AMP1	50	*Dahlia merckii*	[49]
		Animalia	
Invertebrates			
SK84	84	*Drosophila virilis*	[50]
Cecropin A	37	*Hyalophora cecropia*	[51]
Gomesin	18	*Acanthoscurria gomesiana*	[52]
SPP-12	73	*Caenorhabditis elegans*	[53]
Vertebrates			
Human lactoferricin	49	*Homo sapiens*	[54]
Indolicidin	13	*Bos taurus*	[55]
BACTENECIN 5	43	*Bos taurus*	[56]
HNP -1	30	*Homo sapiens*	[57]
Lactoferricin B	25	*Bos taurus*	[58]
Catestatin	21	*Homo sapiens*	[59]
LL-37	37	*Homo sapiens*	[60]
Mouse β-defensin-14	45	*Mus musculus*	[61]
Temporin A	13	*Rana temporaria*	[62]
Pleurocidin	25	*Pleuronectes americanus*	[63]

3.1 General structures properties

Peptides are biomolecules formed from links between amino acid residues, mostly with hydrophobic character. Natural peptides often exhibit positive charges averaging +3.2, however, according to the pH of the solution they may have a negative or neutral net charge [11, 64]. In free solution, some peptides have secondary and tertiary preformed structures,

while others have no defined structure. However, these peptides when in contact with the lipid bilayer of the target cell it fold into an amphipathic structure [65].

Most of the AMPs have an α-helical amphipathic structure with cationic character. But, there are also hydrophobic α-helical structures or even slightly anionic. allowing interaction with the surface of the micro-organisms to perform the antimicrobial activity [66]. Based on the wide diversity of structural demonstrated by AMPs as well as the growing number research of new peptide members, one of the alternative AMPs classification is based on their 3D structure, showed on Figure 3. Thus, they are classified into four families: α, β, αβ, and non-αβ based on the types of secondary structures [64].

AMPs with α-helical structures

More than 100 AMPs have been annotated as having helical structures in the APD. A few AMPs are helical even in aqueous solutions, primarily due to the formation of helix-bundle structures that is further stabilized by three disulfide bonds. Upon association with bacterial membranes, the helix bundle structure may open at a site that has several exposed hydrophobic side chains. In the helical family, AMPs with longer polypeptide chains tend to display toxicity to mammalian cells. This observation laid the foundation for truncating such AMPs to improve peptide selectivity. Some AMPs of this family are amphibian magainin 2, human LL-37.

AMPs with β-sheet structures

Cyclic peptides and stable in aqueous media, mainly composed of β strands linked by intramolecular disulfide becoming more stable when associated with lipid. Little is known of the mechanism of action of these peptides. Some examples are the defensins and lactoferrin.

AMPs with αβ structures

They have extended structure, characterized by a sequence rich in one or more specific amino acids. This group includes, among others, peptides rich in proline (49%) and arginine (33%) or proline (57%) and phenylalanine (19 %), peptides rich in histidine residues, peptides rich in tryptophan and glycine rich peptides. Some important AMPs of this family are catelicidins and tripsin.

AMPs with non-αβ structures

In contrast to other antimicrobial peptides, these are rich in proline and arginine and can not form amphipathic structures, and may adopt β chain type polyproline or cyclic helix. It is easily synthesized, proteolytically stable and small size, thus presenting considerable potential to combat existing and emerging infectious diseases. One of these peptides, nisin, is currently used as an antimicrobial agent for food storage, having high activity against Gram-positive bacteria.

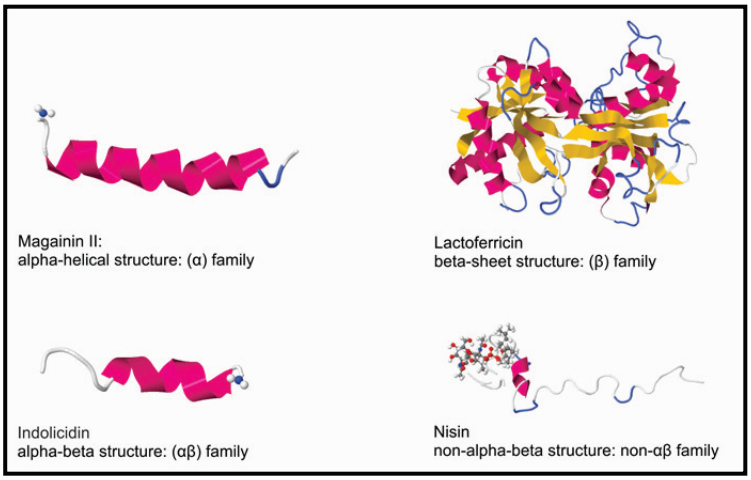

Fig. 3 Structural classification of antimicrobial peptides based on the types of secondary structures into four families: alpha (α), beta (β), alphabeta (αβ), and non-alphabeta (non-αβ). Available at Protein Data Bank (PDB) http://www.rcsb.org/pdb/home/home.do

3.2 Getting antimicrobial peptide ready

The scanning of the therapeutic potential of bioactive molecules starts with detection and isolation from the natural source which it belongs. This process aims at obtaining a substance with purity and high homogeneity, making them suitable for the determination of their physicochemical properties and biological potential applicability. Among the methods of isolation and purification from antimicrobial peptides stand out chromatography techniques. It is frequently applied because of its acuteness separation, identification and quantification of compounds by using a standard method

itself or associated with different biomolecules detection instruments and bioassay, antimicrobial activity, guided chromatography [67].

Once isolated, the following process is the structural characterization and their physicochemical properties. Techniques for two-dimensional electrophoresis (2D), mass spectrometry, circular dichroism spectroscopy (CD) and Nuclear Magnetic Resonance (NMR) are applied to determination of primary characteristics of these molecules such as molecular weight, isoelectric point (pI) , secondary and three-dimensional structure.

Faced with the wide applicability of peptides and the rise of studies involving these biomolecules, the necessity to get them quickly, in high amounts with unchanged structure is indispensable. Synthesis processes have provided a major breakthrough in the study of applicability of these molecules in recent decades [68]. For some biomolecules, such as peptides, the commonly applied methods of chemical synthesis, enzymatic, or even recombinant DNA technology [69].

In chemical synthesis is used a chemical reagent in order to activate the carboxylic acid from Nα-acyl amino acid or peptide Nα-acyl fragment (acyl donor or acylating agent). It becomes accessible nucleophilic attack of the amino group other amino acids or peptide fragment Cα-blockers (nucleophilic agent) forming the peptide bond between them [69].

Chemical synthesis may be employed to classic way (in solution) or on Solid-phase supported polymer synthesis - SPPS [70]. However, the most used method is on solid-phase to be a simple, quick and advantageous process. This system allows use excess to reagents to ensure synthesis cycle of peptides more efficient in each step of reactions [68]. Although having advantages in SPSS has some purification limitation due to intermediate products formation, since the synthetic synthesis cycle cannot reach the value close to the ideal (100%). Besides the large number of steps involved in the process can be obtained contaminants formation, which difficult the peptide separation afterwards [68].

In the enzymatic synthesis, or simply biocatalyzed synthesis, the linear amino acid composition is mediated by an free enzyme into the solution or immobilized form [71]. Proteases, enzymes responsible for breaking the peptide bond *in vivo*, are the most suitable catalysts for this strategy. The most used are the termolisis, pepsins, subtilisin, trypsin, quimotripsina, papain, clotripaínas and carboxypeptidases [69]. The experimental method for obtaining a peptide bond by enzymatic catalysis is based on the inversion of the peptide bond hydrolysis, transpeptidation and aminolysis of esters. This technique offers several advantages over the chemical synthesis. It has a high stereochemistry and structural selectivity that avoid racemic mixtures formation or secondary chemical reactions typical from chemical synthesis. In addition to the specificity of reaction and partial protection of substrates in the reaction media, the process can be produced in biological reactors for large-scale production and consequent cheapening of technique [69].

Relatively less employed than two methods mentioned above, techniques of molecular biology of cloning and gene expression can be used for synthesis of recombinant antimicrobial peptides. In this case, the factory of synthesis factory of the polypeptide chains will be microorganisms modified through gene insertion. Often researchers use bacterial expression system just to be simple and devoid of post-transcription and translation reactions in peptide sequences newly produced. However to shot peptide synthesis, less than 40 amino acid residues, with chemical modifications, like amidation, phosphorylation or cyclization, the classical chemical synthesis appears as the best option.

4. Working on antimicrobial peptides

Despite to broad spectrum of action of AMPs on different aspects, some characteristics, such as cytotoxicity, stability and high production costs, makes hard the large-scale production of the AMPs as well as their applicability as therapeutic agent [9, 72]. Also, peptides generally have a short half-life due to enzymatic degradation at the injection site or during travel to the site of action. [73]. The exploration of structural alternatives from peptides through specific changes have contributed significantly to the enhance of the potential therapeutic these biomolecules in order to overcome the obstacles currently faced [74].

The techniques of structural changes consist in altering the native structure of the peptide. In some cases, this process occurs naturally, such as post translational adjustments, such as adding an amide grouping the C-terminus, which makes the peptide more stable against the proteolytic action [75]. Among the changes we can mention synthetic chemical modifications and replacement or addition of chemical groups or amino acid residues [69].

Chemical modifications may occur for formation of new structures such as cyclic peptides and photo-cross-linking peptides. In the first case, is forming a disulfide bridge between two cysteines to increase the flexibility of the peptide frame. While the approach photo-cross-linking is characterized to biological action is activated by photoinduced binders [73, 76]. Minor changes also can change rationally some properties of AMPs. The addition of the lysine residue to the N-terminus on antimicrobial peptide Hy-a1 increased from three to four fold the antimicrobial effect. While the replacement of a leucine residue for tryptophan on the same peptide can promote a greater pore-forming ability [77].

Furthermore, changes in favor of reducing the hydrophobicity act on the reduction of cytotoxicity. Although hydrophobicity is required for membrane permeabilization, increasing levels this characteristic is strongly correlated with toxicity in mammalian cells and loss of antimicrobial specificity [33].

5. New light to antimicrobial peptides application

In December 2014, over 100 new peptides were recorded at Peptides Antimicrobial Database (APD), bringing the total number of entries to 2493 [38]. Target to numerous therapeutic applications, either just itself or combined with another compound, are alternatives to apply primarily by pharmaceutical and food industry [78]. Featuring antimicrobial activity against numerous bacterial, fungal and viral strains, as well as anti-tumor action, the pharmaceutical industry holds its applicability as possible constituents or therapeutic agents [79]. The interest of the food industry in the application of AMPs aims to manufacture a more healthy and natural products through inhibition of the growth of certain microorganisms as well as contamination risk of food [80]. Differently of the classic antibiotics, AMPs have been noted for presenting broad spectrum of activity, fast action, and rarely induce the development of antimicrobial resistance, as depicted on Figure 4 [81].

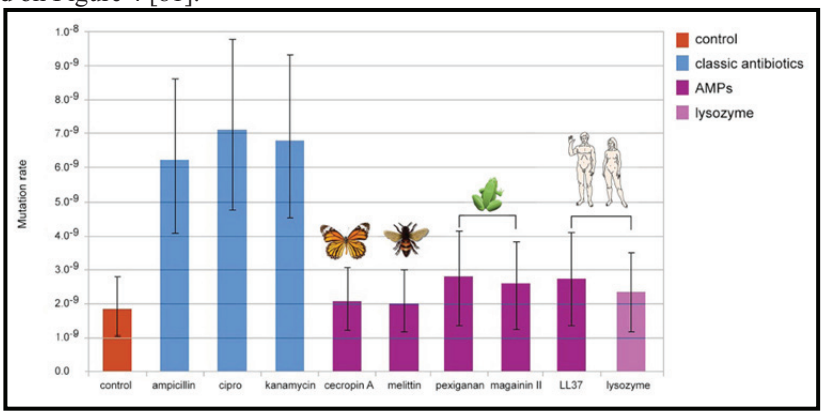

Fig. 4 Changes in mutation rate in *E. coli* MG1655 induced by different antimicrobial treatments. *Image available at: Rodríguez-Rojas A, Makarova O, Rolff J. Antimicrobials, Stress and Mutagenesis.October 2014, Volume 10, Issue 10, e1004445*

Thus, combinations methods among biomolecules that are often applied in order to promote additive or synergistic effects, since this technique of "Combination Therapy" aims potentiate the biological activity of a molecule. However, applications "*per se*", or by itself, are also considered potential in their mechanisms of action [82].

Currently, many peptides have been studied about its structure and function determined for the occurrence of structural modifications, as well as elucidated for potential biological activity *per se* and in combination models.

Table 2 Strategies applied to antimicrobial peptides to enhance the potential application to AMPs

Peptide	Sequence	Combination	Potential application	Reference
Aurein 1.2	GLFDIIKKIAESF*	Clarithromycin, Minocycline	*S. aureus; E. faecalis; S. pyogenes*	[83]
Gramicidin S	VXLFPVXLFP*	-	Gram+; Gram-; Fungus; Protozoa; Cancer	[84, 85]
Hepicidin 20	ICIFCCGCCHRSKCGMCCKT*	Anfotericin B Fluconazol Caspofungin	Clinical isolates of *Candida glabrata*	[86]
Indolicidin	ILPWKWPWWPWRR*	Teicoplanin	Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms	[87]
Magainin II	GIGKFLHSAKKFGKAFVGEI MNS*	Rifampicin	*P. aeruginosa* ATCC 27853; multidrug-resistant *P. aeruginosa*	[88]
Nisin A	ITSISLCTPGCKTGALMGCN MKTATCHCSIHVSK*	Lactoferrin	Food antimicrobial (*Escherichia coli* O157: H7 ATCC 43895; *Listeria monocytogenes* Scott A ATCC 19111)	[89]
Pleurocidin	GWGSFFKKAAHVGKHVGK AALTHYL*	-	Gram +; Gram-; Biofilms; Fungus; Cancer	[90]
Lys-a1	KIFGAIWPLALGALKNLIK**	Ciprofloxacin	*Pseudomonas aeruginosa* ATCC 9027	[91]

Protegrin IB-367	RGGLCYCRGRFCVCVGR**	Imipenem e colistin	*P. aeruginosa; Acinetobacter baumannii; Klebsiella pneumoniae; E. coli*	[92]
CWR11	CWFWKWWRRRRR***	-	Surface coating (Gram+; Gram -; biofilm)	[93]
CAMA	KWKLFKKIGAVLKVL****	Ciprofloxacin	MRSA ATCC 43300	[94]

* Natural peptide; ** Modified peptide; *** De novo peptide; **** Synthetic peptide

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