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REVIEW

Natural antimicrobial peptides

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ABSTRACT

The recent appearance of a growing number of bacteria resistant to conventional antibiotics has become a serious medical problem. To overcome this resistance, the development of antibiotics with novel mechanisms of action is a pressing issue. Endogenous antimicrobial peptides are attractive candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides. Antimicrobial peptides play a key role in the defense against bacterial pathogens, with an increased importance in those species lacking adaptive immunity. Their functions as key members of the innate immunity justify their potentiality as anti-infective therapeutic agents. An essential requisite for any host defense or therapeutic agent is selective toxicity over microbial targets and not to the host, implying a minimum risk for the latter. However, antimicrobial resistance to this sort of compounds must be carefully analyzed. The searching of new alternatives must be guaranteed by the previous knowledge about these molecules mechanism of action and structural determinants for activity. The purpose of this review is to show the main functional features that determine the antimicrobial peptides activity, with an insight in their mechanism of action. Here we expose basic knowledge and considerations about these molecules that must be taken into account for the new researchers in the field.

Keywords: antimicrobial peptides, mechanism of action, cytotoxicity, microbial resistance

RESUMEN

Péptidos antimicrobianos naturales. La reciente aparición de un creciente número de bacterias resistentes a los antibióticos convencionales se ha convertido en un problema serio para el sistema de salud mundial. Para superar esta resistencia es apremiante el desarrollo de antibióticos con nuevos mecanismos de acción. Los péptidos antimicrobianos son candidatos exitosos como nuevos agentes antimicrobianos debido a su amplio espectro de acción, alta selectividad citotóxica y su dificultad para que las bacterias desarrollen resistencia a éstos. Con esta revisión actualizada de la materia pretendemos mostrar conceptos básicos que deben ser dominados en los estudios que se realicen en el descubrimiento de nuevas moléculas de naturaleza peptídica con actividad antimicrobiana. Los péptidos antimicrobianos desempeñan una función primordial en la defensa contra patógenos bacterianos, teniendo un peso mucho mayor en aquellas especies que carecen de inmunidad adaptativa. Su función como moléculas claves de la inmunidad innata justifica su potencialidad como agentes terapéuticos antiinfecciosos. Un requisito esencial para cualquier agente de defensa del organismo o agente terapéutico es la toxicidad selectiva sobre objetivos microbianos más que sobre el hospedero, que impliquen un riesgo mínimo para este último. Sin embargo, la resistencia de los microorganismos a este tipo de compuestos debe ser cuidadosamente analizada. La búsqueda de nuevas alternativas debe estar respaldada por el conocimiento previo de los mecanismos de acción de estas biomoléculas así como los factores estructurales que determinan su efectividad. La literatura actualizada que se refiere en el artículo muestra las principales características funcionales de los péptidos antimicrobianos que determinan su actividad, profundizando además en el mecanismo de acción de los mismos. Se exponen conocimientos básicos y consideraciones sobre estas moléculas a tener en cuenta por los investigadores en el campo.

Palabras clave: péptidos antimicrobianos, mecanismo de acción, citotoxicidad, resistencia microbiana

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Introduction

Living organisms are commonly exposed to microbial infections and environmental pathogens. Consequently, they have developed potent defense mechanisms as part of their innate or adaptive immune systems. One of the most relevant innate mechanisms comprises the production of substances displaying antimicrobial activity, which are mainly small peptides or polypeptides called antimicrobial peptides (AMPs) [1]. This type of peptides can be constitutively expressed or induced and released in response to the interaction with the invading pathogen or its components, the specific mechanism depending on the organism itself.

Since the first isolation of Nisin A from *Lactococcus lactis* as early as in 1947 [2], among the first peptides identified displaying antibacterial activity, the number has grown considerably, and up to 3000 such peptides are reported yearly at specialized databases as in the Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.php), all of them isolated from natural sources. They have shown a wide range of mechanisms of antimicrobial activity against bacteria, fungi and viruses with additional immunomodulatory effects. More precisely, AMPs denomination is currently reserved to those peptides

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3. Hale JD, Hancock RE. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. Expert Rev Anti-infect Therapy. 2007;5(6):951-9.

when referring to their microorganisms-killing functions. In this regard, AMPs can be grouped attending to their specific effect against pathogens, including antiparasitic, antiprotozoal or antibiofilm AMPs. The dbAMP database (http://csb.cse.yzu.edu.tw/dbAMP/) hosts more than 4000 entries for natural AMPs which activity has been demonstrated or presumed by transcriptomic studies. In this database, peptides are differentiated attending to functional criteria, with more than 50 % being classified as antibacterial peptides and 32 % as antifungal (Figure 1).

This differentiates them from those settings in which other alternative functions are discussed, such as immunomodulatory, angiogenic, healing or chemotactic activities, among others, or when such functions determine the mechanism of action of the molecules themselves *in vivo*, being denominated host defense peptides (HDPs) [3].

There are certain significant sources for natural AMPs relevant for their specific activity, despite this type of peptides have been found in all the organisms studied to date. For instance, defensins have been widely assessed in humans, while more than a thousand peptides identified in frog secretions are among the most intensively analyzed ones due to their antimicrobial potential. The most frequently researched biological sources of AMPs are summarized in the table.

The aim of this review is to provide an overview of compounds classified in the group of natural AMPs. Their main structural properties, including those less frequently found, are discussed here. Moreover, their mechanisms of action on the cellular membrane and the characteristics mediating them are also described, in the light of current debates on the possible application of AMPs to circumvent microbial resistance.

AMPs: basic concepts

AMPs comprise a unique and diverse group of molecules which are classified in subgroups or families attending to their structural properties determined by peptide primary sequence and tridimensional conformation. At the same time, a given family groups those peptides showing a specific structural constraint that determines its action on the membrane of a particular set of microorganisms. Noteworthy, there is also a variety of effects on the membrane among the peptides belonging to a single AMP family, broadening their spectrum of action over several antibiotic-resistant pathogens for the treatment of infectious diseases [4].

Up to date, the research and discovery of new structures with associated antimicrobial activity has necessarily and continuously widen the parameters considered for AMPs classification. The well-known family of cationic AMPs (CAPs) constitute the largest set of AMPs molecules characterized in the literature [5]. All of them share as main properties a relatively low number of residues and molecular weight (12 to 50 amino acids), a net positive charge (from +2 to +9) and they are hydrophobic. Moreover, they are encoded in the genome with constitutive and/or inducible expression, the last triggered by signals from infectious and/or inflammatory agents [6].

More recently, new AMPs have been described which structural properties tend to differ from those abovementioned classification parameters while displaying antimicrobial activity. Among them are natural anionic peptides, both anionic and cationic peptides derived from larger protein molecules, and even anionic and cationic peptides of molecular weights higher than 10 kDa [7, 8]. According to their properties, these molecules can be arranged following either the classical or the non-classical classification criteria [9]. Another more practical classification resides on the traditional classification attending to their

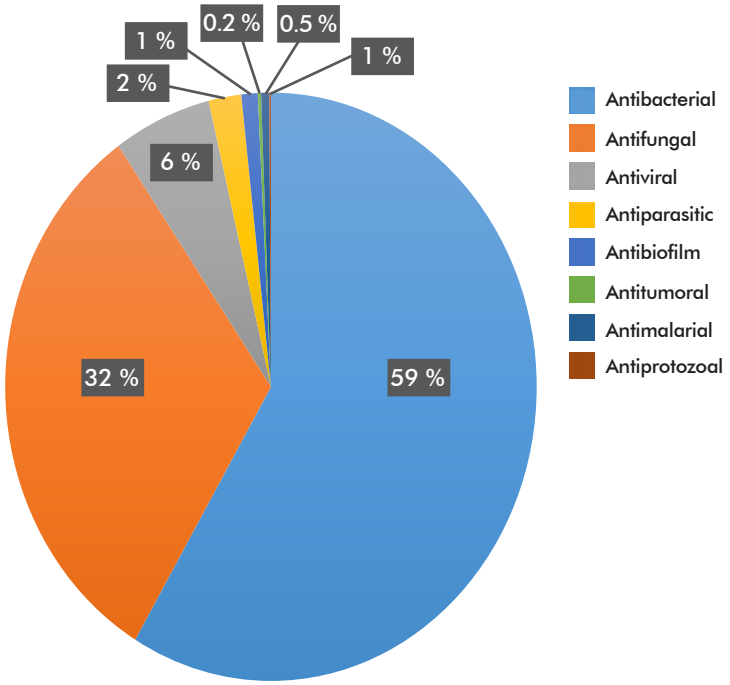

Figure 1. Main functional activities detected in natural antimicrobial peptides. Elaborated from the information retrieved from the dbAMP database (http://csb.cse.yzu.edu.tw/dbAMP/).

Table. Summary of the main natural sources and distinctive structural properties of some natural antimicrobial peptides

Species	Peptide family	Structural properties	Mechanism of action
Humans	Defensins	β-sheets / 3 disulfide bonds	Membrane disruption
	Cathelicidins	α-helix	Membrane disruption
	Dermcidin	α-helix	Membrane depolarization
Amphibia	Buforins	α-helix	Membrane disruption / DNA binding
	Temporins	α-helix	Membrane disruption
	Magainin	α-helix	Membrane disruption
	Dermaseptin	α-helix	Membrane disruption
Crustaceans	Penaeidins	Proline-rich / α-helix domain	Undescribed
	Crustins	Cluster of 3 or 4 disulfide bonds	Undescribed
Insects	Defensins	Csαβ motif	Membrane disruption
	Cecropins	Disordered / α-helix	Membrane disruption/ depolarization
	Apidaecins	Proline-rich	ATPase/Protein folding inhibition
	Melittins	α-helix	Membrane disruption
Plants	Defensins	β-sheets / α-helix	Diverse mechanisms
	Thionins	β-sheets / α-helix	Membrane disruption
	Knottin-type	β-sheets	Vacuolar ATPase inhibition

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mechanism of action on their target cells, being divided in membrane breaking or non-breaking peptides, the later penetrating the cell membrane and binding their target molecules within the cell [10].

Classical cationic AMPs

Most AMPs can be grouped following secondary structure criteria in three main groups: peptides in α-helix, peptides enriched in one specific amino acid, and antiparallel β-sheets conformations stabilized through disulfide bonds (Figure 2). Many CAPs adopt an amphipathic α-helix structure in the cell membrane microenvironment (Figure 2A), which is regarded a prerequisite for lytic activity. Several *in vitro* and *in vivo* studies has demonstrated the antimicrobial activity as mediated by a complex and fine balance of peptide parameters including the peptide chain length, net charge, hydrophobicity, secondary structure, amphypaticity, the size and depth of the polar helix in respect to apolar zones in the peptide, the molecule's flexibility and the resistance to degradation. The specific preponderance of each of these properties tends to vary according to the peptide. Hence, engineering peptides of low toxicity and high antimicrobial activity has to be established case by case [11].

This subgroup comprises around 300 cationic peptides which are short, spanning 40 amino acid residues approximately, lacking Cys and sometimes bearing a sort of molecular hinge in the middle of the amino acid chain. They form complex and disordered structures in water solutions. Particularly, they form complete or partial α-helixes in the presence of trifluoroethanol, in liposome dispersions [12] or Lipid A [13]. This structure correlates with a strong wide-range antimicrobial activity against both Gram-positive or Gram-negative bacteria [14].

There is another subgroup formed by peptides approximately 50 amino acids in length and rich in proline, arginine, tryptophan or phenylalanine (among other residues), these amino acids conferring them its hydrophobic nature and positive charges. This subgroup also lacks Cys residues and are generally linear molecules while forming extended spirals occasionally (Figure 2 B) [5]. Moreover, it completely diverges from α-helix and β-sheets prototypes.

In the case of Cys-bearing peptides and β-sheet formation, they are composed of a quite diverse subset of molecules attending to their primary sequence. They commonly show antiparallel β-sheets stabilized by up to six disulfide bonds [15]. A few years ago, a multidimensional proteomics analysis discovered a common motif for all the Cys-stabilized antimicrobial peptides. It was called 'γ-core' and it is composed by two antiparallel β-sheets, with polar basic residues along the axis (Figure 2C) [16]. In fact, this structural motif is recurrent to all the classes of Cys-stabilized defense peptides found in the organisms [17].

Currently, it is relatively easy to design and create synthetic antimicrobial peptides based on the sequences known, due to their small structure and the number of physical properties observed, combined with their marked natural sequence variability and amino acid chain length [18, 19]. Nevertheless, their sequence homology is rather limited, even for peptides belonging to the same family, something explaining to some extent its successful and prolonged evolution.

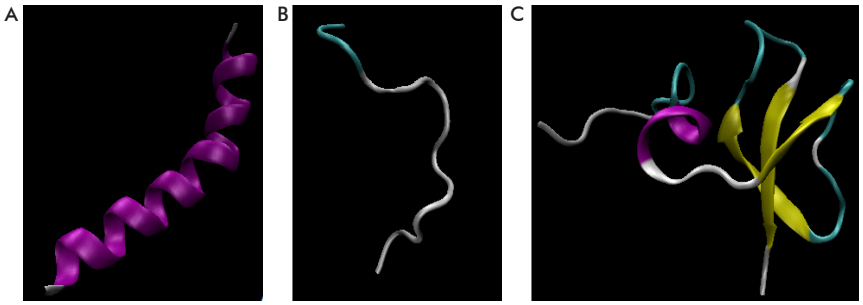

Figure 2. Classic secondary structures of natural antimicrobial peptides. A) α-helix (Melittin). B) Abundance of a single amino acid (Indolicidin). C) Antiparallel β-sheets stabilized by disulfide bonds (hBD3). Loop structures are shown in blue, β-sheets in yellow, α-helixin purple and disordered structure in gray.

Non-classical AMPs

One relevant non-classical AMPs subgroup comprises anionic peptides 5-70 amino acid in length, rich in glutamic and/or aspartic acid, the latter commonly conferring them with a negative –1 or –2 net charge. They are produced in millimolar concentrations, requiring zinc as cofactor for its antimicrobial action and they are effective against both Gram-positive and -negative bacteria [19]. They are similar to the charge quenching pro-peptides found in large zymogen molecules, which display its antimicrobial activity once synthetized separately [7].

In this family, there is another subgroup of nearly 400 peptides both anionic and cationic. They bear Cys residues as disulfide bonds and adopt a stable β-sheet conformation. This is one of the most numerous groups which includes protegrins and a very diverse family of defensins (α, β and θ) found in vertebrates, invertebrates and plants [7, 20].

Finally, there is a group of anionic and cationic peptides which are fragments from larger proteins. They are similar in composition and structure to the previously mentioned peptides, and comprise peptides like lactoferricin B. This molecule acts by altering the ionic equilibrium of the pathogen cell while simultaneously triggering the immune response activation by stimulating the phagocytic activity of neutrophils and its IL-8 secretion [21].

Noteworthy, the structural classification of AMPs can vary attending to the selected properties, mainly their structural features, their natural source [16] or a combination of both. It could include those synthetic AMPs, with the rise of in silico bioactive AMP design tools. Hence, a fourth group has been proposed for classic AMPs denominated 'peptides with looped structure and single bond' [22] in addition to those susceptible to form α-helix or β-sheet structures, or those showing predominance of a given single amino acid. This group comprises peptides with antiparallel β-sheets stabilized by a single disulfide bond.

Other classifications have been proposed for AMPs based on their biological activity [23]. Nevertheless, the most accepted classification criteria based on the structural properties of AMPs are presented here.

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*S*tructural features of AMPs determining its biological activity

Selective cytotoxicity for its microbial target is the main requirement for any defense agent of the organism or therapeutic agent to be developed. In this regard, AMPs selectivity resides on initial recognition of the highly preserved bacterial molecular structures, which are absent on the host cells. These specific structures called 'pathogen associated molecular patterns' (PAMPs) are common to a wide spectrum of microbes [24]. Besides, the recognition of these well preserved structures is further specified by distinctive structural constraints making AMPs able to discriminate among cell types to exert its action.

Amphypaticity [25] is one key structural property of AMPs. It consists on the selective location of hydrophilic amino acid residues along one side of the helicoidal molecule and the hydrophobic amino acid residues along the other side. A standard method to quantitate this property is the hydrophobic momentum (HM), which is calculated as the vectorial sum of normalized hydrophobicities of each independent amino acid on its ideal α-helix [26]. This peptide property highly correlates to the antimicrobial toxicity and efficacy of the peptides. Highly amphypatic molecules, mostly exhibiting segregated hydrophobic domains, tend to break zwitterionic membranes in mammalian cells. In fact, the increased amphypaticity has been correlated to the high efficacy of the antimicrobial activity in studies using helicoidal peptides and others bearing β-sheets of similar charge and hydrophobicity [9].

The amphipathic properties of the peptides resemble those of membrane phospholipids, allowing the former to interact an exploit vulnerabilities inherent to microbial structures as the cell membrane [27]. For this, peptides must have certain tridimensional topological homology which then translate into a structure able to penetrate a hydrophobic environment as the cytoplasmic membrane. Those structures showing these properties support their classification in two major groups: AMPs bearing α-helix or β-sheets, and the others enriched in one or more amino acid residues like Arg or Trp [28], Phe, His, Pro or other more unusual residues as Gly or Asp [29].

Other structural parameters such as conformation, charge and polar angle further influence on each mechanism of action of AMPs [30]. While studying the differential effect of each parameter, their interrelationship must be considered, since modifying one could notably influence on the others. Highly significant changes in a single parameter would affect the behavior of the entire molecule, ultimately determining a change in the properties of the AMP and compromising its antimicrobial activity.

Many AMPs characterized so far show a positive net charge in the range +2 to +9, with a well-defined cationic domain asymmetrically distributed on the peptide's structure. This is a key feature mediating the initial electrostatic attraction between the peptide and the negatively-charged microbial membrane, due to the presence of certain types of phospholipids. The fact that bacterial membranes are enriched of acidic phospholipids as phosphatidylserine (PS), phosphatidylglycerol (PG) and cardiolipin (CL) confers a net negative charge to those membranes [31]. Furthermore, the presence of PAMPs as lipopolysaccharide (LPS) in Gram-negative bacteria, or teichoic acid and teichuronic acid in Gram-positive bacteria imposes an additional negative charge, resulting in a highly ionized membrane. All these properties favor the electroaffinity among membranes and peptides, also aiding on the selectiveness of the latter [32].

There are several examples on the proportional balance between the increase in the net charge of peptides and their antimicrobial activity [33, 34]. However, not all their effectiveness is mediated by their electroaffinity despite the observed correlation. For instance, the temporins found in amphibians are the smaller size AMPs described (10-14 amino acid residues), and, despite their negative net charge, they are among the lowest charge peptides because of having just +1 or +2 cationic residues on their structure [35]. Moreover, the peptide Alamethicin is devoid of charged residues or Trp which could function as anchors when interacting with the interfacial membrane, but it shows a strong antimicrobial activity against Gram-positive and –negative bacteria [36].

AMPs require a moderate level of hydrophobicity for their action following the initial electrostatic attraction. Hydrophobicity determines the range in which they partition within the lipid bilayer. The lipid portion is defined by approximately 50 % of the amino acid residues showing this property [7]. Conversely, the excess of hydrophobicity influences on the loss of antimicrobial activity and increases the peptide's toxicity in mammalian cells [37].

There has been described that hydrophobicity significantly affects the AMP selectivity by modifying the activity on phosphatidylcholine (PC) bilayers [38]. A high membrane permeation efficiency has been reported for AMPs on palmitoyl-phosphatidylcholine (POPC) vesicles. On the contrary, the extension of the polar/hydrophobic angle correlates with the increase activity on 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) [39]. In fact, the increased concentration of peptides on the lipid bilayer destabilizes it through electrostatic interactions with the negatively charged lipid membranes, as the ones formed by (POPG). However, this effect is associated with a decrease in the peptide permeation efficiency. Hence, there is a threshold determined by the combination of AMP hydrophobicity, its hydrophobic momentum and the polar angle, distinctively influencing on the effect over prokaryote membranes, mammalian lipid bilayers and cell membranes [9].

In this regard, the polar angle acts as a measure of the relative ratio of the polar vs. the apolar side of the peptides forming an amphipathic helix. For instance, a hypothetical α-helix peptide with one side comprising hydrophobic residues exclusively and charged residues in the other, the ideal polar angle is 180º. Therefore, a slight separation between these two domains or an increase in the hydrophobic portion of the α-helix could proportionally reduce the polar angle [27]. Several studies with natural and synthetic peptides have shown that a small change in the polar angle is associated to the rise in the membrane permeation capacity of the peptide [40, 41]. The polar angle has

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been correlated to the stability and the half-life time of peptide-induced membrane pores [42].

Mechanisms of action of AMPs

AMPs have been regarded as 'dirty drugs' in the scientific literature [43], due to their amphiphilic and cationic nature mediating their multiple targets. For many years they were assummed to act through the interaction with the microbial membranes affecting its integrity (Figure 3). Nevertheless, alternative mechanisms and/or molecular targets have been postulated which have locations other than the cellular membrane [3]. Therefore, AMPs have been classified into two main functional classes: membrane disrupting peptides and membrane non-disrupting peptides [44]. The differentiation between both types of peptides is quite difficult since all the peptides attack membranes during the initial interaction, but it does not always result in the disruption of the supramolecular assembly of the membrane. A possible outcome could be the increase in permeability leading to peptide translocation within the cell, where it interacts with the final molecular target.

Membrane-disrupting AMPs

The interaction with the cytoplasmic membrane starts when the AMPs get into contact with phospholipids. While the peptide:lipid ratio is low, the AMPs remain associated along the membrane plane, inserted into the hydrophilic interface of lipid heads and the hydrophobic part of the acyl heads [7]. When the peptide:lipid ratio increases, peptide aggregation and/or reorientation towards the membrane begin, disrupting membrane's integrity. This disruption process follows certain models proposed in the literature [3, 7].

Particularly, the 'Barrel-stave' model (Figure 2A) is known as a helicoidal mallet [45]. Following peptide association with the membrane and the rise above the critical threshold peptide:lipid concentration, peptides get reoriented perpendicular towards the membrane, and further expands the lipid bilayer, as in the case of the non-cationic AMPs Alamethicin [45]. The hydrophobic side of the lateral chain reorients towards the hydrophobic core of the membrane, and the polar side of the lateral chain heads inside, creating a hydrophilic pore that expands until reaching both sides of the membrane. Then, cytoplasmic content can be released through the pore. Nevertheless, this model is unable to explain the pore formation process, since the obtained channels are quite irregular in size and they are somehow transient and relatively selective for anions (something relying on the orientation of cationic groups into the lumen of the channel) [3].

A model called the 'toroidal-pore', also known as the 'wormhole' (Figure 2B) has been postulated, in which peptides bind to the membrane and cause the folding of the membrane inside, leading to a channel recovered by the phospholipids polar heads associated to the interface, forming a transmembrane continuous channel. In this structure, the peptides remain attached the most to the lipid heads along the entire process, quite different to the process seen in then 'barrel-stave' model. Consequently, the formed pores release the cellular components, ultimately causing cell death.

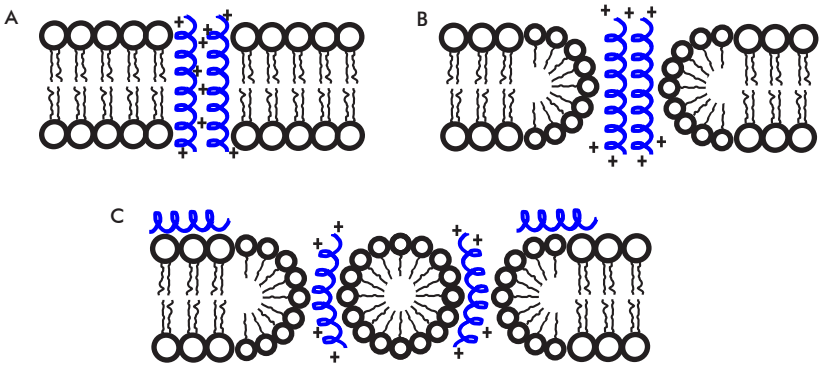

Figure 3. Diagram of the classic mechanisms of cationic antimicrobial peptides' interaction with membranes. A) 'Barrel-stave' model. B) 'Disordered toroidal pore'. C) 'Carpet model'. Peptides are represented in blue.

For instance, peptides magainin-1, melitin and protegrin-1 interacts with the membrane forming this pore type [46, 47]. One variant of this model is named the 'disordered wormhole' or 'disordered toroidal pore', with a less pronounced lipid folding, keeping most of the peptides parallel to the bilayer, with just one or two peptides located near the pore core [48].

A very distinct model called the 'carpet model' (Figure 2C) has been described, in which the peptides do not insert into the membrane, remaining associated to the interface region of the outer layer instead. Once the critical peptide concentration is achieved, peptides forms a carpet capable of weakening the bilayer structure through the destruction of the electrostatic surface, and thereby provoking the collapse of the membrane into a micellar configuration [49]. That is the case of the AMP denominated PMAP-23, which amount of peptide required for such membrane collapse has been successfully quantitated in the range of 10^6-10^7 peptides per cell, enough for destabilizing the lipid bilayer and causing bacterial death [50].

Another model called 'aggregate model' requires a specific concentration of peptides bound to the interface, which reordering forms a micelle-like complex with the lipids extending the lipid bilayer in a lipid-peptide complex. This random aggregation of transmembrane lipids, peptides and water molecules form channels through which ions are released, causing the cellular death by the release of cytoplasmic components. Alternatively, these complexes could spontaneously disintegrate, allowing peptide translocation into the cytoplasm, where they affect the cellular metabolism [51].

Membrane non-disrupting peptides

Recent evidences suggest that a high number of AMPs are able to act on target molecules located within cells, as frequent that there has been considered that their main target molecule is within the cell instead the membrane itself. This type of peptide is able to penetrate the cell directly or as an additive effect of its mechanism of action following an incomplete destabilization of the cell membrane. Possible intracellular targets are the varied number anionic compounds interacting with them, such as enzymes, nucleic acids, proteins involved in the cell division process, among others [3].

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All the previously mentioned models tend to predict the capacity of cationic AMPs to disrupt the cytoplasmic membrane, but only the 'toroidal pore' and the 'aggregate model' successfully explain the action of certain peptides on their cytoplasmic targets. The most relevant studies on the action of these peptides on nucleic acids [52, 53], on protein synthesis [54, 55] and protein translation and folding [56, 57] have demonstrated their use for treating infectious diseases. The activity of these peptides for the interruption of the cell wall synthesis and cell division processes has been reported [58, 59].

Selective cytotoxicity of AMPs

AMPS are highly selective for discriminating among microbial targets and host cells. The rules governing such selective cytotoxicity remain unraveled in full detail. Nevertheless, as already mentioned, there are conserved structural features which relate AMPs with their functional properties against potential targets. They are: a) the divergence between composition and the electrostatic affinities in microbial membranes as compared to those of the host cells; b) the entropy of target cells which accelerates the interaction of peptides with the microbial membranes and slows down such interaction with host cells; c) the limitations of AMPs with low selective toxicity for host cells to potentially access susceptible host cell types [27].

Effective AMPs are extensively characterized, their location for production and its range of activity well documented [60, 61]. Besides, the structure-activity relationship has been well described for many of them, while there is scarce literature on the molecular basis mediating the activity and specificity differences seen among them. For instance, differences found in the susceptibility of a single organism against a panel of AMPs has indicated the coincidence in the abovementioned distinctive properties for each subgroup as determining the AMPs activity [62, 63]. Otherwise, the differences found in susceptibility among a panel of microorganisms against a single AMP indicated that the composition of the microbial surface and the cytoplasmic membrane is relevant for such susceptibility [64].

An effective definition of the activity and specificity of AMPs should consider the in vivo studies conducted under physiological conditions. This includes the concentration of antimicrobial peptides at the infection site, the synergic role of the substances that could be present in tissues and fluids (the presence of bivalent cations, lysozyme, other endogenous peptides), the role of inhibitory substances (physiological concentrations of salts and serum proteins) and the unusual properties of bacterial replication in vivo [65].

Microbial resistance mechanisms against AMPs

The microbial antibiotic resistance phenomenon is increasingly alarming since they comprise the emergence of unprecedented evolutionary adaptations of microorganisms to existing therapies. This increases the vulnerability of any antimicrobial strategy to resistance in the post-antibiotic age. Therefore, many factors have to be taken into account which could influence ahead in the lack of effectiveness of some antimicrobial alternatives. In the case of natural AMPs, their biological success for host defense mechanisms against infections must be considered from the evolutionary point of view. Nevertheless, it is also relevant the natural regulatory pathways for the genetically encoded AMPs, which protects from the unnecessary exposure of tissues and even common microbiota to these peptides, unless challenged with the pathogens, something that protect these molecules from the development of potential resistance mechanisms by pathogens.

Microorganisms have evolved several resistance mechanisms against conventional antibiotics that can interfere with the antimicrobial action of AMPs. In certain cases, microorganisms have even changed their cytoplasmic membrane structural composition, something that could interfere with the initial step of attraction and interaction of AMPs with microbial membranes. An example is found in bacteria as *Staphylococcus aureus*, which incorporated D-Ala residues in lipoteicoic acid, thereby reducing the negative charge of the latter molecule and interfering with peptide binding to the cell surface [66]. Other alterations of the membrane are found in *Salmonella*, where the transference of a palmitate chain to a free hydroxyl group in Lipid A decreased the fluidity of the membrane and hampers the insertion of AMPs in the bilayer [67].

In addition to the modification of the lipids present on the membrane surface, either aimed to modify the net charge or to alter the membrane's fluidity, other resistance mechanisms against AMPs have been reported. Certain bacterial strains are able to produce highly elaborated outer matrixes which encapsulate the cell, providing the microorganisms with protective mechanical barriers that block the interaction of the peptides with the cytoplasmic membrane for their protection. Such extracellular matrixes can function either as electrostatic barriers that reject or sequester AMPs as mechanism of resistance against them, as reported for *Klebsiella* and *Neisseria* strains [68].

It is also known that many bacteria release proteases that degrade AMPs, particularly those linear and less stabilized peptides. The rigidity conferred by disulfide bonds and Pro residues to many AMPs make them more resistant to this type of proteolysis [43]. Lastly, some efflux mechanisms present in certain microbes function as resistance pumps against AMPs [69], expelling them from the cytoplasm or the periplasmic space outside the cell. Noteworthy, it is plausible that the resistance mechanisms mentioned could be found simultaneously in a given pathogen, similarly as demonstrated for conventional antibiotics [70]. For instance, in *Neisseria meningitidis*, the mechanism of modification of surface lipids and an efficient efflux excretion mechanism coexist against AMPs with the polysaccharide encapsulation of the cell. Their combination has been found to confer resistance of this bacteria against certain AMPs [71].

Regardless the abovementioned limitations, the therapeutic potential of AMPs is undeniable. Antimicrobial peptides are able to evade the classic resistance mechanisms expanded among infectious microorganisms and to be effective. In fact, they have proven effective against multi-resistant bacteria refractory to

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conventional antibiotics, leading to consider them as promising therapeutic drugs.

Conclusions

Due to the chemical-physical properties of genetically-encoded AMPs, their mechanisms of action which directly influences on the lipid composition of microorganism' membranes and cell walls, the AMPs described can be regarded as molecules with great potential for the design of new generation antibiotics. Currently, the dynamics for obtaining new generations of antibiotics are overrun by the adaptive capacity of microorganisms, due to the fast development of new resistance mechanisms. Hence, new antimicrobial therapeutic alternatives are needed. In this setting, the finding of new molecules with novel mechanisms of action must be accompanied by new strategies for using them, that can be adapted to conventional therapies. Therefore, AMPs can primarily function as non-classical treatments complementary to established antibiotic therapies, in order to palliate opportunistic and emergent infectious diseases.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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