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ARTÍCULO DE REVISIÓN

Advances in Antimicrobial Peptides with an approach to molecular structure prediction

Actualización en Péptidos Antimicrobianos con un enfoque en la predicción de estructura molecular

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Recibido: 2020-10-25

Aceptado: 2020-12-22

RESUMEN

Antimicrobial peptides (AMP) are molecules involved in the innate immune system of almost all living organisms. They are potent agents with diverse structural and antimicrobial properties, which represent one of the most promising future drug candidate for combating infections and antimicrobial drug resistance. They have also a wide spectrum of activity against a large number of pathogenic microorganisms, multiple mechanisms of action and a low potential for resistance. Numerous AMPs have been isolated from natural sources and many others have been de novo designed and synthetically produced. This review provide insight into antimicrobials properties of AMPs, focusing in antibacterial molecules, that provides better understanding of versatile biological properties of such peptides. Also, provide a short insight into chemical modifications, characterization and the principal methods for protein structure prediction of AMPs.

Palabras clave: antimicrobial activity, host defense peptides, protein structure prediction, Pom-1

ABSTRACT

Los péptidos antimicrobianos (AMP) son moléculas presentes en el sistema inmune innato de casi todos los organismos vivos. Ellos son un potente agente con diversas propiedades estructurales y antimicrobianas, lo cual lo convierte en uno de los más prometedores candidatos para enfrentar infecciones resistentes a drogas antimicrobianas, debido a que presentan un amplio espectro de actividad contra un gran número de microorganismos patógenos, múltiples mecanismos de acción y un bajo potencial de resistencia. Un gran número de AMPs han sido aislados de fuentes naturales y muchos otros se han diseñado de novo y producidos sintéticamente. La revisión proporciona información sobre las propiedades antimicrobianas de los AMPs, enfocándose en antibacterianos, proporcionando un mejor entendimiento de la versátiles propiedades biológicas de estos péptidos. Además, proporciona una breve información sobre modificaciones químicas, caracterización y los principales métodos de predicción de estructura de proteínas de AMPs.

Keywords: *actividad antimicrobiana, péptidos de defensa del hospedero, predicción de estructura de proteínas, Pom-1*

INTRODUCTION

Antimicrobial peptides (AMPs), also known as host defense peptides, are low molecular weight components of the innate immune system of almost all living organisms. They show a wide spectrum of activity against a large number of pathogenic microorganisms such as bacteria, viruses and fungi (Boparai and Sharma, 2020). In addition, they present multiple mechanisms of action, as well as a low potential for resistance (Hancock *et al,* 2000). These characteristics of AMPs have highlighted these molecules as therapeutic alternatives for the treatment of infections caused by multi-drug resistant strains (Gutierrez and Orduz, 2003; Galdiero *et al.*, 2015). However, its use has some disadvantages such as: low stability, cytotoxicity, poor biodistribution and high production costs (Di L. 2015).

The first MPAs were described several years ago, but did not reach relevance until the 1980s when the first insect AMPs were discovered: cecropins (1980), and the first amphibian MPAs: magainins (1987) (Zaslof, 1987) Since then, more than 5000 AMPs have been isolated from various sources (Kumar *et al.*, 2018). One of the most studied sources of AMPs is invertebrates. These organisms lack adaptive immunity and depend almost exclusively on these peptides for their defense, which is why AMPs play a crucial role as effector molecules of their innate immune system (Hancock *et al.*, 2006).

Knowledge of the three-dimensional structure of a protein can provide invaluable hints about its functional and evolutionary features and, in addition, the structural information is useful in drug design efforts (Mihăşan, 2010). Experimental methods for identification and designing of antibacterial peptides are costly, time consuming and resource intensive. Thus, there is a need to develop computational tools for predicting antibacterial peptides, which could be used to design potent molecules against bacterial pathogens (Lata *et al.,* 2007).

This review will provide an overwiew of antimicrobials properties of AMPs, focusing in antibacterial AMPs. Also, provide a short insight into chemical modifications, characterization and the principal methods for protein structure prediction using *in silico* techniques of AMPs.

ANTIMICROBIAL PEPTIDES

Common Properties

AMPs are characterized by their small size of between 10 and 100 amino acids. These have a positive charge (generally between +2 and +13) due to the large number of positively charged amino acids (Kumar *et al.*, 2018) and an amphipathic structure with ≥ 30% hydrophobic residues, allowing them to interact with the membrane lipid of microorganisms (Brandenburg *et al.*, 2012; Huerta-Cantillo and Navarro-García, 2016).

In their antimicrobial action, they select multiple targets, so the development of resistant strains to them is limited (Marr *et al.*, 2006). They are found in almost all living organisms including fungi, bacteria, insects, vertebrates, plants (Gutiérrez and Orduz, 2003), and even viruses (Huerta-Cantillo and Navarro-García, 2016). In addition to their antimicrobial activity, they can exert anticancer activity (Kumar *et al.*, 2018) and stimulate the immune system (Otero-Gonzalez *et al.*, 2010).

These peptides have a complex mechanism of action because they can interact with the pathogen directly but can also modulate the immune response to them. Direct interaction with the pathogen occurs mainly through the lipid membrane, but can also affect intracellular targets. On the other hand, they interact with the adaptive immune system through different routes, such as the regulation of the inflammatory process and healing (Kumar *et al.*, 2018; Tellez and Castaño, 2010).

They are produced by different cell types in a constitutive or inducible way, depending on the organism and the type of tissue where the infection is located (Tellez and Castaño, 2010). Among the different cell types that synthesize them we can find keratinocytes, epithelial cells of the respiratory tract or urogenital tract, Paneth cells of the small intestine, neutrophils, Natural Killer cells, mast cells and endocrines glands. (Castañeda-Casimiro *et al.*, 2009).

They present a great diversity both structurally and in terms of the producer organism, cell target, mechanisms of action, etc. (Alba *et al,* 2012), so they are classified according to these characteristics. According to Brogden (2005), taking into account the structure and composition of their amino acids, AMPs are divided into: 1.) anionic peptides, which include small molecules rich in glutamic and aspartic acid; 2.) α-helical linear cationic peptides, such as cathelicidin LL-37 from humans; 3.) cationic peptides enriched with specific amino acids, such as proline, arginine or glycine; 4.) cationic and anionic peptides that contain cysteine and

form disulfide bonds and 5.) anionic and cationic peptide fragments of larger proteins. However, one of the most recent structural classifications groups them into three main groups (Fig. 1): those that adopt an α-helix structure, those that adopt a β-sheet structure and those that have an extended structure.

Figure 1. Structural diversity of antimicrobial peptides (AMPs). A: α-helical penaeidin peptide family (pdb code: 1UEO) isolated from *Litopenaeus vannamei*. B: β-sheet (hairpin-like) arenicin 1 (pdb code: 2JSB) isolated from *Pinnixa arenicola* C: fragment of random (extended coil) of Cg-Def (pdb code: 2B68) isolated from *Crassostrea gigas*. Taken with permission and with modifications from Otero *et al.*, 2010).

Figura 1. Diversidad structural de los péptidos antimicrobianos (AMPs). A: α-hélice péptido de la familia de las penaedina (código pdb: 1UEO) aislada de Litopenaeus vannamei. *B: hoja β arenicin 1 (código pdb: 2JSB) aislado de* Pinnixa arenicola. *C: estructura extendida de un fragmento aleatorio de Cg-Def (código pdb: 2B68) aislado de* Crassostrea gigas. *Tomado con permiso y modificado de Otero et al., 2010.*

AMPs with α-helical structures are the most abundant and are generally found in the extracellular matrix of insects and toads. Many of these peptides are unstructured in aqueous solutions, but they change their structure to α-helix in contact with environments that simulate the lipid membrane. LL-37 is one of the AMPs belonging to this group. It is the only member of the cathelicidin family of human origin and is one of the most studied AMPs. The cathelicidin family is one of the most diverse in vertebrates, its members have a range between 12-18 amino acids and can adopt other structures. Another group of α-helical AMPs is that of magainins, originally isolated from the African frog *Xenopus laevis*, showing activity against Gram-positive and Gram-negative bacteria, fungi, yeasts and viruses (Galdiero *et al.*, 2015; Kumar *et al.*, 2018).

AMPs which adopt a β-sheet structure, such as protegrins (belonging to the cathelicidin family), defensins and tachypiplesins, show in their structure cysteine residues that form disulfide bonds. Unlike α-helical peptides, these AMPs have a defined structure in solution and do not undergo significant changes when they pass from an aqueous environment to a membrane-mimetic environment. In this group, the defensin family stands out, which has been categorized into subfamilies based on the location of the disulfide bond. These bonds provid them with structural stability and minimizes degradation by proteases. Defensins are involved in the host's antibacterial, antifungal, antiviral, inflammatory, and immune responses (Kumar *et al.*, 2018).

The last group presents a unique extended structure. Many of these AMPs belong to the cathelicidin family and have two or more proline residues, which disrupt the regular α-helix and β-sheet secondary structures. An example of AMP with this structure is indolicidin, a peptide isolated from bovine neutrophils that has 13 amino acids and is rich in tryptophan residues. A representative compilation of mode of action, structure and peptide source of some AMPs is presented in Table 1. The classification of a peptide into a group according to its structure is not indicative of its mode of action or its spectrum of activity. Studies with α-helical analogs of the cecropin-melittin hybrid have revealed that even peptides that have similar secondary structures and minimal differences in primary sequence may possess different antibacterial activities (Jenssen et al., 2006).

Antimicrobial Peptides from invertebrates

AMPs are an essential component in the innate immunity of invertebrates, since the humoral response of these organisms is based on them. The main site of AMP synthesis in insects are fatty bodies, while in the rest of invertebrates they are produced by hemocytes, which migrate towards the point of infection and release peptides. These AMPs not only perform their direct antimicrobial action, but also modulate inflammatory responses, similar to that observed in the innate immune response in mammals (Hancock *et al.*, 2006).

An example of AMP isolated from invertebrates is butynin, a peptide found in the hemocytes of the scorpion *Androctonus australis* and showing activity against Gram-positive and Gram-negative bacteria. Others, such as tachiplesins and polyphemusins, isolated from the hemocytes of the king crabs *Tachypleus tridentatus* and *Limulus polyphemus*, show activity against a wide spectrum of organisms such as: bacteria, protozoa, viruses and fungi; they also have an affinity for lipopolysaccharides (LPS) (Balandin and Ovchinnikova, 2016). Mytilus A and B, obtained from the mollusk *Mytilus galloprovincialis*, show antibacterial activity. On the other hand, miticin B is also active against the fungus *Fusarium oxysporum* and the Gram-negative bacterium *Escherichia coli* (Otero-Gonzalez *et al.*, 2010).

Table 1. Mode of action (Nguyen *et al*., 2011) and structural features and source (Kumar *et al*., 2018) of some AMPs
Tabla 1. *Modo de acción (Nguyen et al., 2011), caracteristicas estructurales y origen (Kumar et al., 2018) de algunos AMPs*

Category	**Peptide**	**Unique Structural/Sequence Feature**	**Specific target/mode of action**	**Source**
α-helix	Melittin	Amidated C-terminus	Toroidal pore and Disordered toroidal pore	Bees
	buforin II	-	DNA, RNA	Toad
	LL-37	Amidated C-terminus	Membrane thinning/thickening	Humans
	Magainins	-	Phospholipase A2 activation and Toroidal pore	Frogs
	cecropins	Amidated C-terminus	Detergent micellization	Insects
β sheet	Protegrins	Cysteine rich	Toroidal pore	Pigs
	Tachyplesin Polyphemusin	Cysteine/arginine rich and amidated C-terminus	DNA	Horse Crab
	Defensins	Disulfide bonds	Lipid II	Mammals
Extended conformation	Tritrpticin	Tryptophan and arginine rich	-	Pigs
	Indolicidin	Tryptophan and amidated C-terminus	Phospholipase A2 activation, Oxidized phospholipid targeting and Anion carrier	Bovine
	PR-39	Proline and arginine rich	Intracellular proteins	Pigs

Antibacterial Peptides

The best-studied AMPs are those that show antibacterial activity, since bacteria are the pathogen that most frequently affects human health (Jenssen *et al.*, 2006). They can also have both antifungal and antiviral activity (Boman, 2003).

Antibacterial peptides can be both bactericidal (if they kill the bacteria) and bacteriostatic (if they inhibit their division). Generally, the minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) of the antibacterial peptides coincide, indicating that these peptides are mostly bactericidal (Marr *et al* ., 2006).

Compared with conventional antibiotics, the bactericidal effect of peptides is extremely rapid and can include multiple cell targets, which represents an advantage over conventional antibiotics. Another advantage is its ability to neutralize bacterial toxins and prevent sepsis or endotoxemia, being common and dangerous complications during infection (Marr *et al.*, 2006).

In addition, they are not affected by the same resistance mechanisms that affect current antibiotics because their interaction with the membrane does not require a specific target (Mookherjee *et al.*, 2020). This is why many MPAs have excellent activity against multi -drug resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) (methicillin is a beta-lactam antibiotic from the penicillin group) and multidrug resistant *Pseudomonas aeruginosa* (Marr *et al.*, 2006).

Mechanism of Action of AMPs

Originally, it was thought that the only mechanism of action of AMPs was the attack to the membrane of pathogens (Kumar *et al.*, 2018). Currently, it is known that the mechanisms of action of AMPs are divided into two categories: direct action on the pathogen and immunomodulation (Lai and Gallo, 2009).

AMPs Immunomodulation.

AMPs can interact with the adaptive immune system through different pathways (Fig. 2). These peptides, when synthesized by various cells of the immune system, such as macrophages and neutrophils, are among the first molecules that face pathogens (Kumar *et al.*, 2018). They can modulate both the pro-inflammatory and anti-inflammatory response by altering signaling pathways, directly or indirectly recruit effector cells such as phagocytes, promote dendritic cell maturation and macrophage differentiation, as well as apoptosis (Martell *et al*., review, submitted to Peptides). They also facilitate the release of neutrophil extracellular traps (NETs), alter the endotoxin-mediated signaling pathway, increase the pro-inflammatory response to nucleic acids and influence dendritic cell differentiation and T-cell polarization (Mookherjee *et al.*, 2020). In addition, they may have other roles in immunity, such as stimulation of angiogenesis, wound healing, and adjuvant action (Brown and Hancock, 2006).

AMPs can also protect the host against partially lethal effects resulting from an excessive anti-inflammatory response induced by Toll-like receptors. Cathelicidins suppress the transcription of pro-inflammatory cytokine genes (TNF-α and IL-6) and the release of pro-inflammatory mediators induced by LPS and other bacterial products. These neutralizing and repairing effects protect the host against the destructive effects of inflammation.

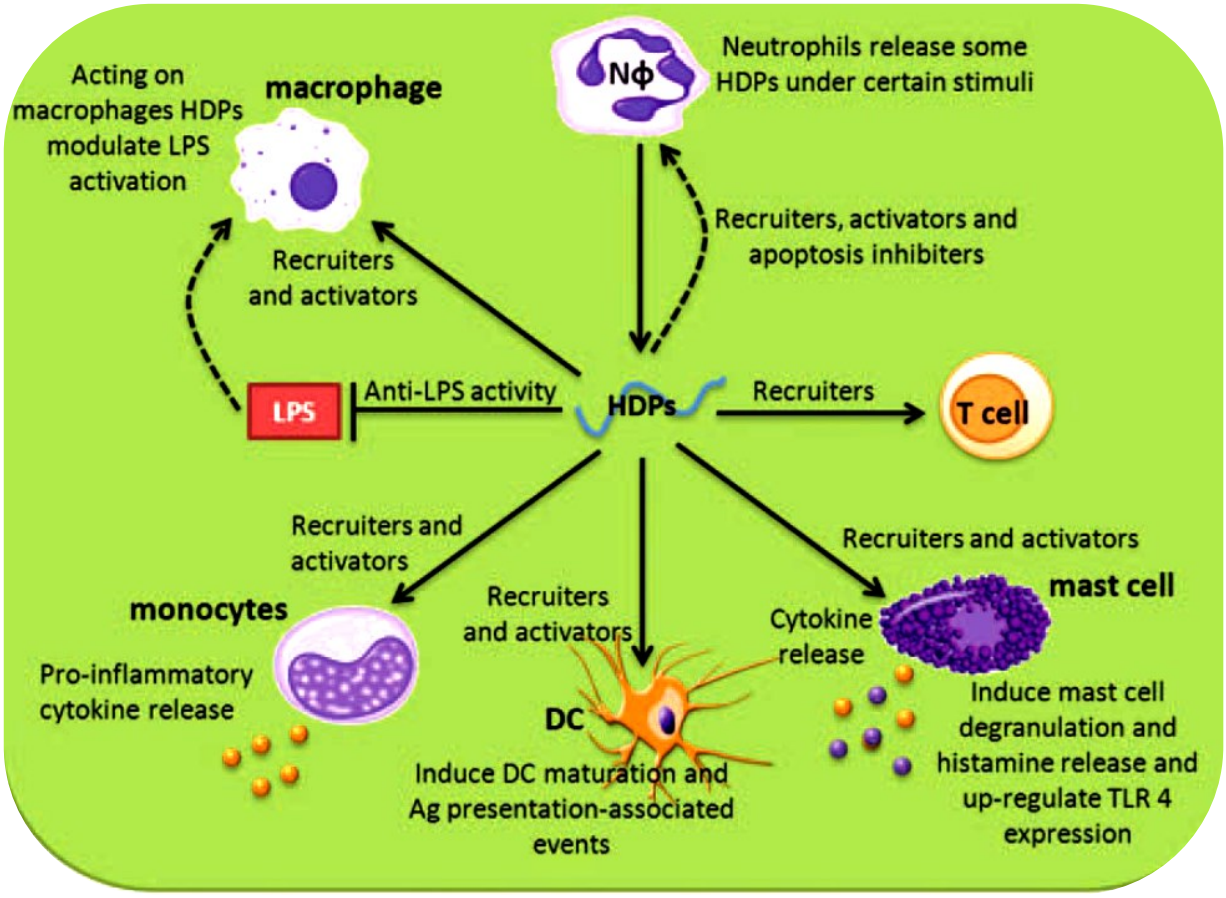

Figure 2. Main mechanisms of immunomodulation of AMPs in addition to wound healing and angiogenesis promoters contributing to the resolution of the infection or damage and maintaining the homeostasis (LPS: soluble lipopolysaccharide; NΦ: neutrophils; DC: dendritic cells; TLR Toll like receptor; Ag: antigen. Taken with permission from Alba *et al.,*2012.

Figura 2. *Principales mecanismos de inmunomodulación de los AMPs, además de la cicatrización y los promotores de la angiogénesis que contribuyen a la resolución de la infección o daño y al manteniento de la homeostasis (LPS: lipopolisacárido soluble; NΦ: neutrófilo; DC: célula dendríticas; TLR: receptor tipo Toll; Ag: antígeno). Tomado con permiso de Alba et al.,2012.*

Mechanism of direct action on the pathogens.

AMPs with direct action on microorganisms are subdivided into three groups: those that exert direct action on the cell membrane, those that exert it on the cell wall, and those that have intracellular targets (Fig. 3).

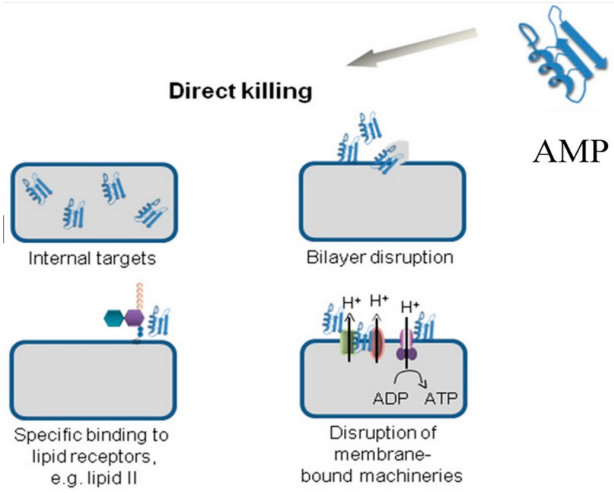

Figure 3. Principal mechanisms of direct action of AMPs (Kumar et al., 2018 adapted with permission from (Ulm, H.; Wilmes, M.; Shai, Y.; Sahl, H.-G. Antimicrobial Host Defensins Specific Antibiotic Activities and Innate Defense Modulation. Front. Immunol. 2012, 3, 249. Authorized by Biomolecules with full citation.

Figura 3. *Principales mecanismos de acción directa de los AMPs. (Kumar et al., 2018 adaptado con permiso de Ulm, H.; Wilmes, M.; Shai, Y.; Sahl, H.-G. Antimicrobial Host Defensins Specific Antibiotic Activities and Innate Defense Modulation. Front. Immunol. 2012, 3, 249). Autorizado por Biomolecules con cita completa.*

Action on intracellular targets

A substantial amount of AMPs, including polyphemusin, can translocate across the membrane and induce pathogen death by affecting cytoplasmic processes such as protein synthesis (Fjell *et al.*, 2012). These AMPs can exhibit several intracellular targets such as nucleic acids, to which they can bind and interfere with their synthesis. They can also inhibit the activity of some enzymes and the synthesis of the cell wall, stop cell division, stimulate autolysis or cause damage to the cell due to accumulation inside, etc. (Huerta-Cantillo and Navarro-García, 2016).

One of the AMPs that follows this mechanism of action is buforin II. In studies by Jenssen *et al.* (2006), it was shown that this AMP is translocated through bacterial membranes without causing permeabilization of the same and binds both DNA and RNA. Similarly, α-helix peptides derived from pleurocidin and dermaseptin inhibit DNA and RNA synthesis without destabilizing the *E. coli* membrane (Jenssen *et al.*, 2006). Another peptide that also inhibits DNA and RNA synthesis is indolicidin, while PR-39 stops protein synthesis and induces the degradation of some proteins necessary for DNA replication (Brogden, 2005).

Pyrrozidin penetrates the target cell and can bind to DnaK, a heat shock protein involved in protein folding. Specifically, this peptide inhibits the ATPase activity exhibited by DnaK, preventing protein folding and causing an accumulation of non-functional polypeptides, which causes cell death (Castañeda-Casimiro *et al.*, 2009).

Action on the cell wall

AMPs can also target the formation of structural components, such as the cell wall. The bacterially produced lantibiotic mersacidin interferes with transglycosylation of lipid II, a necessary step in the synthesis of peptidoglycan (29). Nisin, another lantibiotic, can also bind to lipid II, thus inhibiting cell wall synthesis in addition to its pore-forming activity. Interestingly, this is the same biosynthetic process that is targeted by the antibiotic vancomycin; however, mersacidin and nisin are thought to act by interacting with distinct molecular moieties within lipid II, explaining why these peptides are still active against vancomycin-resistant bacteria (Jenssen *et al.*, 2006).

Action on the cell membrane

The initial interaction of AMPs with the pathogen membrane occurs through electrostatic (positively charged AMPs and negatively charged lipid membrane) and hydrophobic interactions. By interacting with the membrane, they alter its permeability or cause cell lysis through the formation of pores (Huerta-Cantillo and Navarro-García, 2016).

Studies with magainins showed that peptide-induced membrane permeability changes are related to the anionic nature of the lipid. It was shown that in liposomes of phosphatidylglycerol, a lipid abundantly observed in bacterial membranes, the peptide induces effective permeabilization. On the other hand, in liposomes of phosphatidylserine, a characteristic lipid of mammalian cell membranes, the peptide is less

effective. However, other peptides such as melittin, paraxin, dermaseptins, and cecropins are mostly lytic in both bacterial cells and mammalian erythrocytes (Reddy *et al.*, 2004).

The mechanism of membrane permeabilization and pore formation by AMPs has been described by various models. Among the best-studied models are: the carpet model, the barrel model and the toroidal pore model (Tellez and Castaño, 2010). Other pore-forming mechanisms have also been described, such as the Shai-Huang-Matsazuki model (Kumar *et al.*, 2018) and the electroporation model (Nguyen *et al.*, 2011). All these models have in common the initial interaction of the peptides with the negative heads of the lipids on the surface of the membrane, adopting an orientation parallel to this (Jenssen *et al.*, 2006).

In addition to the models already mentioned, there are others that do not involve the formation of pores in the membrane. The presence of peptides in the lipid bilayer can cause its curvature (model of thinning / thickening of the membrane), as well as its remodeling, forming domains rich in anionic lipids around the peptide (anionic lipid clustering model). In some cases, the binding of the peptide to the membrane can increase if they target oxidized phospholipids (oxidized lipid target model). Additionally, the accumulation of AMPs can attract small anions to the other side of the membrane and cause their efflux (anion transporter model) or dissipate the membrane potential without causing any other apparent damage to the membrane (non-lytic depolarization model membrane) (Nguyen *et al.*, 2011).

Chemical modifications of AMPs

The selectivity of AMPs is given by their affinity to the membranes of different cells and is determined by various physicochemical parameters such as cationicity, hydrophobicity, amphipathicity, among others (Kim *et al.,* 2019). The positive charge favors the initial attraction of AMPs with negatively charged microbial surfaces (Kumar *et al*., 2018), so that increasing modifications improve these properties will favor the peptide's selectivity. Among the covalent modifications that can be made to AMPs, mention can be made of cyclization, polymerization, synthesis of multiantigenic peptides and conjugation to carrier proteins, introduction of N-substitutions or peptoid residues, variation of the N and C ends, pegylation, change of amino acids in the sequence, phosphorylation, glycosylation, lipidation or the synthesis of peptide-steroidal hybrids. These changes in peptide structures could result in greater selectivity and resistance to proteases, and increase conformational rigidity or immunogenicity (Morales *et al*., 2016).

The lipidation of peptides and intracellular proteins is carried out by incorporation of fatty acids, isoprenes and glycophospholipids, modifying the flexibility in the position occupied by the lipid, increases hydrophobicity and contributes to the association with the microbial membrane (Nadolski and Linder, 2007).

In addition, larger peptides tend to present greater cytotoxicity at equal concentrations, so it would be convenient to generate fragments from AMPs in order to reduce their cytotoxicity (Galdiero *et al.*, 2015).

An example of a modified peptide is the antifungal peptide Cm-p5; which was obtained by adding two new amino acid residues at the C terminal of Cm-p1. This significantly modified its cationicity, hydrophobicity and Boman index, improving its selectivity (Lopez-Abarrategui *et al*., 2015).

Characterization of AMPs

Many AMPs are isolated from natural sources and others are obtained by chemical design and synthesis. However, no matter their origin, all peptides must be characterized structurally and functionally. For this goal, various methods can be used, including as mass spectrometry, enzymatic cleavage and antimicrobial activity tests.

Structural analysis can be performed using spectroscopic techniques such as Circular Dichroism (DC) and Nuclear Magnetic Resonance (NMR), which allow to obtain the secondary and tertiary structure of peptides at comparable resolutions to those obtained by X-ray crystallography. Additionally, NMR provides information on peptides in solution, such as oligomerization state, lipid-peptide interaction, and peptide dynamics. Furthermore, using the information obtained by NMR it is possible to study the peptide under conditions analogous to those found *in vivo* (Wang and Morden, 1997).

Regarding functional characterization, agar diffusion and microdilution methods are the most used techniques to determine the spectrum of antimicrobial activity and MIC. In both methods, MIC is defined as the lowest concentration of the antimicrobial agent that prevents the visible growth of the microorganism (Wiegand *et al.*, 2008).

The study of the different mechanisms of action of AMPs on the membrane allows the characterization of their antimicrobial activity. This study is crucial because AMPs, regardless of their final target, interact with this cell structure (Ciumac *et al.*, 2019; Kumar *et al.*, 2018). For this, generally, different membrane models are used because they mimic the lipid composition of natural plasma membranes. These models also allow the study of different properties of AMPs, the role of lipids in cell interaction and the process of transport of peptides through the membrane (Ciumac *et al.*, 2019). In this sense, there are three widely used systems: lipid monolayer, lipid bilayer and liposomes (Deleu *et al.*, 2014)

Protein structure prediction

The knowledge of the 3D structure of the peptide could constitute a basis to better rationalize further exploration by focusing on the role of particular positions likely to affect peptide activity (Thévenet *et al.*, 2015).

The three-dimensional structure of a protein can provide invaluable hints about its functional and evolutionary features and the structural information is useful in drug design efforts. Accordingly, theoretical structure prediction methods can be divided in two different groups: template based methods, which include homology modelling and threading; and template free methods, also called *ab initio* methods

Template based methods.

Homology modeling

For proteins, the best option to get a 3D model now days is using comparative protein modeling, also called homology modeling (Thévenet *et al.*, 2015). Homology modeling makes structure predictions based primarily on its sequence similarity to one or more proteins of known structures. Homology modeling, is a class of method based on the fact that proteins with similar sequences adopt similar structures, as most protein pairs with more than 30 out of 100 identical residues were found to be structurally similar. Homology modeling is facilitated by the fact that 3D structure of proteins from the same family is more conserved than their amino acid sequences. When the structure of one protein in a family has been determined by experimentation, other members of the same family can be modeled based on their alignment to the known structure. This high robustness of structures with respect to residue exchanges partly explains the robustness of organisms with respect to gene-replication errors, that allows for the variety in evolution (Mihăşan, 2010).

Comparative modeling consists of five main stages: (a) identification of evolutionary related sequences of known structure; (b) aligning of the target sequence to the template structures; (c) modeling of structurally conserved regions using known templates; (d) modeling side chains and loops which are different than the templates; (e) refining and evaluating the quality of the model through conformational sampling (Floudas, 2007).

The accuracy of predictions by homology modeling, however, strongly depends on the degree of sequence similarity. If the target and the template share more than 50% of their sequences, predictions usually are of high quality and have been shown to be as accurate as low-resolution X-ray predictions. For 30–50% sequence identity more than 80% of the C^{α}-atoms can be expected to be within 3.5 ˚A of their true positions, while for less than 30% sequence identity, the prediction is likely to contain significant errors (Floudas *et al.*, 2006).

Protein threading

Also known as fold recognition, protein threading is a class of methods that aims at fitting a target sequence to a known structure in a library of folds. Generally, similar sequence implies similar structure but the converse is not true: similar structures are often found for proteins for which no sequence similarity to any known structure can be detected (Floudas *et al.*, 2006). This means that the actual number of different folded protein structures is significantly smaller than the number of different sequences generated by large scale genome projects (Floudas, 2007). An optimistic view is that the number of existing folds is a few orders of magnitudes smaller than the number of different sequences, possibly ranging from a few hundred to a few thousand.

The basic idea of protein threading is to literally “thread” the amino acids of a query protein, following their sequential order and allowing for insertions and gaps, into the structural positions of a template structure in an optimal way measured by a scoring function. This procedure is repeated for each template structure in a database of protein folds. The quality of a sequence-structure alignment is typically assessed

using statistical-based energy and the "best" sequence -structure alignment provides a prediction of the backbone atoms of the query protein (Mihăşan, 2010).

The main drawback of this class of methods is the fact that it is very demanding on the computing power and also, that there is still a need for target identification. Currently, the Protein Data Bank contains enough structures to cover small single domain protein structures up to a length of about 100 residues, so the method has the best chances of success with proteins within this limit (Mihăşan, 2010), like AMPs.

Template free methods.

Ab initio methods, also known as *de novo* methods, seeks to predict the native conformation of a protein from the amino acid sequence alone (Chivian *et al.*, 2003). AMPs are generally small in size which makes them particularly suitable for *ab initio* modeling (Kozic *et al*., 2018). Unlike the comparative protein modeling, a successful *ab initio* modeling procedure could help address the basic questions on how and why a protein adopts the specific structure out of many possibilities (Lee *et al.*, 2017).

These methods assume that the native structure corresponds to the global free energy minimum accessible during the lifespan of the protein, and attempt to find this minimum by an exploration of many conceivable protein conformations. These methods primarily utilize the fact that, although we are far from observing all folds used in biology, we probably have seen nearly all substructures. Structure fragments are chosen based on the compatibility of the substructure with the local target sequence, and then assembled into one new structure (Lee *et al.*, 2017).

Typically, *ab initio* modeling conducts a conformational search under the guidance of a designed energy function. This procedure usually generates a number of possible conformations (also called structure decoys), and final models are selected from them.

Therefore, a successful *ab initio* modeling depends on three factors: (1) an accurate energy function with which the native structure of a protein corresponds to the most thermodynamically stable state, compared to all possible decoy structures; (2) an efficient search method which can quickly identify the low-energy states through conformational search; (3) a strategy that can select near-native models from a pool of decoy structures (Lee *et al.*, 2017).

Even though the methods from this last class are computationally very demanding and still lack accuracy, they are continuously used and developed for several reasons (Mihăşan, 2010). Firstly, in some cases, even a remotely related structural homolog may not be available. In these cases, *ab initio* methods are the only alternative. Secondly, new structures continue to be discovered which could not have been identified by methods that rely on comparison to known structures. Thirdly, knowledge- based methods have been criticized for predicting protein structures without the necessity to obtain a fundamental understanding of the mechanisms and driving forces of structure formation. In contrast template-based structure prediction methods, base their predictions on physical models for these mechanisms. As such, they can therefore help to deepen the understanding of the mechanisms of protein folding (Floudas *et al.,* 2006).

Recently, González-García *et al.* identified AMPs related to the freshwater mollusk the endemic *Pomacea poeyana* (Gastropoda: Ampullariidae, Pilsbry, 1927). One of these peptides, named Pom-1 (González-García *et al.,* 2020), is a 34 amino acid cationic peptide

(KCAGSIAWAIGSGLFGGAKLIKIKKYIAELGGLQ). Bioinformatics analysis showed that Pom-1 is a fragment of the protein Closticin 574 (Keperman *et al.*, 2003), which is a bacteriocin produced by *Clostridium tyrobutyr* ADRIAT 932 (González-García *et al.*, 2020).

González-García and co-wokers (González-García *et al.*, 2020) predicted the 3D structure of Pom-1 by *ab initio* modeling on the *de novo* QUARK protein structure prediction server (https://zhanglab.ccmb.med.umich.edu/QUARK/) using the default parameters (Xu and Zhang, 2013). To validate the obtained models, the MolProbity server (http://molprobity.biochem.duke.edu/) (Williams *et al.*, 2018) was used. The analysis included angle, length, and Ramachandran evaluations.

The search for homologous proteins of Pom-1 in the SwissModel server could not find any related protein with known 3D structure and more than 30% sequence identity. So, there was no template protein to be used for predicting the peptide structure. In this context, *ab initio* modeling was used. This method has been useful in predicting the 3D structure of small proteins without the use of a template protein (Bradley *et al.*, 2005).

Table 2. A list of ab initio modeling algorithms is shown along with their energy functions, conformational search methods, model selection schemes and typical CPU time per target. Taken from Lee et al., 2017.

Tabla 2. *Lista de algoritmos de modelados ab initio junto con sus funciones energéticas, métodos de búsqueda conformacional, esquemas de selección de modelo y tiempo de CPU por objetivo. Tomado de Lee et al., 2017.*

Algorithm	Force-field type	Search method	Model selection	References
AMBER/CHARMM/OPLS	Physics-based	Molecular dynamics (MD)	Lowest energy	Brooks *et al*., 1983 Jorgensen and Tirado-Rives, 1988 Zagrovic *et al*. 2002
UNRES	Physics-based	Conformational space annealing (CSA)	Clustering/free-energy	Liwo *et a*l., 1999 Liwo *et al*., 2005 Oldziej *et al*., 2005
ASTRO-FOLD	Physics-based	aBB/CSA/MD	Lowest energy	Klepeis and Floudas, 2003 Klepeis *et al*., 2005
ROSETTA	Physics- and knowledge-based	Monte Carlo (MC)	Clustering/free-energy	Simons *et al*., 1997 Das *et al.,* 2007
TASSER/Chunk-TASSER	Knowledge-based	MC	Clustering/free-energy	Zhang *et al*., 2004 Zhou and Skolnick, 2007
I-TASSER	Knowledge-based	MC	Clustering/free-energy	Roy et al., 2010 Yang *et al*., 2015
QUARK	Physics- and knowledge-based	MC	Clustering/free-energy	Xu and Zhang, 2012

The QUARK algorithm has been selected as one of the best *ab initio* prediction programs (Moult *et al.*, 2009). This algorithm involves several complex steps. First,multiple fragments with continuously distributed lengths are identified at each position from unrelated protein structures. Then, contact maps are collected from distance profiles of the structural fragments, which are used to assist the fragment assembly simulations. Next, possible models are generated by replica -exchange Monte Carlo simulations under the guide of a composite physics and knowledge-based force field, with the best model(s) selected by structure clustering (Lee *et al.*, 2017). Since the protein fragments can be assembled in different ways, the program generates several possible models for the target protein and usually reports only the five best models.

The selection of the final model is made by comparing the model to experimental evidence and/or assessing the model quality by other computational algorithms.

In the case of Pom-1, the five models (Fig. 4) were similar, with a tertiary structure composed of two α-helices linked by a small loop. The quality of the models was also checked using the MolProbity server and the model that best predicts the Pom-1 structure was selected. Most models have several isolated Ramachandran points and bad angles, indicating problems in modeling. However, Pom-1_5 did not exhibit these difficulties and had the best number of favored residues in the Ramachandran plot. All this suggests that this model has no structural problems.

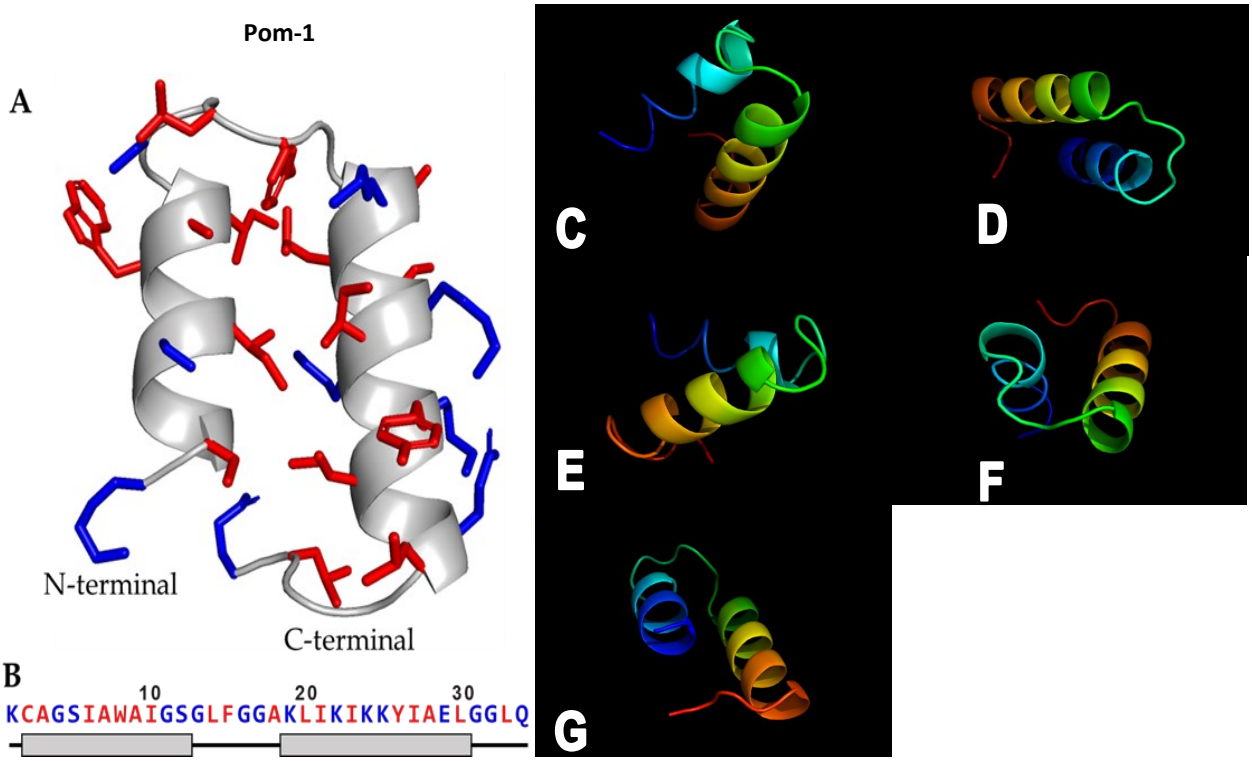

Figure 4. A) Cartoon diagrams of the predicted structure for Pom-1 modelled using QUARK. Residue side chains are represented in sticks. B) Amino acid sequence of Pom-1 and its associated secondary structure. The gray boxes represent alpha helix residues; the black lines represent unordered residues. Hydrophobic residues are highlighted in red, hydrophilic residues in blue. C) Model Pom-1_1. D) Model Pom-1_2 E) Model Pom-1_3 F) Model Pom-1_4 G) Model Pom-1_5.

Figura 4. *A) Diagramas de Cartoon de la estructura de Pom-1 modelado usando el servidor QUARK. Las cadenas laterales de los aminoácidos se representan como barras. B) Secuencia de aminoácidos de Pom-1 y su estructura secundaria asociada. Las cajas grises representan α-hélices y las líneas negras residuos desordenados. Los residuos hidrofóbicos se muestran en rojo y los hidrofílicos en azul. C) Modelo Pom-1_1. D) Modelo Pom-1_2 E) Modelo Pom-1_3 F) Modelo Pom-1_4 G) Modelo Pom-1_5.*

On the other hand, the MolProbity score represents the statistical quality of the protein by combining the clashscore, rotameters and Ramachandran evaluations (Table 2). For Pom-1 the best score was for Pom1_5 (1.99). This means that the exhibited structure by this model it is similar in 2.0 Å to the resolution of the protein in database, which is an acceptable result. Taking this analysis into account, the POM 1_5 model was considered adequate for representing the structure of this peptide. However, it must be taken into account that a model with high quality refers to a model without serious structural problems related to modeling, but may have deviations from the structure of the native protein.

As mentioned, the structure represented by the models is helical in nature, with an arrangement of two helixes joined by a small loop. These helices are amphiphilic with several positively charged residues, a common feature in many helical AMPs and considered essential for their mechanism of action on microbial membranes (Lai *et al*., 2019).

It has been speculated that structures similar to Pom-1 structure with strongly amphiphilic a-helix at the N-terminus only are likely to be functional through the carpet mechanism, while structures with N- and C-termini that are both strongly amphiphilic are more likely to act via the pore-forming mechanism (Kozic *et al*., 2018).

Table 3. Structure evaluation of the five predicted models for Pom-1. The final selected model is highlighted in grey. Molprobity score: combination of the clashscore, rotamer, and Ramachandran evaluations into a single score. Clashscore: number of < 0.4 Å steric overlaps per 1000 atoms. Poor rotamers: number of rotamers that are outside the bounds of a rotamer definition. Favored rotamers: number of rotamers that are close to the most favored rotamer conformations. Rama outliers/Rama favored: number of residues in the disallowed/favored regions in the Ramachandran analysis. Cβ deviations: Number of Cβ deviation of 0.25Å or more from the ideal position. Bad bonds/angles: number of bonds length/angles that deviate more than 4σ from average. A more detailed explanation of every evaluation can be found in the MolProbity webpage (Gonzalez-Garcia *et al.*, 2020, with permission).

Tabla 3. *Evaluación estructural de los cinco modelos predictivos de Pom-1. El modelo seleccionado está señalado en gris. Molprobity score: combinación de la clashscore, rotámeros y evaluaciones de Ramachandran en una sola evaluación. Clashscore: números de superpociciones estéricas < 0,4 Å por 1000 átomos. Poor rotamers: números de rotámeros que están fuera de los límites de una definición de rotámero. Favored rotamers: números de rotámeros que se acercan a las conformaciones de rotámeros más favorecidas. Rama outliers/Rama favored: número de residuos en las regiones favorecidas y no permitidas en el análisis de Ramachandran. Bad angles: número de ángulos que se desvían más de 4σ del promedio. Una explicación más detallada de cada evaluación puede ser encontrada en la página wed de MolProbity (con permiso de Gonzalez-Garcia et al., 2020).*

Model	MolProbity score	Clash score	Poor rotamers	Favored rotamers	Rama. outliers	Rama. favored	Cβ deviations	Bad bonds	Bad angles
Pom-1_1	2.72	5.9	2 / 22	17 / 22	2 / 32	27 / 32	3 / 27	0 / 246	2 / 326
Pom-1_2	3.1	7.8	7 / 22	14 / 22	2 / 32	29 / 32	1 / 27	0 / 246	0 / 326
Pom-1_3	2.98	3.9	3 / 22	15 / 22	3 / 32	18 / 32	0 / 27	0 / 246	1 / 326
Pom-1_4	2.86	2.0	7 / 22	11 / 22	2 / 32	25 / 32	1 / 27	0 / 246	3 / 326
Pom-1_5	1.99	0.0	4 / 22	16 / 22	0 / 32	29 / 32	2 / 27	0 / 246	0 / 326

http://molprobity.biochem.duke.edu/help/validation_options/validation_options.html

This structure is common in various AMPs such as sarcotoxin IA and Pd (Iwai *et al*., 1993), melittin (Buhroo *et al*., 2018), papiliocin (Kim et al., 2011), pardaxin and cecropins (Oñate-Garzón *et al*., 2017). Compared to the cecropin family, the structure of Pom-1 is very similar in terms of sequence length and the absence of cysteines. However, the N- and C-terminal helices are almost parallel in Pom-1 while in the cecropins they present an angle between 45–80 °. This may be due to the differences in the force field used. In the case of Pom-1, the QUARK server was used, using an optimized force field for proteins in water, while the structure of the cecropins was determined in phospholipid micelles (Kim *et al*., 2011). In water, the packing of the helix favors hydrophobic contacts, but in micelles, the peptide can adopt an extended conformation to interact with the membrane.

CONCLUSIONS

In recent years, the AMPs have stepped forward as a considerable alternative to conventional antibiotics. As a result of the continuous increase of bacterial pathogens resistant to many antibiotics, search for drugs that use alternative mechanisms of action has become an urgent imperative. The AMPs are a group of unique and incredible diversity of compounds that are derived from the immune system of all living species and may be directed to a therapeutic use. In general, AMPs show different folding and a high dynamic capability that allows them to structure in the presence of lipids, modifying their mechanism of action. The biological properties are determined by their noticeable amphipathic behavior and structure-activity relationship studies have shown that it is possible to chemically modify natural peptides to obtain AMPs with improved antibacterial activity and, at the same time, a lower cellular toxicity.

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