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Natural Antimicrobial Peptides from Eukaryotic Organisms

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1. Introduction

Antimicrobial peptides (AMP) are usually described as being short (less than 100 a.a.), gene encoded, ribosome synthesized, polypeptide substances that have antimicrobial activity. For simplicity reasons, we will exclude peptaibol and other non-ribosomaly synthetized antibiotic from our classification.

The first peptidic antibiotic was described in 1968 coming from the *Manduca sexta* and was of linear nature; since then the number of antimicrobial peptide discovered have grown asymptotically. Though loose homology has been found between certain set of antimicrobial peptides; it has proven difficult to classify the AMP through their primary structure. Antimicrobial peptides show a great diversity of primary structures, and their short size do not permit robust evolutionary classification, but for the most close related peptides. The primary structures signature of the different AMP families may have arisen independently, and in some case these structures homology are the result of convergent evolution rather than a common ancestry. Nevertheless in order to classify the new components, general classification methods have been established. So far this has been done regardless of evolutionary relationship, source or activity. The criteria that have been commonly used are the number of disulfide bridges and particular amino-acid composition. In 2005 P. Bullet and co-workers suggested a 3 categories classification namely: α-Helical host defense peptides (HDPs), β-Sheet HDPs, Flexible HDPs rich in certain amino acids (Bulet et al., 1999). Though most AMP would fit in this classification, little insight about function can be inferred from the class relation; nor does it give any comparative information between peptides belonging to the same class.

More recently Tomas Ganz proposed a structural classification of the AMP based on their secondary structure (Ganz, 2003b). The classes proposed included antimicrobial peptides with 4 disulfide bridges with alpha helix and beta sheet mixed structures, 3 disulfide bridges with alpha helix and beta sheet mixed structures, 3 disulfide bridges with beta sheet motif, 3 disulfide bridges with two alpha helix and beta sheet mixed structures, 2 disulfide bridges with beta-sheet structures, one disulfide bridge cyclic peptide and alpha helical peptides.

The classification proposed here contains 9 different peptide structure families. The last group consider hybrid structure peptide possessing structural features of more than one AMP class.

1.1 The lineal amphipathic alpha-helix antimicrobial peptide

1.1.1 General properties

The first peptide of this family discovered is the cecropin A from the pupae of the moth *Hyalophora cecropiae* (Steiner et al., 1981; Hultmark et al., 1982). This structure of AMP has been encountered in virtually all the multi-cellular organisms. Even if their sequences show some similarity, they are not all evolutionary linked. As such, they cannot be aligned as a whole, and are commonly separated in structural subclasses: Cecropin, magainin and dermaseptin AMP. This AMP class do share general common features: the lack of cysteine bridges, the tendency to form alpha helical secondary structure in relatively hydrophobic solvent, the net positive charge at neutral pH and hydrophobic residues interspersed every 3 amino acid, giving them an amphipatic nature. Indeed, basic amino acid side chains face predominantly one side of the alpha helix and hydrophobic residues are generally on the other side of the molecule. A global alignment of the linear peptides separates three different classes that could be broadly characterised as Dermaseptin, magainin and cecropin class of lineal amphipathic AMP. Most of these peptides share the present a glycine near the middle of their peptidic sequence.

1.1.2 Dermaseptins peptides

These peptides were extracted from Phyllomedusa genus frog skin secretions. Some of them present a proline-induced kink in the middle of the alpha helix (Shin et al., 2001). Others have a glycine in the same relative position that has been suggested to give the flexibility needed for the membrane lysis activity (Xiao et al., 2006). This structural plasticity has been defined as a molecular determinant for the antimicrobial vs eucaryont membrane specificity (Shin et al., 1999; Shin et al., 2000; Shin et al., 2001), together with overall net positive charge and hydrophibic moment. They present hydrophobic and positively charged amino acid in an alternate pattern. Though mature dermaseptin amino acid sequences are highly variable, their acidic pro-peptides are strikingly conserved (Azevedo Calderon et al., 2010).

1.1.3 Cecropin peptides

Cecropin peptides were first purified from insect hemolymph, and their expression is usually inducible. Cecropins structural conformations were determined by NMR. Circular dichroism and NMR (Nuclear Magnetic Resonance) data have shown that in aqueous solvent the cecropin structure is largely disordered; but they adopt a stable alpha-helical secondary structure in more hydrophobic environment. This makes the insertion of these peptides in the lipidic membranes entropically favorable. Cecropin usually present a glycine in the middle of their amino acid sequence. This glycine has been proposed to induce a kink between the alpha helical structures that these peptides form in hydrophobic solvent. In turn the deletion of this glycine or its replacement by another amino acid do not eliminate the antimicrobial activity; instead it endows these mutant peptides with hemolytic and cytolytic activity (Moore et al., 1996). The cecropin usually present a tryptophan in one of the first two amino acid position as well as a glycine in the first position.

1.1.4 Magainin/scorpions AMP/cathelicidin peptides

Magainin peptides come from the frog genus Xenopus (Duclohier et al., 1989). In this AMP class arthtropod AMP like Opistoporin-2 from Scorpion *Opistophthalmus carinatus* are also

included (Moerman et al., 2002) as well as cathelicidin peptides (Travis et al., 2000) and the fly cecropin from *Stomoxys calcitrans* (Boulanger et al., 2002). This phylogenetically heterogeneous group present lysine/argine doublet repeats that could be considered as a structural signature. These peptides do not present the conserved glycine present in the other 2 lineal amphipatic AMP subclasses. Their positive general formal charge at neutral pH is higher than the one of the cecropin and dermaseptin AMP. They also present a conserved aspartic acid residue at the amino side of these peptides.

1.2 Proline rich peptides

This AMP class has been first described in mammals, in the intestine of *Sus scrofa,* (Agerberth et al., 1991). They are also present in Hymenoptera, Lepidoptera and diptera. Some of these peptides from this AMP class have been studied extensively, like drosocin from *D. melanogaster* (Bulet et al., 1993), pyrrhocoricin from the European sap- sucking bug *Pyrrhocoris apterus* (Cociancich et al., 1994) apidaecins from the *Apis mellifera* (Casteels-Josson et al., 1993), and formaecin from the ant *Myrmecia gulosa* (Mackintosh et al., 1998). Mature proline-rich antimicrobial peptides vary in length from 12 to 54 amino acid, and have few common structural feature. Some authors distinguish between glycine/proline rich and alanine/proline rich peptides. The variety of sequence does not allow for straightforward sequence signature recognition for these peptides. Therefore, their antibacterial activity/specificity is not deducible from the sequence analysis.

On the other hand, some of these peptides are able to penetrate the microbial cytoplasm without inducing bacterial lysis, and do not present hemolytic or cytolitic activities (Knappe et al., 2010). Model PR peptides have been designed, using the Ac-(Arg-Pro-Pro-Phe)n-NHCH3 framework, and some essential structural feature, necessary for antimicrobial activity have been determined. The ability to form poly(proline)-II structure in aqueous solution, as well as a critical peptide length are essential for antimicrobial activity (Niidome et al., 1998).

1.3 Glycine/arginine rich peptides

The first purification of glycine rich peptide was done in 1991 (Bulet et al., 1991). As for the proline rich antimicrobial peptide class, the glycine rich peptides have variable sizes and do not show clear sequence signature, apart from the high proportion of glycine in their primary sequence. These peptides are in general longer than AMP from other classes. Between 25 to 50% of their amino acid are glycines. They have disordered structure in water, and tend to self-order when in contact with artificial membranes (Bruston et al., 2007). The structure of bombinin H resembles the influenza hemagglutinin fusion peptide (Zangger et al., 2008). When binding to DPC micelles, a helix is formed that have a glycine ridge on one side. There is an helix-helix interaction that leads to a multimerization process in the bacterial membrane (Zangger et al., 2008).

1.4 Brevinin (hook structure) peptides

This class of peptides is characterized by having a short amino acid sequence, and a carboxyloterminus disulfide bridge. Some brevinin peptides show post-translational modification. The amino acids included in the carboxyloterminus loop are determinant for the specificity of the antimicrobial activity as well as the length of the loop (Lee et al., 2002) .

The brevinin show alpha helical structure in sodium dodecyl sulfate solution (Lee et al., 2002). Antibacterial activity is favored by structure that group the cationic amino acids of the molecule with on one side an hydrophobic stretch of amino acids and on the other side streches of apolar amino acids (Kumari and Nagaraj, 2001). Liposome disruption activity of the brevinins correlates with the anti-Gram positive bacterial activity, suggesting a lytic activity. None of the peptides showed hemolytic activity making brevinins attractive prospects for broad-spectrum antimicrobial peptide design (Lee et al., 2002).

1.5 Defensins (cysteine knot structure)

1.5.1 General properties

This class of peptide is characterized by its rigid structure, given by the presence of 3 to 4 disulfid bridges. They are sub-classified through their cysteines connectivity and their secondary structure.

By virtue of the cysteine knot motif that stabilize them, all these peptides show a rigid secondary structure. Defensin are amphipatic molecules with a common defined beta sheet motif secondary structure. Indeed there is a gamma-core motif (GXCX(3-9)C), considered the structural signature of the disulfide-stabilized antimicrobial peptides that present two beta strands with an interposed loop (Sagaram et al., 2011). This motif has one hydrophobic and one hydrophilic side. The hydrophilic side of these peptides is usually constituted by several lysine or arginine aminoacids. This gives them a general positive charge at physiological pH. They are resistant to degradation and peptidase digestion because of their compact structure. As for the other antimicrobial peptide classes, there are few phylogenetic relationships even within each defensin subclass. The first three defensins class described were found exclusively in mammals.

1.5.2 Alpha defensins

This type of defensins is found in mammals. Their cysteine are connected between the cysteines 1-6 2-4 3-5. They show a structure of triple-stranded beta-sheet stabilized by a conserved triple disulfide bridges array (Hadjicharalambous et al., 2008). Alpha defensin sequence present more arginine than lysine residues, and it has been suggested that this high arginine content endows the alpha-defensin with a higher antibacterial activity in high salt conditions (Llenado et al., 2009).

P. hamadryas alpha-defensin ACYCRIPACFAGERRYGTCFYLGRVWAFCC

1.5.3 Beta defensins

The beta defensin have a cysteine connectivity of 1-5 2-4 3-6. They present the consensus sequence of Xn-C-X2-4-G-X1-2-CX3-5CX9-10CX5-6CCXn (Ganz, 2003a) (C=cysteine and G=Glycine). They present a tri dimensional structure of a triple stranded beta sheet. The glycine invariant is also present in alpha defensin. This glycine is necessary for the beta bulge structure to be formed, and the protein is unable to fold if it is replaced by any other natural amino acid (Xie et al., 2005).

Sus scrofa beta-defensin 1 SVSCLRNKGVCMPGKCAPKMKQIGTCGMPQVKCCKRK

1.5.4 θ defensins

θ defensin are macrocyclic octadeca peptides connected head to tail. These peptides are present in monkeys but absent in human. There are θ-defensin ortholog pseudo genes in the human genome but the Theta-defensin genes contain a premature stop codon that aborts translation. (Cole et al., 2002) (Cole et al., 2004). Their synthesis implies a head to tail circularization of an octa-peptide (Selsted, 2004). They were found to be active against *S. aureus, E. coli, and C. albicans* as well as HIV virus (Cole et al., 2002). This type of defensins also present a disulfide bridges stabilized amphipathic beta sheet structure.

P. hamadryas theta-defensin-1 RCVCRRGVCRCVCTRGFC

1.5.5 Insect defensins

The insect defensin class have an alpha-helix secondary structure bound to the beta sheet. Their structures are similar to another arthropod peptide class: the scorpion potassium channel blocker toxins (Bontems et al., 1991). Study of the structural features involved in the antimicrobial activity of longicin, a defensin from the hard tick *Haemaphysalis longicornis*, showed that the beta sheet alone was sufficient for antimicrobial activity. This part of the insect peptide is positively charged at neutral pH, as does the alpha helix of the same, nevertheless the role of the alpha-helix, at the antimicrobial level, seems to be restricted to maintain the globular shape of the peptide (Rahman et al., 2009).

R. prolixus insect defensin B
ATCDLLSFRSKWVTPNHAGCAAHCLLRGNRGGHCKGTICHCRK

1.5.6 Plant defensins

Plant defensin antimicrobial peptide class present 4 disulfide bridges with a cysteine connectivity of 1-8, 2-5, 3-6, and 4-7. Their three-dimensional structures are similar to the insect defensin structure in that they have a disulfide bonds stabilized alpha-helix. Their structure also show a triple anti-parallel beta-strands. (Sagaram et al., 2011). The strong antifungal activity of the plant defensin has been associated with their alpha helix motif, in a similar fashion as for insect defensin (Lamberty et al., 2001). Conversely, heliomycin, a defensin from *Heliothis virescens*, has more structural communality with plant defensin than with insect defensin (Lamberty et al., 2001).

P. sylvestris defensin 1

RMCKTPSGKFKGYCVNNTNCKNVCRTEGFPTGSCDFHVAGRKCYCYKPCP

1.6 Tachyplesin

This class of AMP was first found in horseshoe crab (*Polyphemus litoralis*). Gomesin is a tachyplesin type of antimicrobial peptide found in tarantula hemocyte (Silva et al., 2000); and androctonin has been extracted from scorpion hemolymph (Mandard et al., 1999). This type of antimicrobial structure is broadly distributed amongst the genus. The AMP related to this class of peptide present a beta sheet secondary structure stabilized by two disulfide bridges (Nakamura et al., 1988). Tachyplesin have a rigid conformation of antiparallel beta-sheet connected by a beta-turn (Iwanaga et al., 1994). The tachyplesin family of AMP adopts

beta-hairpin-like structures when in contact with hydrophobic solvent. NMR studies revealed a largely unordered structure in water, but a transition to a regular beta-hairpin backbone conformation in the presence of dodecylphosphocholine micelles. The cysteine null mutant of protegrin, a mammal tachyplesin type peptide, revealed that the cystein bridges were not necessary for antimicrobial activity. Aside from their antimicrobial activity, tachyplesin have also a scavenger capability. They bind lipo-polysacharide with high affinity (Niwa et al., 1990). The structure of tachyplesin I also interacts with Vesicular stomatitis virus envelope, inactivating the virus (Murakami et al., 1991).

Tachyplesin III *Tachypleus gigas*: KWCFRVCYRGICYRKCR

1.7 Tryptophan rich antimicrobial peptides

The archetypical W rich peptide is the indolicin, this peptide comes from *Bos Taurus* neutrophils and is the result of proteolysis. Unlike the amphipathic alpha helical structure of the cecropin class of peptides, their linear structure (no disulfid bridges) has no particular secondary structure in water. Indolicin is globular and amphipathic in aqueous solution, while it adopts a wedge shape when in contact with micelles. Indolicin shows a high affinity for neutral POPC and anionic POPG vesicles(Hsu et al., 2005). The author suggests that the structure changes and the strong membrane affinity are key to the antimicrobial activity of indolicin (Ladokhin and White, 2001). Tryptophan rich AMP contains more than 25% of the aminoacid. Indolicin, the archetypical tryptophan rich antimicrobial peptide, has a globular secondary structure in water, but show a wedge shape when in contact with in lipid micelles (Rozek et al., 2000). This peptide has the ability to permeate bacterial membranes and, depending of its tridimensional shape, inhibits DNA synthesis by binding to it (Hsu et al., 2005).

Indolicin: H-ILPWKWPWWPWRR-NH2

1.8 Histidine rich glycoprotein peptides

Histidine-rich amphipathic cationic peptides are peptides with ¼ of their amino acids represented by histidine. They show a global cationic amphipathic helical structure. They trigger microorganism membrane disruption when the peptide adopts an alignment parallel to the membrane surface. Even though, pore formation is not essential for their high antimicrobial activity (Mason et al., 2009). Clavinin and daptomycin are other studied members of this antimicrobial peptide class. Some Histine rich peptide, like LH4, also have the capability to enhance transfection, a feature that is related to membrane perturbation capability (Georgescu et al., 2010).

1.9 Mixed structure peptides

Some peptides share structural communality with more than one class of AMP; the sum of the different AMP part activity are not additive; and these peptides classes do show unique activity not present in the separated structural part of the molecule. Scorpine type AMP represent a class on its own, as several homologous proteins have been found. The structural defensin part of the molecule resembles the insect peptides. It has been determined in later studies that once separated from the linear cecropin-like amino

terminus, this peptide could effectively block these channels. Even though, the antimicrobial activity of the complete molecule was dependent of the presence of this toxin/defensin motif (Diego-Garcia et al., 2008).

The penaeidin class of peptide consist in proline-rich N-terminus and of a C-terminus containing six cysteine residues engaged in three disulfide bridges (Destoumieux et al., 2000). The proline-rich domain of penaeidin class AMP suffices to confer target specificity and antimicrobial activity of penaeidin (Cuthbertson et al., 2004). The carboxyl end cysteine-rich domain consists of an amphipathic helix linked to the upstream and the downstream coils by two disulfide bonds. The peptide shows a highly hydrophobic core of globular and compact structure, that has 2 arginines exposed on each side (Yang et al., 2003).

Another example of hybrid antimicrobial peptide is Hyastatin, isolated from the spider crab (*Hyas araneus*) hemocytes (Cuthbertson et al., 2008). This AMP combines a Glycine rich motif N-terminal region, a short Pro/Arg-rich region, and a panaeidin like C-terminal region containing 3 disulfid bridges (Sperstad et al., 2009).

The chicken beta defensin 11 is formed by the repeat of two defensin motif, therefore having 6 disulfid bridges. This defensin show a nanomolar range of anti *E.coli* activity, being one of the most effective antimicrobial peptide for this microorganism (Herve-Grepinet et al., 2010).

Microplusin, is a *Rhipicephalus* (*Boophilus*) microplus anti-microbial peptide (AMP). Microplusin has a cysteine-rich AMPs structure with histidine-rich regions at the N- and C-termini. Microplusin consists of five alpha-helix and has been shown to bind copper and iron (Silva et al., 2009).

2. Antimicrobial mechanisms of AMP

The activity of AMPs must start at the cytoplasmic membrane since most AMPs permeabilize microbial membranes. Several models have been proposed on how AMPs insert into the membrane leading to the formation of ion channels, transmembrane pores or extensive membrane rupture. These models are: 1) transmembrane pore models and 2) nonpore models activity. Here we will also review other antimicrobial mechanisms that have been found. For example, the antimicrobial mechanisms of apidaecin do not rely on pore forming activity, this peptide does have antimicrobial activity at a concentration at least four order of magnitude below the concentration that disturbs the bacterial membrane. Peptides from each structural family have been reported to rely on antimicrobial mechanism that would not imply membrane depolarization of the target microorganism, suggesting internal molecular determinant. Certain peptides are unable to cause membrane depolarization at the minimal inhibitory concentration, while other cause maximal depolarization well below the MIC (Minimal Inhibitory Concentration) value. Evidences are mounting that involve particular macromolecules as well as intracellular functions as the final target for antimicrobial activity of the AMP. Even though, bacterial membranes are a necessary entry path for the AMP, therefore determining part of the AMP selectivity as well as efficiency.

2.1 Transmembrane pore models of AMPs

There are more than 1,000 known AMPs (Brahmachary et al., 2004; Wang and Wang, 2004; Fjell et al., 2007), and for the majority of them, there is little or no evidence for

transmembrane pores (Wimley, 2010). Instead, there is compelling evidence that many AMPs function by binding to membrane surfaces and disrupting the packing and organization of the lipids in a nonspecific way. The simplest models of membrane permeation by peptides involve the formation of membrane-spanning pores. These pores have been studied in lipid bilayers and have been proposed to be the major cause of bacterial membrane depolarization. As bacteria ATP synthesis is linked to the trans-membranal potential of the cell, any perturbation of the ion partition may potentially lead to the cell death. The structure of these peptides induced pores have been analyzed and several pore structure were proposed.

The barrel stave pore model (Rapaport and Shai, 1991) involves a mechanism where the peptides interact laterally with one another to form a specific structure enclosing a water-filled channel, much like a protein ion channel. Bioinformatic analysis of protegrin 1 insertion in membranes concluded that this model was most consistant with the observed energy of insertion of the peptide in artificial membranes (Langham et al., 2008). Furthermore the electrophysiology record analysis of Ceratotoxin and pleuricidin peptides inserted in lipid bilayers show that they form a peptide filled pore isolated from the lipids from the membranes. The pore formation dynamics correlates with their antimicrobial activity (Bessin, 2004).

In the toroidal pore model (Ludtke et al., 1996), specific peptide–peptide interactions are not present Instead, the peptides affect cooperatively the local curvature of the membrane, forming a peptide lipid toroid pore in the membrane. The cathelicidin peptide LL 37 (an amphipathic, alpha-helical, antimicrobial peptide) appears to form such kind of pore in the microbial membranes. NMR spectra studies of LL 37 shows that its pore channel is filled with the membrane lipids phosphate heads. The resulting ionic leakage ultimately leads to bacterial membrane depolarization (Henzler Wildman et al., 2003). The pore formation of cateslytin, a beta sheet conformation peptide, has been studied through patch clamp and NMR experiments, and has been found to fit a pore formation model involving transient dissymmetry between the phospholipid leafs of the membrane as a key ingredient to explain the formation of the pore (Jean-Francois et al., 2008)

The carpet model for AMP antimicrobial activity , originally described by Shai (Gazit et al., 1996), is the most commonly cited model of membrane destabilization by AMPs. The carpet/detergent model proposes that the accumulation of the peptides imbedded in the microbial membrane provoques perturbation in the membrane integrity. Antimicrobial peptides accumulate on the membrane surface with an orientation that is parallel to the membrane untill peptide concentration has reached a critical level (i.e., a peptide-rich ‘‘carpet’’ has formed on the membrane surface). Then permeabilization occurs, via global bilayer destabilization. PMAP-23, a cationic peptide member of the cathelicidin family, is considered to induce membrane permeability according to the Shai-Matsuzaki-Huang "carpet" model (Bocchinfuso et al., 2009). Cecropin P1, another alpha helical AMP, imbedded in reconstituted phospholipid bilayer is preferentially oriented nearly parallel to the surface of the lipid membranes, a position that is incompatible with the proteinaceous pore model, as demonstrated by polarized ATR-FTIR spectroscopy analysis(Gazit et al., 1996) The detergent model is also often cited to explain the catastrophic collapse of membrane integrity, observed with some AMPs at high peptide concentration (Ostolaza et al., 1993; Hristova et al., 1997; Bechinger and Lohner, 2006). Some authors combine the

carpet and detergent models into a single idea in which the catastrophic collapse of membrane integrity includes membrane fragmentation. Others distinguish between the two models based on whether or not the peptide-induced leakage efficiency depends on the size of the entrapped solutes (Ostolaza et al., 1993). Bechinger and Lohner (Bechinger and Lohner, 2006; Salnikov et al., 2009) recently discussed molecular shape models in which AMP lipid interactions could be depicted with phase diagrams to describe the propensity of an AMP to permeabilize a membrane by disrupting the lipid packing. Epand and colleagues have proposed a lipid clustering model in which AMPs induce clustering or phase separation of lipids, with leakage occurring due to boundary defects (Epand et al., 2009; Epand et al., 2010). Almeida and colleagues have described AMP activity in terms of binding, insertion and perturbation using the sinking raft model, which they recently augmented by adding a formal thermodynamic analysis to predict activity (Pokorny and Almeida, 2004; Gregory et al., 2008; Almeida and Pokorny, 2009; Gregory et al., 2009; Almeida and Pokorny, 2010). Interfacial activity is defined as the propensity of an imperfectly amphipathic peptide to partition into the bilayer interface and drive the vertical rearrangement of the lipid polar and non polar groups. The disruption of the normally strict segregation of polar and nonpolar groups causes membrane permeabilization.

While these models, and others, have been useful in discussing AMPs, the molecular organization of the lipid bilayer undergoing solutes leakage in the presence of AMPs is still very much unknown. Without this knowledge, it remains challenging to predict structure–sequence activity relationships; thus, it also remains challenging to engineer or *de novo* design AMPs.

2.2 Non membrane mediated models of AMP activity

In addition to the pore models described above, AMP activity has been described using antimicrobial mechanism that do not involve bacterial membrane permeability impairement. For instance PR-39, a porcine proline-arginine-rich antibacterial peptide was found to lyse the microbes through a non pore forming mechanism, altering the microbe division and septum formation (Shi et al., 1996). Small Anionic antimicrobial peptides have been found in ovine lungs, that appear to function without the need for the initial electrostatic interaction with the bacterial membranes, and kill the bacteria through intracellular content flocculation (Brogden et al., 1998). Mersacindin, a lantibiotic, has been shown to inhibits bacterial cell wall formation (Brotz et al., 1997). Some antimicrobial peptides like the pleurocidin and dermaseptin inhibit bacterial DNA synthesis while buforin II and tachyplesin bind to nucleic acid in general (Yonezawa et al., 1992). The proline rich AMP family target proteicious or nucleic acid intracellular molecules (Park et al., 2008), indeed members of the proline-rich AMP family like Drosocin, pyrrhocoricin, and apidaecin have been shown to act on bacterial GRO EL and Dna K proteins(Otvos et al., 2000).

3. AMP classical functions

The classical function of AMP has been their role as major effectors of the innate immune system; AMPs complement the highly specific but relatively slow adaptive immune system. Unlike the acquired immune mechanisms, endogenous AMPs, which are constitutively expressed or induced, provide fast and effective means of defense. Most of these gene-encoded peptides are mobilized shortly after microbial infection and act rapidly to

neutralize a broad range of microbes (bacteria, virus and protozoa). The ubiquitous nature of antimicrobial peptides suggests that their role in nature has been long standing and must have contributed to an organism's fitness. Many of these molecules exert mechanisms of action that appear to be unique and highly complex. However, AMPs exhibit varying, and in some cases, significant degrees of host cytotoxicity, reflecting non-selective cell targeting (Shin et al., 1999). It is likely that distinct antimicrobial peptides have evolved to function within specific physiologic and anatomic contexts to minimize their potential to concomitantly injuring the host cells. An intensive area of focus regarding antimicrobial peptide biochemistry relates to the precise mechanisms by which these molecules cause cell death. A long-held paradigm for microbicidal action has been that AMPs kill microorganisms by initiating multiple injuries in target microbial cell membranes. The principal theory suggest that peptides may create membrane pores in the organism, making a leakage of some metabolites, ensuing depolarization, loss of membrane-coupled respiration and biopolymer synthesis, and ultimately cell death. However, other authors suggest additional mechanisms, , where membrane permeabilization alone appears to be insufficient to cause cell death. These evidences come from studies documenting a clear dissociation between membrane perturbation and cell death. In these cases, cell killing may proceed in the absence of significant disruption in membrane architecture, due rather to disruptions in cellular function (Zhang et al., 2000). The functional integrity of the cytoplasmic membrane is crucial to essential functions of microbial pathogens, including gradient formation and selective permeability, cellular energetics, and synthesis of bio-molecules (Yeaman et al., 1998).

The general membrane effects of AMP are the membrane perturbation however alone may be insufficient for microbicidal effects of certain peptides. Permeabilization alone does not invariably result in staphylococcal death due antimicrobial peptides. Different peptides with varying staphylocidal potencies exhibited disparate capacities of membrane permeabilization and cell killing (Koo et al., 2001). Similar studies showed that gramicidin S rapidly depolarizes *Pseudomonas aeruginosa*, but did not kill it, suggesting that the concept of membrane perturbation and eventual cell killing may be independent (Zhang et al., 2000). Bacterial membrane energetic also appears to be involved in AMP mechanisms of action (Yeaman et al., 1998). It is now widely recognized that the AMP concept could play a promising role in fighting the presently raging microbial resistance to conventional antibiotics.

4. Unconventional function of AMP

4.1 Regulatory activities of AMPs.

Besides the role of endogenous antibiotics, the antimicrobial peptides have other functions in the inflammation; wound healing and regulation of the response immune response, of which are described below.

Microbial infection of the mucosa and skin induces production of large quantities of small antimicrobial peptides, including defensins and cathelicidins, (Zasloff, 2002; Ganz, 2003a; Yang et al., 2004). They can act as chemokines, such as some β-defensins chemoattract immature iDC and other effector cells through the CCR6 receptor (Biragyn et al., 2002); (Niyonsaba et al., 2004) or human cathelicidin LL-37 recruits neutrophils, monocytes and mast cells via human formyl peptide receptor-like 1, FPRL1 (De et al., 2000) (Agerberth et

al., 2000; Niyonsaba et al., 2002). Defensins can also activate effector cells that can work together with the complement system to destroy microbial invaders. The α-defensins HNP1~3 have been reported to increase the production of TNFa and IL-1 while decreasing the production of IL-10 by monocytes (De et al., 2000). Some α-defensins enhance expression of adhesion molecules including ICAM-1, CD11b, and CD11c by neutrophils and facilitate the recruitment and enhance the microbicidal activity (Van Wetering et al., 1997; Chaly et al., 2000; Di Nardo et al., 2003) (FÈger et al., 2002). β-defensins induce mast cell degranulation and release of histamine and prostaglandin D2 (Yamashita and Saito, 1989; Befus et al., 1999; Niyonsaba et al., 2001) increase the expression of CXCL8 and CXCL5 (Van Wetering et al., 1997; van Wetering et al., 2002). Furthermore, murine β-defensin 2 has been shown to act directly on immature DCs as an endogeneous ligand for Toll like receptor 4 (TLR-4), inducing up regulation of co-stimulatory molecules and DC maturation, triggering robust, Th1 polarized adaptive immune responses in vivo (Biragyn et al., 2002). However, the mechanisms that regulate these functions are not well studied. Defensins attract inflammatory cells as neutrophils, B lymphocytes and macrophages. All these cells release inflammatory mediators such as IL-8, IFNγ, IL-6, IL-10 and LTB4. It is interesting that defensins may also have anti-inflammatory activity by the induction of IL-10 or SLPI.(Durr and Peschel, 2002; Zasloff, 2002).The synthesis of β-defensins by epithelial cells and the recruitment of peripheral blood granulocytes α-defensin-rich site of inflammation generates a high concentration of them. Also have direct antimicrobial effects, defensins facilitate and amplify the subsequent immune response. Indeed, spleen cells stimulated with α-defensins increase the production of human cytokines and lymphocyte proliferation. This same type of defensins, when administered to mice, produces increased serum IgG1, IgG2 and IgG2b. In addition, small amounts of HNP extend the antibody response against a singenic tumor (Tani et al., 2000).

These results indicate, without doubt, the AMPs have a role in the regulation of the immune response. On the other hand, recent studies have identified several structurally diverse endogenous mediators of innate immunity with certain features: firstly, they are rapidly released in response to infection or tissue injury; secondly, they have both chemotactic and activating effects on APCs; and thirdly, they exhibit particularly potent *in vivo* immunoenhancing activity and enhance DC differentiation from DC precursors. This subset of mediators alerts host defenses by augmenting innate and adaptive immune responses to tissue injury and/or infection. On the basis of their unique activities, they are called 'alarmins' (Oppenheim and Yang, 2005). Innate-immune mediators possessing alarmin activity include defensins, cathelicidin, eosinophil-derived neurotoxin (EDN), and high mobility group box protein 1 (HMGB1) (Oppenheim and Yang, 2005). The concept of alarmins is very interesting. This has only been observed in mammals, but is likely to exist in other groups of animals including insects. It has been observed over-expression of antimicrobial peptides during infection with various pathogens and even damage to the cuticle. Many groups of insects have been used to understand the basic characteristics of the innate immunity. However, surprisingly, the study of AMPs in insects has been limited study of their bactericidal or antiparasitic activity and virtually no information on the alternative role that could have the AMPs on the immune response in these organisms.

Differential analyses after bacterial or fungal challenge showed the regulation of more than a 100 molecules in adult *Drosophila* hemolymph (Levy et al., 2004). Using differential

MALDI-TOF MS, 28 peptides with a molecular mass below 15 kDa and belonging to different structural families were identified and could be classified into two groups. The first group contains the AMPs and their different isoforms. DIMs belonging to this group are likely to be effectors molecules of the immune response through their antimicrobial activity. The second group contains molecules for which the lack of similarity to any peptide prevents the proposition of any precise function. These peptides are suspected to serve as chemokines during the *Drosophila* immune response but the different approaches for investigating their role have so far been unsuccessful (Levy et al., 2004). On the other hand, our group has analyzed the peptides in the hemolymph of mosquitoes *An. albimanus* infected with malaria parasites. We found a complex pattern of peptides, including cecropin, which are released into the hemolymph. Similarly, gambicin, cecropin, and defensin are over-expressed in the intestinal epithelium and fat body of mosquitoes infected with *Plasmodium*. However, it is unknown whether these peptides participate in the elimination of the parasite. Cecropin has been consider an important AMP against Plasmodium, but in vitro assays with synthetic cecropin did not affect *Plasmodium* viability (unpublished results), but this peptide is over-expressed in mosquitoes infected with the parasite (Herrera-Ortiz, A. et al., 2010.). It would be interesting to analyze the peptides released into the hemolymph of these insects and their role in regulating the immune response.

4.2 Anti-inflammatory (Anti-endotoxin) roles of host defense peptides

Bacterial lipopolysaccharides (LPS), also known as endotoxins, are major structural components of the outer membrane of Gram-negative bacteria that serve as a barrier and protective shield between them and their surrounding environment. LPS is considered to be a major virulence factor as it strongly stimulates the secretion of pro-inflammatory cytokines which mediate the host immune response and culminating in septic shock.

Early experiments determined that a number of host defense peptides from various sources bound to LPS from diverse Gram-negative bacteria and reduced LPS-induced release of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) and nitric oxide from monocyte or macrophages and protected mice from LPS lethality (Larrick et al., 1994; Larrick et al., 1995; VanderMeer et al., 1995; Kirikae et al., 1998). Initial studies focused on the unprocessed form of cathelicidin, hCAP- 18 (Kirikae et al., 1998); however, it was later found that the LPS-binding properties of the peptide were contained within the processed 37-amino acid C-terminal domain, LL-37 (Turner et al., 1998). It has been proposed that the anti-endotoxic properties of these peptides are the result of the inhibition of binding of LPS to CD14 (Nagaoka et al., 2001) and lipopolysaccharide binding protein (LBP) (Scott et al., 2000), and/or indirect effects on cells (Scott et al., 2002). LL-37 has been shown to block a number of LPS-induced inflammatory responses, including contractility and (nitric oxide) NO release in aortic rings (Ciornei et al., 2003), pro-inflammatory cytokine production in a macrophage cell line and in animal models (Scott et al., 2000; Ohgami et al., 2003), suppression of leukocyte infiltration in a model of endotoxin-induced uveitis (Ohgami et al., 2003) and lethality in animal models of sepsis (Scott et al., 2002). These effects occur at concentrations in the physiological range for LL-37 (1–5 μg/ml) and may reflect a natural role for LL-37 in the body (e.g. balancing of the potential stimulus by endotoxin from commensals). This anti-endotoxin activity appears to correlate with an ability to dampen the

pro-inflammatory effects of the Gram-positive surface molecule lipoteichoic acid (Scott et al., 2002; Gutsmann et al., 2010) designed a new class of peptides synthetic anti-LPS peptides (SALPs). SALPs were originally based on the LPS-binding domain of the *Limulus* anti-LPS factor (LALF) but were substantially changed in length and primary sequence for optimal binding to the lipid A portion of LPS. They observed that these peptides are highly efficient in neutralization of LPS and blockage of its immunopathological consequences *in vitro* and *in vivo*. SALPs combine excellent selectivity for LPS, with high neutralizing activity *in vitro* and potent protection to septic shock using the murine model *in vivo*. They also demonstrate the biological efficacy of rationally designed new synthetic antilipopolysaccharide peptides (SALPs) based on the *Limulus* anti-LPS factor for systemic application. Efficient inhibition of LPS-induced cytokine release and protection from lethal septic shock *in vivo* was analyzed, whereas cytotoxicity was not observed under physiologically relevant conditions and concentrations. It seems that the lipid A part of LPS is converted from its "endotoxic conformation," the cubic aggregate structure, into an inactive multilamellar structure. These observations suggest a novel therapeutic role of AMPs.

4.3 Anti-viral activity

Apart from the antibacterial activity, AMPs also possess antiviral activity. For example, the α-defensins target the human immunodeficiency virus (HIV) activity by directly inactivating viral particles and affecting the ability of the virus to replicate within CD4 cells. Human α-defensins HNP-1 to -3 and HD-5 have been shown to block papillomavirus infection. Retrocyclin 2, a synthetic θ-defensin peptide that humans do not synthesize due to a mutation in the corresponding human gene, has the capacity to block influenza virus infection. Human β-defensins can also block HIV-1 replication, and interestingly, a single nucleotide polymorphism in a β-defensin gene has been associated with clinical manifestation of HIV-1 infection, suggesting that the human β-defensins play an important role in host defense against HIV. Cathelicidins, in contrast, have an inhibitory effect on lentiviral replication in vitro, and LL-37 appears capable of interfering with vaccinia virus replication in vitro and in mice. Dermaseptin S4, a 28-residue AMP isolated from frog skin, attenuates HIV infection in vitro. Other AMPs from frog skin including caerin 1.1, caerin 1.9, and maculatin 1.1 have also demonstrated inhibition of HIV in vitro(Albiol Matanic and Castilla, 2004).(Daher et al., 1986; Sinha et al., 2003; Yasin et al., 2004). Our group has worked with the peptide named scorpine from the venom of *Pandinus imperator* scorpion, where we observed a very interesting anti-virus dengue and anti-plasmodium activity (Carballar-Lejarazu et al., 2008). Scorpine is an antimicrobial peptide whose structure resembles a hybrid between a defensin and a cecropin. It exhibits antibacterial activity and inhibits the sporogonic development of parasites responsible for murine malaria. The recombinant expressed scorpine (RScp) in *Anopheles gambie* cells showed antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae*, at 5 and 10 μM, respectively. It also produced 98%mortality in sexual stages of *Plasmodium berghei* at 15 μM and 100% reduction in *Plasmodium falciparum* parasitemia at 5 μM. RScp also inhibited virus dengue-2 replication in C6/36 mosquito cells. In addition, we generated viable and fertile transgenic *Drosophila* that over-expresses and correctly secretes RScp into the insect hemolymph, suggesting that the generation of transgenic mosquitoes resistant to different pathogens may be viable. However, there is no knowledge of their mechanics, action. It is necessary to extend these studies with other peptides during infection induced with virus dengue and other pathogens.

5. Evolutionary perspective on antimicrobial peptides

In this chapter, we proposed a structural classification of antimicrobial peptide families considering the diversity of their structures and then we reviewed the traditional function and biological activities. Finally, we propose new insights into the functions of antimicrobial peptides that could provide a large body of research to create new classes of antimicrobial therapeutics. AMPs are widespread molecules throughout the animal and plant taxa, this fact suggest its relevance in the evolution of immune response. Traditionally, their basic molecular and biochemical nature is related to the disruption of membrane potential and/or structure with the ensuing cell death. However, the diversity in the structure and biological properties (above mentioned) of AMPs within and between species suggest that these molecules have different functions in immune response.

The immune system of living organisms is formed by a set of cells, molecules and reactions. All of these features are continuously evolving to resist (attack and eliminate) pathogen invasion and to limit the negative (in terms of host survival and reproduction, i.e. fitness) consequences of the infection (Hoffmann and Reichhart, 2002). On the other hand, pathogens success depends upon overcoming the selective immune pressures brought about by the host. As a consequence, both, host and pathogens, evolve traits and strategies to increase the fitness of each one. Van Valen (Van Valen, 1973) proposed this co-evolutionary arm races as an evolutionary theory called "The Red Queen Hypothesis". The theory was proposed citing Lewis Carrol's Red Queen, where it takes all the running somebody can do to keep in the same place. Given this situation, immunologically, there is never a "best" solution to pathogens infection. To understand this scenario, we must consider (1) pathogen's short generational cycles, that may provide enough time to adapt to the host's immune response. As an outcome there will be grounds for high variability in immune response. (2) Differences in the kind and burden of pathogens, where divergent host-pathogen interactions for each species are possible and can be reflected in the course of action of taxa immune response (Read and Taylor, 2001). (3) Virulence differences, where there can or cannot be a harm imposed on a host (for example *Bacillus anthracis* vs. commensal microbiota). As long Hypothesis, an immune effecter that has the ability to be produced under different infection circumstances could have a selective advantage, antimicrobial peptides could have this property, as the host has to deal with the problem of maximizing fitness under the Red Queen pressure.

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