Antimicrobial peptides: To membranes and beyond

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1. Abstract

Background: Antimicrobial peptides (AMP) are widely recognized as promising alternatives to the current use of antibiotics and fungicides. Amino acid sequences of a vast majority of AMP share cationic and amphipathic biophysical properties that allow their insertion into lipid bilayers, and can lead to alteration of biological membrane functions. Initial characterization studies linked these properties to antimicrobial killing activity. However, additional data indicate that this is not the sole mode of action and that more subtle mechanisms might mediate the interaction with and effect to target microbes, as well as the specificity and toxicity of peptides. As such, antimicrobial peptides are increasingly viewed as powerful multifunctional drugs.

Objective: This review will summarize findings on these alternative non-lytic modes of antimicrobial action that go beyond membrane disruption, with an emphasis on the specific interaction with microbial cell wall/membrane components, signaling of AMP exposure, and intracellular targets of peptide action. We will also explore how novel technologies can help to reveal, characterize and exploit these antimicrobial properties.

Conclusion: Detailed knowledge on non-lytic modes of action of antimicrobial peptides will help in the design and discovery of novel antibacterial and antifungal compounds.

Keywords: Antibacterial peptide, antifungal peptide, antimicrobial mechanism, apoptosis, cell-penetrating peptides, chaperone, glycan, MAPK signaling, membrane permeation, two-component system.

2. Introduction

Antimicrobial peptides and/or small antimicrobial proteins (AMP) have been characterized from a vast number of organisms, from bacteria to insects, plants and humans [1]. The increasing problem of antibiotic resistance in clinic [2,3] and the pressure to reduce antibiotic and fungicide use in agricultural and food industry [4-7] has put AMP at the edge front as promising compounds to fight microbial infections and contaminations. Over 1000 natural AMP are currently known to their amino acid sequence. Moreover, the advent of amenable synthetic procedures, wide offer of biotech companies with peptide synthesis facilities, and high throughput (HT) approaches to synthesize and screen large collections and libraries of peptides have increased substantially the number and diversity of non-natural synthetic peptides with antimicrobial activity. Thus an enormous amount of peptides endowed with antimicrobial activity are currently known. However, only a minor proportion of them have been characterized in detail and studied in relation to their antimicrobial mode of action.

AMP share common biophysical properties. They are small, from just 5-6 amino acid residues in some synthetic peptides to about 50 -and even up to 100- in natural ones; most of them (although not all) are cationic having positive charge at physiological conditions due to the presence of arginine and lysine residues, also have a high proportion (up to 50%) of hydrophobic residues, and are capable to fold or arrange into a variety of amphipathic structures and conformations. Cationic charge and amphipathic arrangement are on the basis

of their propensity of in vitro interaction with anionic lipid bilayers. In fact, initial studies after discovery of the first AMP were coincident in concluding that antimicrobial activity was a primary consequence of the capacity of cationic amphipathic peptides to interact and disrupt biological membranes, thus resulting in direct cell killing. Treatment of microorganisms with above minimal inhibitory AMP concentration resulted in microbial cell permeation that correlated with microbicidal potency. As consequence, many of the contributions that dealt with AMP mechanism relied on models of peptide interaction with -and disruption of- lipid bilayers.

However, a key weakness point was that AMP cationic charge led to the early recognition that salts -at concentrations close to physiological- blocked AMP interaction with negatively charged microbe surface groups, and also diminished the *in vitro* inhibitory activity of microorganism growth. It was difficult to reconciliate this apparent limitation with the evidence that the high number and diversity of AMP in living organisms is indicative of an important physiological role, presumably in host defense.

A way out of this dilemma derives from the recognition of multilayer roles of peptides in the regulation of host response, also acting on specific cells as effectors of the adaptive immune system [8]. Representative examples are human defensins and the cathelicidin LL-37 [9,10]. In particular, human defensins have emerged as an evolutionary link that bridges innate and adaptive immune responses [10]. But there are also additional alternatives to justify the prevalence of AMP in nature.

If specific AMP can participate in complex processes such as regulation of the mammal immune response, it is not senseless that can also exert multifaceted lethal actions on microbes. In fact, alternative mechanisms have been increasingly considered as part of AMP action against microbes [11-13]. Reports have analyzed peptide action towards microbes (not under *in vitro* membrane mimicking lipid environments) in greater detail, and accumulated convincing evidence that specific peptides do not exert antimicrobial activity by primarily permeabilizing cell membranes. Among these are apidaecin [14] or dermicidin [15] acting on bacterial cells, and specific human defensins and histatin-5 [16,17] or synthetic peptides [18,19] on fungal cells. Therefore, alternative non-lytic modes of action are recognized. Recent reviews have addressed this question in the case of antibacterial peptides [20,21] or the antifungal action of defensins [22]. This contribution aims to provide an updated and broader view to these alternative AMP modes of action or interactions with microbes, to the approaches to further investigate the extent and significance of these, as well as to discuss how this knowledge can be incorporated to the design of novel and improved AMP with higher antimicrobial potency and lower unspecific toxicity. The use of (model) microorganisms for which genomic-scale tools are available and the identification of genes that modulate the microbial susceptibility will be pivotal in the understanding of peptide antimicrobial action. We will not address, however, the responses of microbes to counteract peptide action, that recent reviews summarize in detail [23].

3. Interaction with microbial surface.

Before reaching cell membranes, the first layer of contact for surrounding peptides is the outer microbial surface. There was no evidence of specific and canonical receptors linked to the interaction of peptides with target microorganism and microbial killing. Early reports on the similar activity of stereoisomeric AMP sustained the conclusion that interaction was not stereo-specific [24]. Recently, non-chiral interaction has even been shown for peptides acting intracellularly [25]. However, there were also examples for which stereospecificity was shown [26], and in some cases correlated with an antimicrobial mechanism that was not pore-forming [14].

Several AMP bind bacterial lipopolysaccharide (LPS), and such binding is linked with the differential antimicrobial activity of peptide analogs with lower affinity for LPS [27,28] (Table 1). On the cell side, changes in cell wall/lipid bilayer composition can alter interaction and thus activity of peptides. A mechanism of bacterial resistance to peptide exposure involves the alteration of cell envelope/membrane, to increase the net charge thus reducing the electrostatic interaction with cationic antimicrobial peptides [23,29-31] (Table 2). More intriguingly, biophysical properties of specific synthetic peptides [32], or distinct combinations of temporins acting synergistically [33] can indeed modulate the translocation of peptides across bacterial outer envelopes and allow their access to and interaction with bacterial membranes.

In addition to electrostatic attraction, specific cell membrane/wall components have been shown to promote the interaction with peptides. A significant

example is the antibacterial peptidic lantibiotic nisin, for which it is established that the membrane-bound peptidoglycan precursor Lipid II acts as a docking moiety to attract the peptide to the bacterial membrane and promote peptide insertion into membrane and cell permeation [34-36]. Interestingly, nisin is active at nanomolar range against bacteria containing Lipid II, roughly three orders of magnitude more active than peptides that only act through permeation. Nisin is not active against yeast or filamentous fungi; however, yeast spheroplasts are rapidly lysed when incubated in the presence of nisin at concentrations which do not affect intact cells [37]. This latter activity is presumed to be a consequence of the intrinsic capacity of nisin to disturb lipid bilayers. Thus, even in the case of true membrane-perturbing peptides, additional factors such as their propensity to interact with specific compounds might modulate (and enhance) their (membrane) activity. Interestingly, the related peptidic lantibiotic mersacidin also binds Lipid II, albeit at its terminal N-acetylglucosamine, and inhibits bacterial growth with no cell permeation [38,39]. Because of these binding capacities nisin, as well as mersacidin, also interfere with the peptidoglycan biosynthesis of bacterial envelope [35] (Table 1), being one clear example of multilayer actions among AMP.

Nisin is a well known example, but analogous situations may occur with specific defensins, whose antifungal activity is linked to the presence of distinct classes of membrane glycolipids. Hence, the activity of the plant defensin DmAMP1 and the cyclic lipopeptide syringomycin E are dependent on the biosynthesis of the acidic sphingolipid mannosyl diinositolphosphoryl ceramide, since the presence of functional *IPT1* and *SKN1* genes were linked to an enhanced resistance

phenotype of the yeast *S. cerevisiae* [40-42] (see Table 1 and Table 2). Also, the plant RsAFP2 or the insect heliomycin are coincident in requiring the presence of the neutral sphingolipid glucosyl ceramide [43,44]. The *F. graminearum GCS1* gene involved in the biosynthesis of glucosyl ceramide also mediates the susceptibility to the plant MsDef1 defensin but, interestingly, not to the related MtDef4 [43]. It has been postulated that sphingolipid-rich lipid rafts could promote interaction with membranes of a subclass of defensin AMP [22]. However, other related defensins do not specifically require such membrane lipids, and in fact additional defensin-interacting molecules have been demonstrated [45]. On the other hand, plant PSD1 seems to operate through a distinct mechanism since it has been shown to be internalized and act on intracellular targets (see below) [46]. Therefore, although structurally related, the broad class of defensin peptides do comprise AMP with distinct interacting partners and effects on target cells.

A repeated issue in several of these examples is that sugar moieties (mostly as part of more complex molecules) seem to act as interacting/docking partners for a variety of distinct AMP. In addition to the examples described above, phosphomannans of yeast cell wall mannoproteins increase toxicity of the antifungal osmotin, probably by serving also as docking structures that facilitate the interaction and diffusion across the cell wall [47] and numerous antifungal proteins are known for their ability to bind chitin [48,49]. Mutants of filamentous fungi with specific chitin synthase genes disrupted show alterations in their sensitivity to antifungal proteins [48] (Table 2). In this regard, specific cell wall components, including glycans and glycoproteins, have been recognized as

potential targets for selective antifungals [50-54]. It would be desirable the detailed characterization of the interaction of selected AMP with such glycan structures, in order to obtain information on both the potential microbial targets and the peptide structural requirements for activity.

Additional studies report on the involvement of cell wall proteins on the fungal susceptibility to AMP. In several examples, the relevance of specific cell wall proteins is likely due to a general strengthening of cell wall that increases thickness or reinforces its structural resistance after exposure to peptides, as is the case of yeast PIR proteins in the interaction with tobacco osmotin [55] or other CWP proteins with nisin [37].

A remarkable example studied in detail is that of SSA1 and SSA2, cell surface proteins from *Candida* that mediate the activity of distinct AMP as human β-defensins and histatin, but not of human neutrophil defensins [45,56]. These proteins are highly conserved in organism from distinct phylogenetic scale as part of the ATPase heat shock protein 70 (HSP70) family. In *Saccharomyces*, they have been localized to the nucleus, cytoplasm and cell wall, and are chaperones pivotal in active protein refolding as response to stress. Binding of histatin-5 to *Candida* cell wall SSA1/2 proteins was demonstrated and also linked to fungicidal activity [56,57]. This binding is necessary but not sufficient for the cell killing activity of the peptide [57], which also requires peptide cell internalization (see below). Histatin-5 binding has been recently mapped to the ATPase domain of SSA2 protein and showed to be enhanced by protein-bound nucleotides [58].

Finally, there are peptides whose effect is expressed at the microbial surface/membrane but are known as non-lytic. The synthetic BM0 was identified as an inhibitor of the activity of a yeast plasma membrane ATPase (Pma1) that impairs *in vitro* growth with no permeation at growth-inhibitory concentrations [18]. The peptide locates and is reported to be active at the cell surface.

4. Signaling of peptide exposure

An antimicrobial action solely based on permeation is expected to be quick, not allowing a coordinated cell response to peptide exposure. However, a variety of different observations indicate that microorganism respond to antimicrobial peptides in different ways. For instance, transcriptome analyses have shown global changes in gene expression after exposure to distinct AMP in both bacteria [59-61] and fungi [62-64] (see below). These studies underline a response of microorganism that might be related to peptide mechanism of antimicrobial action and/or be part of the mechanisms to counteract peptide deleterious effect. In any case, some of these reports also indicate the existence of cell signaling components that coordinate such responses.

In bacteria, two-component and related sensor systems participate in AMP recognition and induction of transduction cascades that modulate the bacterial response to peptide exposure [59,65]. The two-component system PhoPQ is a determinant of virulence in a number of Gram-negative bacteria and mediates the interaction with AMP [30,66,67], and the membrane bound sensor kinase

PhoQ is activated by cationic AMP binding to an acidic surface domain [65]. The additional PmrAB two-component system also responds and mediates the response and resistance to AMP [61,68,69]. An analogous three-component system could operate in Gram-positives and is involved in the signaling and coordinated expression of different responses to AMP [59]. These sensor systems are part of the bacterial defensive armor and a general response mediated by them is the modification of the anionic charge of the bacterial surface to reduce peptide interaction (see below). Therefore, they would not be directly linked with the killing mechanism of AMP. However, the detailed knowledge of their involvement in the response to peptides and the structural characterization of the interaction could lead to the design of AMP capable of by-passing or disturbing these bacterial surveillance systems.

In yeast, a transmembrane receptor-like protein is required for sensitivity to osmotin, and functions upstream of RAS2 in a signaling pathway that induces apoptosis after exposure to the AMP [70,71]. Different fungal protein kinase signaling cascades mediate the response to distinct antimicrobial peptides and proteins, and mutations in the corresponding genes resulted in increased sensitivity [63,72,73] (Table 2). At least in some examples, the involvement of each pathway seems to be dependent of the specific peptide as was nicely demonstrated in the case of two related plant defensins [73]. The increased sensitivity of fungal cells deleted in components of these signaling cascades is indicative that they are part of the microbial response to peptide exposure and damage, and not necessarily linked to the peptide antimicrobial action.

On the other hand, independent studies indicate that specific AMP (such as frog dermaseptins, human lactoferrin, tobacco osmotin or fungal AFP) induce apoptosis markers in yeast [71,74,75] or filamentous fungi [76]. Contrary to the above discussed data, mutation of involved genes enhance resistance to peptides, which supports that induced microbial suicide is indeed part of the fungal killer mechanism in these examples [71,74,76]. Reactive oxygen species (ROS) are known as markers of cell suicide [77], and additional studies broaden the number of AMP for which their effect is associated with intracellular ROS production, which also suggest an induction of intracellular signaling pathways [71,78,79]. However, the role of ROS in antimicrobial action remains controversial for specific peptides such as histatin [80,81].

5. Cell internalization

Distinct AMP have been demonstrated to translocate across the cell membrane in a non-disruptive mechanism, and examples exist in bacteria and fungi. Insect apidaecin was one of the first AMP for which a non-pore forming mechanism was invoked [14], and early uptake assays with radiolabeled peptide indicated retention by *E. coli* cells [82]. The use of fluorescently labeled peptides and proteins coupled to confocal microscopy has boosted the demonstration of cell internalization of this and other AMP [19,46,83-89]. Additional techniques such as immunodetection were used to show that antimicrobial proteins enter fungal hyphae [87]. In several of these reports, peptide internalization was shown at peptide concentrations and/or times of exposure at which no obvious growth alteration or cell membrane damage could be observed. For instance, the

synthetic hexapeptide PAF26 was internalized at sub-inhibitory concentrations by fungal hyphae and caused severe morphological alterations in the absence of mycelium permeation, which was finally produced at higher completely inhibitory concentrations [19]. Another noteworthy example is that of the synthetic NK-2 peptide, which is selectively internalized by *Plasmodium* infected red blood cells due to the increase in the negative charge of the membrane that occurs in infected cells, and subsequently kills the protozoan pathogen intracellulary [90].

Targeting of peptides to specific cell compartments has also been shown and in some cases linked to the antimicrobial activity. Thus, the pea defensin PSD1 was shown to locate inside the nucleus of the model fungus *Neurospora crassa*, wherein it might alter cell cycle progression [46]. Also, histatin-5 at low (inactive) concentrations is directed towards yeast vacuoles, while inhibitory higher concentrations locate in the cytosol [17].

A paradigmatic example of cell penetration is that of antimicrobial peptides derived from bovine lactoferrin, which have been shown to be internalized by both bacterial and yeast cells [86,91]. It remains to be determined, however, whether cell internalization share common mechanism for both classes of microorganisms.

The capability to penetrate target cells is therefore increasingly viewed as a common property of distinct cationic AMP (Table 1). AMP having cell translocation activity share biophysical properties with the so-called cell

penetrating peptides (CPP), which has brought into question the differences between antimicrobial and cell penetrating peptides [92]. CPP have been proposed as shuttle vehicles for the delivery of killing or therapeutical molecules into (mammalian) cells [93]. Peptides initially known as CPP onto mammalian cells have been later demonstrated to have antimicrobial effects on distinct microorganisms, and this activity correlated with internalization into microbial cells [94,95]. An attractive hypothesis is therefore that this class of peptides are in fact dual molecules in which internalization determinants do not necessarily overlap with antimicrobial [21]. Interestingly, for selected AMP such as the proline-rich apidaecin the microbial internalization and killing activities seem to be separated, and peptide uptake was necessary but not sufficient for antibacterial activity of selected analogs [82]. Experiments should address the identification of these (separate) determinants in additional model AMP, in order to determine the minimum amino acid sequence requirements for these activities, if any, and help in the design of “modular” domains with distinct functional capabilities.

An still unsolved question relates to the actual mechanism(s) of non-lytic peptide internalization [96,97]. Despite numerous efforts, the underlying mechanism of AMP/CPP uptake is still unclear and controversy exists, which likely reflects the involvement of distinct pathways and processes depending on the peptide and cells under study. Several peptides have been demonstrated to be internalized in an active, energy-dependent process [82,88]. Internalization may follow multiple and simultaneous endocytic pathways, and even at high peptide concentrations a non-lytic endocytosis-independent uptake [97]. Initial

interaction seems to depend on cell surface complex glycans [98]. Deciphering the mechanism of peptide cell penetration including the related similarities/differences among CPP acting on mammalian cells and AMP on microbes, will undoubtedly help to design improved antimicrobial peptides endowed with higher specificity.

AMP sequence analogs will surely help to characterize the above described open questions such as the internalization mechanism/pathways, and determine structure activity relationships [84]. An study showed the importance of a single proline residue at the hinge region of buforin, required for bacterial membrane translocation [83]. This residue is also critical for maintaining both antibacterial and antifungal activities of the peptide. Interestingly, buforin analogs with the proline substituted did not penetrate bacterial cells but rather remained at the surface and seemed to kill bacteria through permeation.

6. Intracellular targets

It is expected that AMP that translocate into cells might disturb cell homeostasis in different ways determined by their intrinsic properties as well as their targeting/interaction with cellular organelles. Due to their cationic nature, most AMP readily bind nucleic acids (DNA and RNA) *in vitro* which might result in a broad inhibition of DNA synthesis, transcription and/or mRNA translation inside cells [82,85,99-101]. Even for some peptides known to cause permeation, an inhibition of DNA, RNA and/or protein synthesis was reported [102,103], indicating that disruption of cell membranes might be combined with inhibition of

intracellular targets. However, it is obvious for nearly all the examples analyzed, that nucleic acid binding by known AMP is quite unspecific, at least *in vitro*. Therefore, specificity of inhibition of such peptides would be derived from the interaction with outer microbial envelopes or cell membrane components that enable internalization.

More specific intracellular mechanisms have also been proposed. Distinct approaches have been used to identify host protein partners of AMP. Affinity purifications identified *S. cerevisiae* DNA binding proteins involved in DNA repair, as partners of the AMP dermaseptin S3 [74], and contributed to the understanding that its mode of action is related to the induction of apoptosis in yeast. Similarly, immunoaffinity purification of proline-rich AMP incubated with *E. coli* proteins identified the DnaK and GroEL bacterial chaperones with the ability to bind AMP, and DnaK binding was shown to be related to bacterial killing [104,105] (Table 1). DnaK is an ATPase HSP that is similar to the class of SSA proteins from yeast that bind to histatin (see above), and in fact the AMP pyrrhocoricin binding inhibits ATPase activity and protein refolding by the chaperone, which would led to accumulation of misfolded proteins and lethality [104]. DnaK and related chaperones are also overexpressed in *Lactococcus lactis* strains with enhanced resistance to nisin [106]. These chaperones are biologically selected for recognition and binding of aberrantly folded proteins/peptides, and as such are critical for the cell response and survival to various types of stress (for instance, heat stress). Therefore, it seems that distinct classes of AMP are prone to interact with specific cell chaperones. It

remains to be determined whether this is a behavior that extends to other AMP, and how this is related to cell killing.

A unique example of intracellular target is that of the pea defensin PSD1, which in a yeast two hybrid approach interacted with several fungal nuclear proteins, including cyclin F with which also *in vitro* binding was demonstrated [46]. The defensin translocated to the fungal nucleus (see above) and further analysis indicated that affects normal cell cycle progression.

Several members of the mammalian superfamily A of RNases have been shown to have a direct antimicrobial function, and be involved in the innate immune system [107]. Plant antimicrobial PR-10 proteins also have ribonuclease-like properties. The peanut RNase AhPR-10 has been shown to locate inside hyphae and kill susceptible fungi [108]. Interestingly, a point mutation devoid of RNase activity internalizes into hyphae but does not inhibit fungal growth or disrupt membrane permeability, thus separating the cell penetration from the ribonuclease activity and, further, linking this and the antimicrobial properties. Although not related with nuclease activity, there are previous examples in which cell penetration has been separated from peptide antimicrobial activity [82], thus confirming that penetration and killing can be separated steps of the antimicrobial mechanism of peptides.

Also, there are examples of AMP that are not internalized although elicit an intracellular action, as specific plant defensins that induce extended

citosqueleton disorganization despite being retained at the cell surface of yeast cells [72].

7. Genome-wide analyses of microbial response to peptide exposure.

The use of genomic tools is expected to help in the characterization of alternative modes of AMP action, including the effects on microorganisms, the determinants of susceptibility to peptide action and the identification of potential microbial targets. These approaches will lead to the identification of microbial genes that modulate sensitivity to peptides (Table 2), as a critical part of the detailed knowledge of AMP action. However, few studies have been reported yet, so as to draw general conclusions. Most of these have been conducted by exposing model organisms such as *E. coli* or *S. cerevisiae* to sub-lethal peptide concentrations, and analyzing the transcriptomic response or testing the susceptibility of genome-wide mutant collections. An alternative approach is to compare the transcriptional profile of AMP-resistant bacterial strains with that of the corresponding parentals [106].

These types of studies have been carried out both in Gram-negative (i.e., *E. coli*) and Gram-positive bacteria [59-61,106]. A major transcriptional change relates to stress response, and also to the attempt to reduce peptide interaction and exposure, namely by increasing the net charge of the bacterial surface (explained above), strengthening of exopolysaccharide envelope, and inducing extrusion/transporter systems [59,61,106,109]. Interestingly, it was found that selected *E. coli* genes respond differently to distinct AMP as occurs with the

sensor *BasS* of the AMP responsive two-component BasRS [61] (see also above), reflecting a specificity in AMP sensing and/or action that would depend on the peptide sequence. This latter work also showed that non-active sequence analogs do not exert the same transcriptional response as the parental antimicrobial peptide. In any case, it is noteworthy that most of the genes that respond significantly to AMP correspond to proteins of unknown function [60,61], implying the existence of a part of the microbial response that clearly needs to be characterized in detail and might be related to the activity/specificity of the peptides.

There is also a limited number of reports on the analysis at a genome scale of the effect of AMP on fungi, and these have been focused on unicellular yeasts [62-64]. Conclusions need to be critically tested for their relevance to filamentous fungi. For instance, a common report in yeast is the induction by distinct AMP of the osmotic stress response regulated by the HOG MAPK pathway, involved in osmotic tolerance and cell wall maintenance [62,63]. Indeed, it seems to be a cross-tolerance phenotype between AMP and osmotic stress [62]. However, the HOG pathway does not participate in the response of *F. graminearum* to specific defensins [73].

A direct comparison of a *S. cerevisiae* genome-wide screen between two unrelated AMP that had been previously known to kill by permeation -a dermaseptin derivative and magainin 2- indicate that their actions are more complex than membrane disruption, and also demonstrated common and -interestingly- unique effects of each peptide [64]. Thus, treatment with either

peptide elicits responses related to DNA damage that would be part of a general reaction to damage/stress. Likewise, a significant functional annotation related with ribosome assembly and rRNA processing was found among genes repressed by both peptides. However, and considering changes in gene expression and alterations of susceptibility of deletant strains, only a minor proportion of the nearly 5,000 genes analysed showed common behaviours among the two peptides. For instance, no gene deletions were found that conferred increased resistance to both peptides. Deletion of genes involved in vacuolar transport and protein targeting to the vacuole increased sensitivity to the dermaseptin peptide [64]. An independent work showed that over-expression of a different vacuolar H+-ATPase increased resistance to a distinct AMP [110], and targeting of histatin-5 to vacuoles from the surrounding cytoplasm seems to be a survival mechanism in *Candida* [17]. Thus, different approaches that include genomic screens identified transport to fungal vacuoles as a common mechanism of defence to AMP.

Also, comparisons among genomic screens based on collections of mutant strains or on transcriptomic data indicate that both approaches are complementary and required for a deep characterization of AMP mechanisms, since they do not necessarily led to the identification of the same gene sets [64]. An additional approach is also the screening of cDNA expression libraries to identify genes whose over-expression can influence the sensitivity of fungi to AMP [110], which permitted the identification of genes that led to increased either resistance or susceptibility to a plant defence peptide. Authors report that these would have not been identified in deletant mutant screens since the

corresponding knockout strains did not show alterations of sensitivity to peptides.

A limitation of the above studies on fungi is that they use unicellular yeasts, namely *S. cerevisiae*. Although it has obvious advantages, *S. cerevisiae* is also limited as a model organism. AMP active against laboratory yeast strains may have no effect on pathogenic yeast or filamentous fungi and viceversa. Also, *S. cerevisiae* has undergone gene duplication during evolution that may produce function redundancy and bias the analysis of peptide antifungal activity. Despite these disadvantages, the use of yeast as model for drug (antimicrobial peptide) characterization and development has a wide potential, albeit similar studies should be extended/confirmed onto filamentous fungi and must be encouraged.

A significant conclusion in all these genome-wide studies with both bacteria and fungi is that genes involved in known resistances to nowadays antibiotics and fungicides are not usually identified, indicating that AMP are indeed compounds of novel modes of action that could aid in the development of novel antimicrobial strategies.

8. Expert opinion

Researchers have agreed over the last years that non-lytic modes of AMP action exist, and even co-exist with membrane disrupting properties of well known lytic peptides [11,20-22]. In our view such alternative mechanisms go beyond membrane interaction and in a broad sense might explain the specific

properties of selected peptides, including their specificity towards certain microbes or cells and also their potency not always correlated with their membrane permeation capacity. As summary, Figure 1 shows a conceptual model of the steps of AMP action onto microbes that includes, but also goes beyond, their interaction with biological membranes. Three major steps are envisioned as relevant to accommodate the current knowledge of AMP mechanisms in a general model of peptide antimicrobial action: (1) Interaction with outer microbial structures, (2) interaction with cell membrane that can result in peptide sensing that signals peptide exposure, internalization/translocation to cell interior, and/or disruption of lipid bilayer, and (3) intracellular targets that are related to/explain peptide activity. In this review we have discussed examples on how specific the activity of selected peptides can be, regarding all these steps. Previously not foreseen examples are the significance of stress-related chaperones observed with distinct microorganisms and peptides at the level of protein:peptide interactions [57,58,105], inhibition of activity [104], and responsive genes [106]. Also, the importance of vacuolar transport and peptide targeting to the vacuole as suggested by overexpression/deletion of related genes [64,110], and localization data [17]. Detailed knowledge of the molecular and cellular bases of each one of these steps in an increasing number of peptides/microbes (including clinical and agronomically relevant pathogens) might allow the design of AMP with increased potency and lower unspecific toxicity.

In our view, studies on antimicrobial peptide action must be shifted from the peptide structural requirements/biophysical properties to the cell determinants

of sensitivity to peptides. The use of model organisms, genomic approaches, and screening/selection of collections of mutants will be pivotal to unravel the mechanism of action of selected peptides. We have given some relevant examples of this [61,64,106], but they are still scarce. These types of studies are identifying relevant genes modulating sensitivity to AMP (Table 2). With an increasing number of reports we will be in good position to ask the fundamental question on whether there are non-lytic modes of AMP action “common” to distinct peptides and whether each peptide class has specific properties not shared by others. Also related, we will be able to classify AMP in relation to their effect on target cells, as well as to identify those cell targets that are more promising in terms of potency and specificity.

The use of synthetic peptides and their sequence analogs will be critical in establishing structure/activity relationships between peptide sequence, antimicrobial activity and effects on microorganisms, with the potential for extrapolation to the genetic/molecular determinants of peptide susceptibility of the latter. It is intriguing, for instance, that highly related plant defensins seem to interact with distinct cell components/signaling cascades [43,73]. A consequence is that minor amino acid changes in AMP could be responsible for differences in the specific modes of action [35,57]. Due to their small size and feasibility of synthetic production, small AMP can be used to dissect the molecular basis of such differences and specificities.

It is conceivable that an holistic combination of detailed knowledge of modes of action, genome screens, high-throughput peptide identification technologies,

and amino acid sequence requirements, could lead to the development of novel rationally designed AMP to be used as drugs against functionally important microbial targets.

Among the very interesting potential of AMP, the elucidation of specific modes of action constrained in relatively small peptide molecules might allow the combination of more than one killing mechanisms in one single AMP, as multitarget drugs, and could potentiate the antimicrobial activity while diminishing the probability of developing resistance in the susceptible microbes [35].

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Figure Legend

Figure 1.

General model of the antimicrobial mechanism of AMP. The figure shows a schematic view of an eukaryotic cell, although the conceptual framework can be also applied to bacterial cells. A major difference would rely on peptide import to nucleus, which has been demonstrated for specific AMP in fungal cells (see text for details). Three major steps are depicted in the antimicrobial mechanism of AMP (blue line): (1) Interaction with outer microbial structures, (2) interaction with cell membrane components that results in disruption of lipid bilayer/pore-formation (2a), internalization/translocation to cell interior (2b), and/or signaling of peptide exposure (2c); and (3) intracellular targets (as detailed, 3a-3d) that have been demonstrated in distinct examples as related to peptide activity (see text for details). The relevance of glycan structures for peptide interaction is highlighted by hexagons within cell wall/envelope glycans, membrane lipids, and glycoproteins. AMP folding (thick blue line) may change upon each step.

Table 1. Selected examples of non-lytic antimicrobial peptide mechanisms

Peptide	Microorganism	Interaction/Binding	Cell Uptake	Mechanism	References
Nisin	Bacteria	Lipid II		Inhibition of peptidoglycan synthesis	[35,36]
Buforin II	Bacteria		Yes	Binding DNA/RNA	[83,85]
Magainin 2	Bacteria	LPS	Yes		[91,111]
Indolicidin	Bacteria	LPS	Yes	Binding to nucleic acids/Inhibition of proteins and nucleic acid synthesis	[99,112]
Pyrrhocoricin	Bacteria		Yes	HSP (DnaK) binding / Prevention of chaperone protein folding	[84,104]
Lactoferricin B	Bacteria	LPS	Yes	Binding to nucleic acids / Inhibition of macromolecular synthesis	[28,91,113]
DmAMP1	Fungi/yeast	M(IP)2C sphingolipid			[42]
RsAFP2	Fungi/yeast	GlcCer sphingolipid		ROS	[79]
Psd1	Fungi		Yes	Binding to Cyclin F / Cell cycle impairment	[46]
Osmotin PR5	Yeast	Membrane Receptors / Phosphomannoproteins		Apoptosis	[47,70]
BM0	Yeast		No	Inhibition of Plasma membrane H+-ATPase	[18]
Dermaseptin S3	Yeast			Apoptosis, ROS and DNA damage	[74]
Histatin-5	Yeast	SSA1/2 proteins	Yes	ROS (¿?), Subcellular targeting	[17,58,80]

Table 2. Representative examples of genes that modulate the sensitivity of microorganisms to antimicrobial peptides.

Peptide	Microorganism	Genes	Functional annotation/category	References
Different AMP	*S. typhimurium*	*pagP*	Lipid A acylation	[30]
Different AMP	*S. aureus*	*Dlt* Operon	D-alanine esterification of LTA	[29]
Different AMP	*S. aureus*	*MprF*	Lys modification of PG	[31]
Nisin	*L. lactis*	*DltD*	D-alanine esterification of LTA	[106]
		AhrC	Transcriptional repressor of Arc operon (Arginine catabolism)	[106]
Protamine	*S. meliloti*	*ExoT, ExoU, NdvB*	Exopolysaccharide and glucan biosynthesis	[109]
		Y01361, HutU, UreF	Nitrogen metabolism	[109]
		Y01826	ABC membrane transporter	[109]
DmAMP1	*S. cerevisiae*	*IPT1*	Sphingolipid biosynthesis	[42]
		SKN1	Sphingolipid biosynthesis	[40]
Syringomicyn E	*S. cerevisiae*	*IPT1, ELO2, ELO3, CSG1, CSG2*	Sphingolipid biosynthesis	[41]
MiAMP1	*S. cerevisiae*	*YGL191W*	Mitochondrial Cyt. c ox. sub.	[110]
		VMA11	Vacuolar H^+-ATPase sub. c	[110]

		SKS1	Serine/Threonine protein kinase	[110]
		PTC7	Protein phosphatase type 2C	[110]
Dermaseptin S3	*S. cerevisiae*	*Izh2, Izh3, Stm1, Aif1*	Regulation of Apoptosis	[74]
MsDef1	*F. graminearum*	*GCS1*	Glucosylceramide synthase	[43]
MsDef1 / MtDef2 / RsAFP2	*F. graminearum*	*STE11, STE7, GPMK1, MGV1*	MAPK signaling cascade	[73]
AFP	*A. oryzae*	*ChsB, CsmA*	Chitin synthases, classes III and V	[48]
AFP	*F. oxysporum*	*ChsV*	Chitin synthase, class V	[48]

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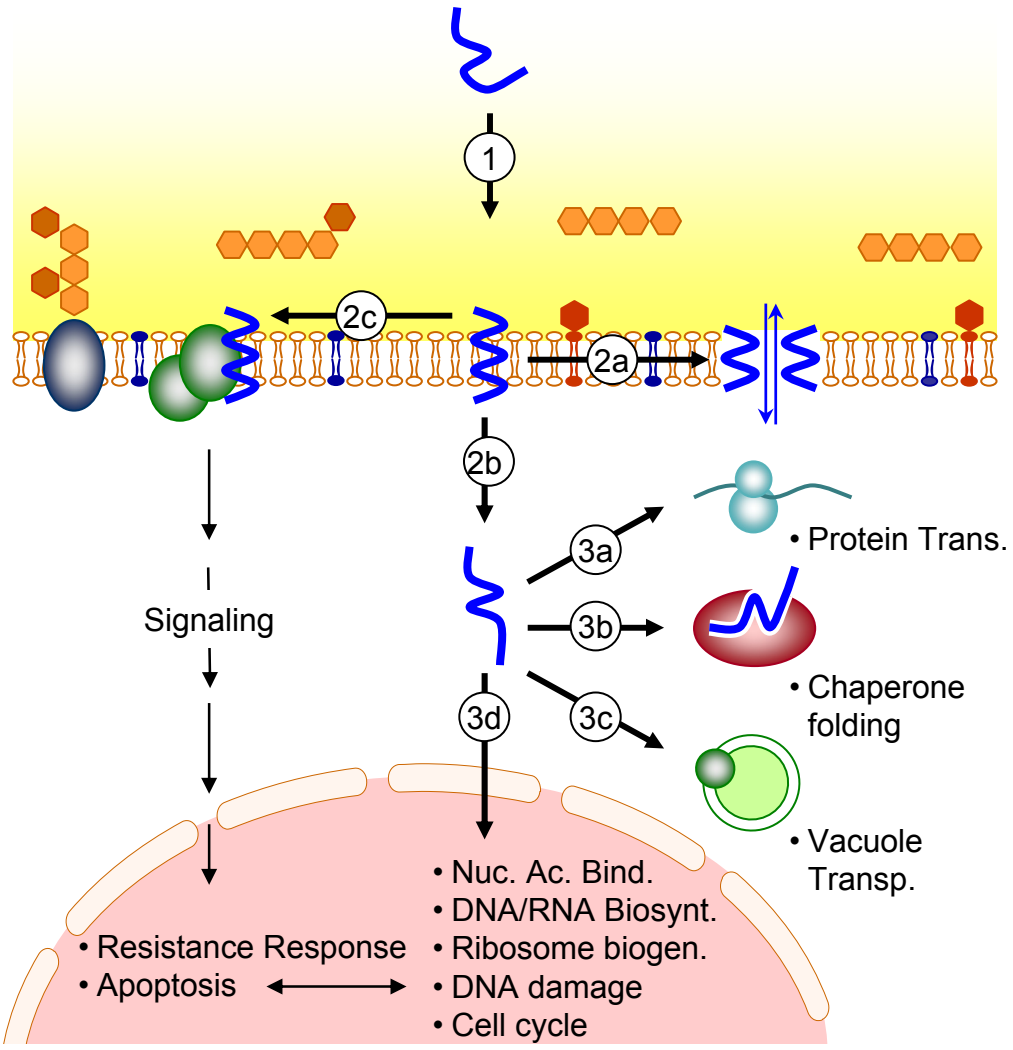
Figure 1


1
2c
2a
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Signaling
• Protein Trans.
• Chaperone folding
• Vacuole Transp.
• Nuc. Ac. Bind.
• DNA/RNA Biosynt.
• Ribosome biogen.
• DNA damage
• Cell cycle
• Resistance Response
• Apoptosis