

1 **Antimicrobial peptides: To membranes and beyond**

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1 1. Abstract

2

3 Background: Antimicrobial peptides (AMP) are widely recognized as promising

4 alternatives to the current use of antibiotics and fungicides. Amino acid

5 sequences of a vast majority of AMP share cationic and amphipathic

6 biophysical properties that allow their insertion into lipid bilayers, and can lead

7 to alteration of biological membrane functions. Initial characterization studies

8 linked these properties to antimicrobial killing activity. However, additional data

9 indicate that this is not the sole mode of action and that more subtle

10 mechanisms might mediate the interaction with and effect to target microbes, as

11 well as the specificity and toxicity of peptides. As such, antimicrobial peptides

12 are increasingly viewed as powerful multifunctional drugs.

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14 Objective: This review will summarize findings on these alternative non-lytic

15 modes of antimicrobial action that go beyond membrane disruption, with an

16 emphasis on the specific interaction with microbial cell wall/membrane

17 components, signaling of AMP exposure, and intracellular targets of peptide

18 action. We will also explore how novel technologies can help to reveal,

19 characterize and exploit these antimicrobial properties.

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21 Conclusion: Detailed knowledge on non-lytic modes of action of antimicrobial

22 peptides will help in the design and discovery of novel antibacterial and

23 antifungal compounds.

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

1 2. Introduction

2

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

18

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

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

10
11 However, a key weakness point was that AMP cationic charge led to the early
12 recognition that salts -at concentrations close to physiological- blocked AMP
13 interaction with negatively charged microbe surface groups, and also
14 diminished the *in vitro* inhibitory activity of microorganism growth. It was difficult
15 to reconcile this apparent limitation with the evidence that the high number
16 and diversity of AMP in living organisms is indicative of an important
17 physiological role, presumably in host defense.

18
19 A way out of this dilemma derives from the recognition of multilayer roles of
20 peptides in the regulation of host response, also acting on specific cells as
21 effectors of the adaptive immune system [8]. Representative examples are
22 human defensins and the cathelicidin LL-37 [9,10]. In particular, human
23 defensins have emerged as an evolutionary link that bridges innate and
24 adaptive immune responses [10]. But there are also additional alternatives to
25 justify the prevalence of AMP in nature.

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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.

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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 stereospecific [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

1 example is the antibacterial peptidic lantibiotic nisin, for which it is established
2 that the membrane-bound peptidoglycan precursor Lipid II acts as a docking
3 moiety to attract the peptide to the bacterial membrane and promote peptide
4 insertion into membrane and cell permeation [34-36]. Interestingly, nisin is
5 active at nanomolar range against bacteria containing Lipid II, roughly three
6 orders of magnitude more active than peptides that only act through
7 permeation. Nisin is not active against yeast or filamentous fungi; however,
8 yeast spheroplasts are rapidly lysed when incubated in the presence of nisin at
9 concentrations which do not affect intact cells [37]. This latter activity is
10 presumed to be a consequence of the intrinsic capacity of nisin to disturb lipid
11 bilayers. Thus, even in the case of true membrane-perturbing peptides,
12 additional factors such as their propensity to interact with specific compounds
13 might modulate (and enhance) their (membrane) activity. Interestingly, the
14 related peptidic lantibiotic mersacidin also binds Lipid II, albeit at its terminal N-
15 acetylglucosamine, and inhibits bacterial growth with no cell permeation
16 [38,39]. Because of these binding capacities nisin, as well as mersacidin, also
17 interfere with the peptidoglycan biosynthesis of bacterial envelope [35] (Table
18 1), being one clear example of multilayer actions among AMP.

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

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

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

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

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

12
13 A remarkable example studied in detail is that of SSA1 and SSA2, cell surface
14 proteins from *Candida* that mediate the activity of distinct AMP as human β -
15 defensins and histatin, but not of human neutrophil defensins [45,56]. These
16 proteins are highly conserved in organism from distinct phylogenetic scale as
17 part of the ATPase heat shock protein 70 (HSP70) family. In *Saccharomyces*,
18 they have been localized to the nucleus, cytoplasm and cell wall, and are
19 chaperones pivotal in active protein refolding as response to stress. Binding of
20 histatin-5 to *Candida* cell wall SSA1/2 proteins was demonstrated and also
21 linked to fungicidal activity [56,57]. This binding is necessary but not sufficient
22 for the cell killing activity of the peptide [57], which also requires peptide cell
23 internalization (see below). Histatin-5 binding has been recently mapped to the
24 ATPase domain of SSA2 protein and showed to be enhanced by protein-bound
25 nucleotides [58].

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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

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

13
14 In yeast, a transmembrane receptor-like protein is required for sensitivity to
15 osmotin, and functions upstream of RAS2 in a signaling pathway that induces
16 apoptosis after exposure to the AMP [70,71]. Different fungal protein kinase
17 signaling cascades mediate the response to distinct antimicrobial peptides and
18 proteins, and mutations in the corresponding genes resulted in increased
19 sensitivity [63,72,73] (Table 2). At least in some examples, the involvement of
20 each pathway seems to be dependent of the specific peptide as was nicely
21 demonstrated in the case of two related plant defensins [73]. The increased
22 sensitivity of fungal cells deleted in components of these signaling cascades is
23 indicative that they are part of the microbial response to peptide exposure and
24 damage, and not necessarily linked to the peptide antimicrobial action.

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1 On the other hand, independent studies indicate that specific AMP (such as frog
2 dermaseptins, human lactoferrin, tobacco osmotin or fungal AFP) induce
3 apoptosis markers in yeast [71,74,75] or filamentous fungi [76]. Contrary to the
4 above discussed data, mutation of involved genes enhance resistance to
5 peptides, which supports that induced microbial suicide is indeed part of the
6 fungal killer mechanism in these examples [71,74,76]. Reactive oxygen species
7 (ROS) are known as markers of cell suicide [77], and additional studies broaden
8 the number of AMP for which their effect is associated with intracellular ROS
9 production, which also suggest an induction of intracellular signaling pathways
10 [71,78,79]. However, the role of ROS in antimicrobial action remains
11 controversial for specific peptides such as histatin [80,81].

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13 5. Cell internalization

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

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

9
10 Targeting of peptides to specific cell compartments has also been shown and in
11 some cases linked to the antimicrobial activity. Thus, the pea defensin PSD1
12 was shown to locate inside the nucleus of the model fungus *Neurospora crassa*,
13 wherein it might alter cell cycle progression [46]. Also, histatin-5 at low (inactive)
14 concentrations is directed towards yeast vacuoles, while inhibitory higher
15 concentrations locate in the cytosol [17].

16
17 A paradigmatic example of cell penetration is that of antimicrobial peptides
18 derived from bovine lactoferrin, which have been shown to be internalized by
19 both bacterial and yeast cells [86,91]. It remains to be determined, however,
20 whether cell internalization share common mechanism for both classes of
21 microorganisms.

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

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

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

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

6
7 AMP sequence analogs will surely help to characterize the above described
8 open questions such as the internalization mechanism/pathways, and
9 determine structure activity relationships [84]. An study showed the importance
10 of a single proline residue at the hinge region of buforin, required for bacterial
11 membrane translocation [83]. This residue is also critical for maintaining both
12 antibacterial and antifungal activities of the peptide. Interestingly, buforin
13 analogs with the proline substituted did not penetrate bacterial cells but rather
14 remained at the surface and seemed to kill bacteria through permeation.

15 16 6. Intracellular targets

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

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

6
7 More specific intracellular mechanisms have also been proposed. Distinct
8 approaches have been used to identify host protein partners of AMP. Affinity
9 purifications identified *S. cerevisiae* DNA binding proteins involved in DNA
10 repair, as partners of the AMP dermaseptin S3 [74], and contributed to the
11 understanding that its mode of action is related to the induction of apoptosis in
12 yeast. Similarly, immunoaffinity purification of proline-rich AMP incubated with
13 *E. coli* proteins identified the DnaK and GroEL bacterial chaperones with the
14 ability to bind AMP, and DnaK binding was shown to be related to bacterial
15 killing [104,105] (Table 1). DnaK is an ATPase HSP that is similar to the class of
16 SSA proteins from yeast that bind to histatin (see above), and in fact the AMP
17 pyrrolicin binding inhibits ATPase activity and protein refolding by the
18 chaperone, which would lead to accumulation of misfolded proteins and lethality
19 [104]. DnaK and related chaperones are also overexpressed in *Lactococcus*
20 *lactis* strains with enhanced resistance to nisin [106]. These chaperones are
21 biologically selected for recognition and binding of aberrantly folded
22 proteins/peptides, and as such are critical for the cell response and survival to
23 various types of stress (for instance, heat stress). Therefore, it seems that
24 distinct classes of AMP are prone to interact with specific cell chaperones. It

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

3

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

9

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

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

1 cytoskeleton disorganization despite being retained at the cell surface of yeast
2 cells [72].

3 4 7. Genome-wide analyses of microbial response to peptide exposure.

5
6 The use of genomic tools is expected to help in the characterization of
7 alternative modes of AMP action, including the effects on microorganisms, the
8 determinants of susceptibility to peptide action and the identification of potential
9 microbial targets. These approaches will lead to the identification of microbial
10 genes that modulate sensitivity to peptides (Table 2), as a critical part of the
11 detailed knowledge of AMP action. However, few studies have been reported
12 yet, so as to draw general conclusions. Most of these have been conducted by
13 exposing model organisms such as *E. coli* or *S. cerevisiae* to sub-lethal peptide
14 concentrations, and analyzing the transcriptomic response or testing the
15 susceptibility of genome-wide mutant collections. An alternative approach is to
16 compare the transcriptional profile of AMP-resistant bacterial strains with that of
17 the corresponding parentals [106].

18
19 These types of studies have been carried out both in Gram-negative (i.e., *E.*
20 *coli*) and Gram-positive bacteria [59-61,106]. A major transcriptional change
21 relates to stress response, and also to the attempt to reduce peptide interaction
22 and exposure, namely by increasing the net charge of the bacterial surface
23 (explained above), strengthening of exopolysaccharide envelope, and inducing
24 extrusion/transporter systems [59,61,106,109]. Interestingly, it was found that
25 selected *E. coli* genes respond differently to distinct AMP as occurs with the

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

10
11 There is also a limited number of reports on the analysis at a genome scale of
12 the effect of AMP on fungi, and these have been focused on unicellular yeasts
13 [62-64]. Conclusions need to be critically tested for their relevance to
14 filamentous fungi. For instance, a common report in yeast is the induction by
15 distinct AMP of the osmotic stress response regulated by the HOG MAPK
16 pathway, involved in osmotic tolerance and cell wall maintenance [62,63].
17 Indeed, it seems to be a cross-tolerance phenotype between AMP and osmotic
18 stress [62]. However, the HOG pathway does not participate in the response of
19 *F. graminearum* to specific defensins [73].

20
21 A direct comparison of a *S. cerevisiae* genome-wide screen between two
22 unrelated AMP that had been previously known to kill by permeation -a
23 dermaseptin derivative and magainin 2- indicate that their actions are more
24 complex than membrane disruption, and also demonstrated common and -
25 interestingly- unique effects of each peptide [64]. Thus, treatment with either

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

16
17 Also, comparisons among genomic screens based on collections of mutant
18 strains or on transcriptomic data indicate that both approaches are
19 complementary and required for a deep characterization of AMP mechanisms,
20 since they do not necessarily led to the identification of the same gene sets [64].

21 An additional approach is also the screening of cDNA expression libraries to
22 identify genes whose over-expression can influence the sensitivity of fungi to
23 AMP [110], which permitted the identification of genes that led to increased
24 either resistance or susceptibility to a plant defence peptide. Authors report that
25 these would have not been identified in deletant mutant screens since the

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

3
4 A limitation of the above studies on fungi is that they use unicellular yeasts,
5 namely *S. cerevisiae*. Although it has obvious advantages, *S. cerevisiae* is also
6 limited as a model organism. AMP active against laboratory yeast strains may
7 have no effect on pathogenic yeast or filamentous fungi and viceversa. Also, *S.*
8 *cerevisiae* has undergone gene duplication during evolution that may produce
9 function redundancy and bias the analysis of peptide antifungal activity. Despite
10 these disadvantages, the use of yeast as model for drug (antimicrobial peptide)
11 characterization and development has a wide potential, albeit similar studies
12 should be extended/confirmed onto filamentous fungi and must be encouraged.

13
14 A significant conclusion in all these genome-wide studies with both bacteria and
15 fungi is that genes involved in known resistances to nowadays antibiotics and
16 fungicides are not usually identified, indicating that AMP are indeed compounds
17 of novel modes of action that could aid in the development of novel
18 antimicrobial strategies.

19
20 8. Expert opinion

21
22 Researchers have agreed over the last years that non-lytic modes of AMP
23 action exist, and even co-exist with membrane disrupting properties of well
24 known lytic peptides [11,20-22]. In our view such alternative mechanisms go
25 beyond membrane interaction and in a broad sense might explain the specific

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

23

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

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

12

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

23

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

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

4
5 Among the very interesting potential of AMP, the elucidation of specific modes
6 of action constrained in relatively small peptide molecules might allow the
7 combination of more than one killing mechanisms in one single AMP, as
8 multitarget drugs, and could potentiate the antimicrobial activity while
9 diminishing the probability of developing resistance in the susceptible microbes
10 [35].

11 12 9. Acknowledgements

13
14 We apologize to all the investigators whose research could not be appropriately
15 cited due to space limitations. Our work on antimicrobial peptides has been
16 supported by grants BIO2003-00927 and BIO2006-09523 (Spain). M.G. is
17 recipient of a research contract from the JAE-DOC Program (CSIC, Spain).

1 **Figure Legend**

2

3 Figure 1.

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

17

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

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

1

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Figure 1

