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Non-Membrane Permeabilizing Modes of Action of Antimicrobial Peptides on Bacteria

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Abstract: Antimicrobial peptides (AMPs) are a large class of innate immunity effectors with a remarkable capacity to inactivate microorganisms. Their ability to kill bacteria by membranolytic effects has been well established. However, a lot of evidence points to alternative, non-lytic modes of action for a number of AMPs, which operate through interactions with specific molecular targets. It has been reported that non-membrane-permeabilizing AMPs can bind to and inhibit DNA, RNA or protein synthesis processes, inactivate essential intracellular enzymes, or affect membrane septum formation and cell wall synthesis. This minireview summarizes recent findings on these alternative, non-lytic modes of antimicrobial action with an emphasis to the experimental approaches used to clarify each step of their intracellular action, i.e. the cell penetration mechanism, intracellular localization and molecular mechanisms of antibacterial action. Despite the fact that such data exists for a large number of peptides, our analysis indicates that only for a small number of AMPs sufficient data have been collected to support a mode of action with an authentic and substantial contribution by intracellular targeting. In most cases, peptides with non-lytic features have not been thoroughly analyzed, or only a single aspect of their mode of action has been taken into consideration and therefore their mechanism of action can only be hypothesized. A more detailed knowledge of this class of AMPs would be important in the design of novel antibacterial agents against unexploited targets, endowed with the capacity to penetrate into pathogen cells and kill them from within.

Keywords: Antibiotic, antimicrobial peptide, intracellular target, mechanism of action, metabolic inhibition, non-membranolytic.

1. INTRODUCTION

The spread of antibiotic-resistant bacteria becomes of increasing concern as more and more antibiotics are rendered ineffective [1]. The World Health Organization has identified antimicrobial resistance as one of the three greatest threats to human health. The problem is so serious that in some cases antibiotic resistance now represents a potential public health disaster, with a very real threat that infectious diseases may soon be untreatable in certain circumstances [2]. In this situation, an urgent need to develop new bactericidal agents which target resistant pathogens is evident.

A number of new antibiotics have recently been approved or are in advanced development to try to meet this demand. Unfortunately most of them belong to existing classes with similar mechanism of action to known antibiotics, and this can raise problems in terms of cross-resistance [3]. Other than to design new antibiotics, a key to future development is to understand the mode of action of new antibacterial agents.

Antimicrobial peptides (AMPs) represent a promising class of such new compounds, especially as they have already proven their efficacy as part of innate immunity [4]. They are an evolutionarily conserved component of the innate immune response and have been found among all kingdoms of life, ranging from animals to plants [5] (For a comprehensive list of antimicrobial peptide databases see [6]). AMPs are multifunctional molecules that have a central role in infection and inflammation. Besides direct antimicrobial activity against bacteria, viruses, fungi and parasites, several AMPs influence diverse cellular processes. Some AMPs stimulate cytokine release, chemotaxis, antigen presentation, angiogenesis and wound healing (see the review [7, 8]), others have been shown to be cytotoxic for certain tumors [9].

AMPs are generally small in size (9 to 100 amino acids) and most of them, but not all, are cationic at physiological conditions due to a high content of arginine and lysine residues. They also usually have a high proportion (up to 50%) of hydrophobic residues and can fold or arrange into a variety of amphipathic structures and conformations. Their sequence diversity in so high that it is difficult to classify them except on the basis of their amino acid composition and secondary structure. They have been divided into subgroups on the basis of four major classes: β-sheet, α-helical, β-hairpin and peptides with extended conformation [10, 11].

Positive charges and amphipathic residue arrangement explain their high propensity for *in vitro* interaction with anionic lipid bilayers [12]. Extensive studies on endogenous AMPs, as well as on artificial peptides derived from them, indicate that antimicrobial peptide-mediated permeabiliza-

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tion/disruption of the microbial cytoplasmic membrane is the main mechanism of cell killing for most AMPs [13]. The details of the actual membrane permeation process are still not clear completely. Although several models were proposed in recent years, essentially all of them suggest that the membrane permeation process takes place via two major consecutive steps: i) peptides bind onto the membrane surface until a threshold concentration occurs and ii) they organize to form a permeation pathway [5, 12]. The modes of action of AMPs based on membrane permeabilization are described in a number of excellent reviews [10-12, 14].

In the last decade increasing data have been collected suggesting that some, or even most, antimicrobial peptides affect microbial viability also by other mechanisms, in addition or in alternative to their membrane-permeabilizing/disrupting properties [10, 15-17]. These modes of action include interactions with intracellular targets or the disruption of key processes without an immediate membranolytic effect.

Aim of this review is to summarize the different aspects which characterize non-lytic mechanisms of activity of AMPs, specifically against bacteria, focusing also on the main techniques used and the principal results.

2. ANTIBACTERIAL PEPTIDES WITH NON-MEMBRANE-PERMEABILIZING ACTIVITY

It is largely recognized that certain AMPs inactivate bacteria without extensive membrane-permeabilizing (non-lytic) action so that other mechanisms, including molecular interactions with internal targets, have been proposed [10, 15].

Virtually all AMPs have a high affinity for microbial membranes, leading to a certain degree of perturbation [5, 16]. For this reason, it is not facile to determine if a peptide shows a mainly non-lytic mechanism of killing, or if the lethal step is different from membrane damage. There are very few examples of peptides which do not damage the target membranes under any conditions, or at any concentration. Insect apidaecin-type peptides completely lack membrane permeabilization capacity up to concentrations that exceed lethal doses by four orders of magnitude [18, 19]. However, most peptides for which a different killing mechanism has been documented, permeabilize the bacterial membranes when their concentrations are increased well above their MIC values. Peptides derived from pleurocidin, an α-helical cationic peptide from winter flounder, applied at five-fold its MIC value did not permeabilize *E. coli* cytoplasmic membrane, even though it caused a decrease in the viable colony count. Conversely, at 10-fold the MIC it caused an immediate maximal membrane depolarization [20]. The proline-rich peptide Bac7, at near-MIC concentrations, inactivates bacteria via a mechanism based on a specific uptake that is followed by its binding to intracellular targets, but it can also kill bacteria through a secondary membranolytic mechanism when applied at concentrations several folds its MIC value [21]. For these reasons it is important to study the mode of action of AMPs in relation to the concentrations applied.

An important clue of a non-lytic mode of action is the temporal dissociation between cell death, measured as inhibition of colony formation, and changes in membrane permeability. For lytic antimicrobial peptides, permeabilization of the microbial membrane and cell killing are rapid and concomitant events [22]. On the contrary, non-lytic AMPs often show a lag period before observing membrane damage: in this case the increase of permeabilization may be a secondary effect of already non viable bacteria [23], as also observed with some classical antibiotics having intracellular targets [24, 25]. These observations suggest that in a non-membranolytic killing, membrane damage and cell death are independent events that occur at different times and/or concentrations.

3. MECHANISMS OF BACTERIAL INTERNALIZATION

Despite increasing evidence that several AMPs have intracellular targets, the precise mechanism whereby some AMPs enter bacterial cells is not clear. A main mechanism is likely a spontaneous translocation, as contemplated by the Shai-Matsuzaki-Huang model [12, 26, 27]. Peptides first bind to the membrane surface and then, by virtue of their amphipathic structure, insert into the membrane, breaking up lipid chain associations and forming transient pores. Upon disintegration of these pores, some peptides become translocated to the inner leaflet of the membrane. Below the critical peptide concentration that can cause a collapse of the membrane itself, peptide passage preserves the integrity of the membrane, which is only transiently breached.

A second mechanism based on membrane translocation mediated by a bacterial protein has been observed only for the proline-rich group of antimicrobial peptides (PR-AMPs) [28, 29]. Different PR-AMPs expressed in mammals and insects, including PR-39, Bac7, apidaecin 1b exploit the inner membrane protein SbmA to efficiently penetrate into *E. coli* and other gram-negative bacteria [30-32]. Mutants lacking this peptide transporter show a certain decrease of susceptibility to many mammalian and insect PR-AMPs [30, 33, 34].

4. TECHNIQUES USED TO ASSESS THE EXTENT OF MEMBRANE INTEGRITY

A variety of techniques are used to assess membrane integrity and eventually to establish that an AMP has a different mode of action from direct membrane damage. Microbial membrane permeabilization may be studied using model membranes, liposomes, large unilamellar vesicles (LUV) as well as live cells. Each single method provides a different view of peptide activity and it is thus important to consider that no single technique is capable of adequately determining the precise mechanism of action [10]. Techniques based on membrane-mimetic systems have been described in detail elsewhere [13, 35, 36]. A simple example is given by the use of the fluorescent dye calcein or other fluorophores to evaluate leakage from LUVs or liposomes as an indicator of membrane permeabilization. This approach has been largely used to assess the membrane activity of a number of AMPs including magainins [27], cecropins [37], defensins [38], and others. Membrane mimetic systems allow simulation of particular characteristics of the biological membranes, but they cannot adequately represent the highly complex and heterogeneous bacterial membranes. Thus, to establish or to ex-

clude the capacity of peptides to depolarize or to permeabilize the bacterial membranes, assays using whole bacteria should also be performed.

Methods to measure inner and outer membrane permeabilization on the bacteria include the detection of molecules (enzyme substrates, fluorophores) which are released or internalized into the cells, giving thus information on the permeability of the membranes. One popular assay is based on the *E. coli* ML-35 pYC strain (see examples in [39-43]), which constitutively expresses the periplasmic β-lactamase and cytoplasmic β-galactosidase, but not the lactose permease, and thus allows to follow the unmasking of the activity of the two enzymes using specific impermeant extracellular substrates, giving colored products. To expand this assay also to different microbial species, uptake of cell impermeant dyes, such as propidium iodide (PI) [44, 45], and Sytox green [46], calcein and FITC-dextran [47] have been successfully used to assess cytoplasmic membrane integrity or to obtain data about the extent of the damage and measure the diameter of pores formed by the peptide [47].

Concurrent cell killing assays with membrane permeabilization test allows to establish whether the two effects are connected. The proline-rich peptide Bac7(1-35), for example, rapidly killed *E. coli* at micromolar concentrations while PI remained out of the cells [21]. The different capacity to damage the bacterial membrane of buforin II analogues with changed proline position has been revealed by a simultaneous assessment of permeabilized PI-positive cells, DNA binding and antibacterial activity [48].

Membrane depolarization of gram-positive or gram-negative bacteria may be evaluated using potential-sensitive fluorescent distributional probes. Cells that have a membrane potential (negative inside) accumulate the cationic cyanines, such as DiOC6(3) and diSC3(5), whereas oxonols such as DiBAC4(3) are excluded. The oxonol dyes only enter the cell in case of membrane potential depletion (see examples in [20, 49, 50]). diSC3(5) distributes between the cells and the medium, and self-quenches when it is concentrated inside the bacterial cells. If the membrane is depolarized by an AMP, the probe is released into the medium causing a measurable increase in fluorescence.

It is important to take into account that membrane potential does not reflect only membrane integrity, but also the energy status as well as viability of cells [51], thus a comparative analysis that evaluates other parameters may help to give an explanation. For example, the mode of action of human lactoferrin peptide 2 against pathogenic *E. coli* strains was established by monitoring membrane potential together with membrane integrity and metabolic processes by using the fluorescent probes DiBAC4(3), propidium iodide, and carbonyl cyanide m-chlorophenylhydrazone (CCCP), respectively [52].

A multiparametric flow cytometric analysis may be carried out to monitor variations of morphological parameters and use, at the same time, a combination of different fluorescent probes. For example, information on the permeabilization, intracellular enzymatic activity and cell injury and death caused by tachyplesin I on *E. coli* cells were investigated by flow cytometric analysis using single -or double-staining with carboxyfluorescein diacetate or PI [53].

To observe the intracellular localization of peptides and, at the same time, to exclude membrane-permeabilizing effects [44], fluorescent derivatives of AMPs and confocal laser scanning microscopy (CLSM) or flow cytometry have been used (see examples in [46, 54, 55]). To quench the extracellular fluorescence due to the possible binding of the peptide on the bacterial surface, a quencher such as trypan blue or TAMRA should be added to the medium [56].

Biotin-labeled AMPs have also been used as alternative to fluorescent derivatives [57], e.g. to evaluate by CLSM peptide translocation into *E. coli* cells of buforin II [58] and some histone-derived antimicrobial peptides [45, 59]. Subcellular localization of peptides was detected using a fluorescent conjugate of streptavidin, after Triton-X permeabilization of the cells.

Although this procedure is widely used, it should be considered that fixation of the cells might change the cellular distribution of the peptide, this being especially true if the peptide has not been washed out before fixation. To overcome this drawback, subcellular localization has been carried out with fluorescent derivatives of peptides using protocols for unfixed cells [44, 53, 55, 60].

Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) are very powerful techniques to provide insights on bacterial morphology and surface modifications caused by AMPs [49, 61, 62]. The localization of immunolabelled antimicrobial peptides have also been studied using transmission electron microscopy (TEM) [21, 63]. In this case, complementary techniques should be performed to verify the integrity of the bacterial membranes.

Recently, ruthenium-substituted derivatives of AMPs have been used for an interesting development of TEM which allows to omit the lead-staining step being these derivatives electron-dense and thus directly observable. Combining this procedure with graphite furnace atomic absorption spectrometry it is possible to provide absolute quantitation of the ruthenium-labeled peptide in subcellular fractions. Because ruthenium does not occur naturally in bacteria, it is ideally suited for peptide tracing in the cellular environment [64]. The intra-bacterial concentration of peptide has also been estimated by using radioactive derivatives of AMPs, by measuring the amount of radioactivity stably retained by the microorganism after extensive washing of a radioactive peptide-treated bacterial culture [18, 65].

5. INTRACELLULAR MODE OF ACTION

The much lower number of studies reporting data on intracellular targets of AMPs in comparison with those referring to membranolytic effects seems to reflect the relative importance of these two different mechanisms in the mode of action of AMPs. However, the number of reports describing non-lytic killing mechanisms of AMPs has been rapidly increased in the last years [17], underlining the complexity of the action of these molecules.

Both single and multiple targets have been proposed for different non-lytic AMPs. Inhibition of protein synthesis,

DNA binding affecting transcription/replication, cell wall biosynthesis inhibition, and inactivation of fundamental enzyme activities are among the principal mechanisms of action proposed (Fig. **1**). Unfortunately in several studies the state of integrity of the membranes has not been carefully checked. Therefore, it is not known whether the killing activity may be only partially or totally due to intrabacterial action or to secondary effects caused by membrane permeabilization. Examples of those that are the most documented studies are now reported and briefly summarized in Table **1**.

5.1. Inhibition of Protein Synthesis

Inhibition of protein synthesis has been indicated as the killing mechanism in several studies, although in most cases a description of the mechanism of inhibition has not been provided. Studies with the indolicidin variant CP10A showed that this peptide inhibits incorporation of amino acid precursors in *S. aureus,* at twofold the MIC [66]. A similar observation was obtained with a pleurocidin derivative [20]. Inhibition of protein synthesis was reported also for the partially α-helical lactoferricin *in vitro,* at high concentration, and *in vivo* on *E. coli* and *B. subtilis* [67], even though also other biosynthetic pathways were influenced by the peptide.

The most detailed information on inhibition of protein synthesis derived from the studies with insect and mammalian proline-rich antimicrobial peptides (PR-AMPs), a group of molecules presenting high content of proline and often arginine in their sequence that may be arranged in repeated motifs. The high content of proline of PR-AMPs restricts the conformations that they can assume, so that they generally show extended conformations or, in some cases, a polyproline helix II structure [29]. In 1993 Hans Boman showed that the porcine proline-rich peptide PR-39 determined a drop in radioactive amino acid incorporation in *E. coli* without showing lytic activity, suggesting that this peptide interfered with translation (but also with DNA synthesis) [100]. Later, it was shown that another PR-AMP, $Bac7_{1-35,}$ induced in *E. coli* the expression of stress genes related to protein synthesis at sub-lethal concentrations [105]. More recently, proline-rich peptides from both insects and mammals were shown to bind ribosome subunits and inhibit protein synthesis.

After *in vivo* photo-crosslinking, the apidaecin-derived Api88 was found to bind ribosomal proteins. A stereospecific binding of a large panel of apidaecins and oncocins derivates to bacterial ribosome was evidenced by fluorescence polarization and the influence of these peptides on protein synthesis evaluated by *in vitro* cell-free coupled transcription/translation assays [99]. PR-AMP $Bac7_{1-35}$ was also shown to specifically inhibit protein synthesis without significantly affecting RNA transcription and DNA synthesis. Both *in vitro* experiments by cell-free transcription and coupled transcription/translation, DNA binding assays, and *in vivo* assays by incorporation of radioactive isotopes into *E. coli* were performed [65]. This peptide co-localized with purified *E. coli* ribosomes and was shown, by photo-crosslinking mediated affinity chromatography, to bind *in vitro* two different ribosomal proteins whose identity still has to be elucidated [65].

Interestingly, affinity chromatography assays showed that also the plant cysteine-rich antimicrobial peptide NCR247 from *Medicago truncatula* interacts with ribosomal

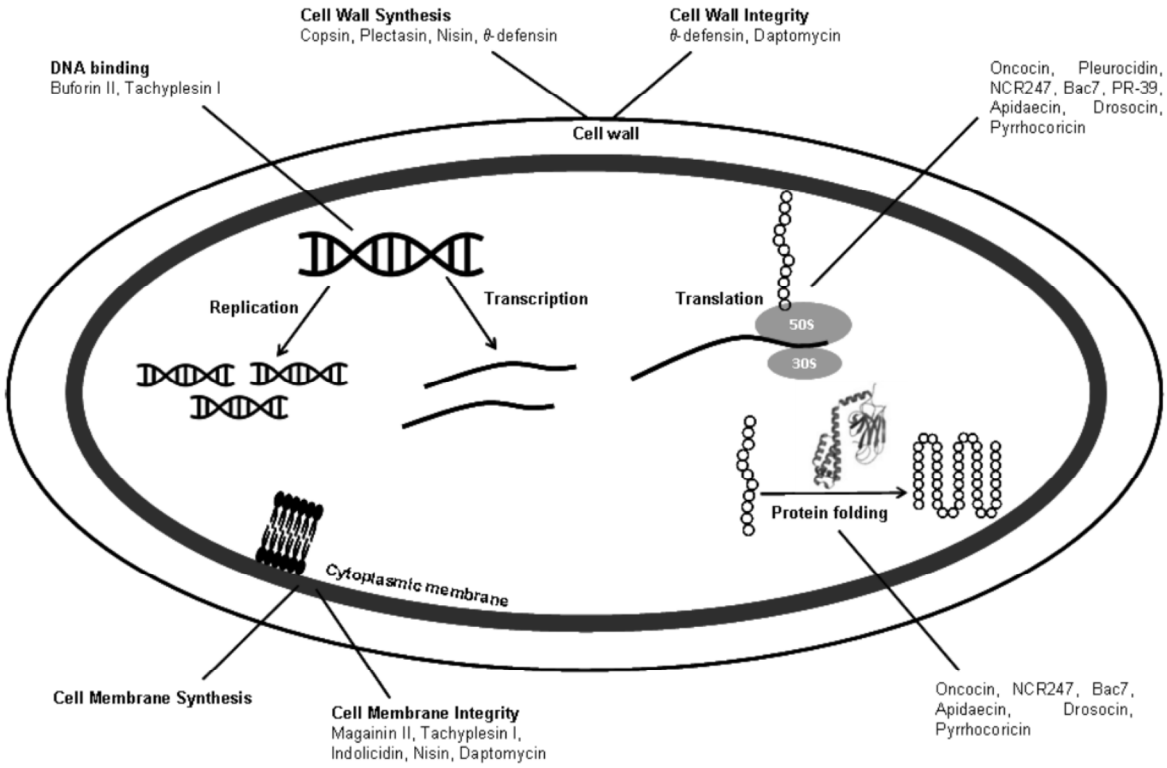

Fig. (1). Schematic representation of proposed targets for membrane-active and non-membrane permeabilizing AMPs

Table 1. List of AMPs that evidenced non-membrane permeabilizing modes of action.

Name	Origin	Structure	Sequence	Target/Mode of Action	Ref.
Buforin II	*Bufo bufo gargarizans*	α-helical in membrane and extended when bound to DNA	TRSSRAGLQFPVGRVHRLLRK	DNA binding after translocation inside the cell without membrane damage	[58, 68-70]
Magainin II	*Xenopus laevis*	α-helical both in membrane and bound to DNA	GIGKFLHSAKKFGKAFVGEIMNS	Cell membrane permeabilization and/or induction of apoptosis-like death	[27, 68, 71, 72]
Tachyplesin I	*Tachypleus tridentatus*	β-sheet and β-turn structures when bound to LPS	KWCFRVCYRGICYRRCR	Cell membrane permeabilization and interaction with DNA *in vitro*	[41, 53, 73, 74]
Indolicidin	*Bos taurus*	Extended	ILPWKWPWWPWRR	Cell membrane depolarization and lysis, inhibition of DNA synthesis	[66, 75-79]
CRAMP	*Mus musculus*	α-helical	GLLRKGGEKI-GEKLKKIGQKIKNFFQKLVPQPE	Cell membrane permeabilization, possibly inhibition of cytokinesis	[80-82]
Nisin	*Lactococcus lactis*	Polycyclic, β-turns complexed to membrane-mimicking micelles	ITSISLCTPGCKTGALMGCNM-KTATCHCSIHVSK	Cell membrane permeabilization and/or inhibition of cell wall synthesis via binding to lipid II	[83-87]
Plectasin	*Pseudoplectania nigrella*	α-helix and a β-sheet contributed by two antiparallel β-strands	GFGCNGPWDEDDMQCHNHCKSI KGYKGGYCAKGGFVCKCY	Inhibition of cell wall synthesis	[23, 88]
Copsin	*Coprinopsis cinerea*	α-helix and two β-strands stabilized by six disulfide bonds	QNCPTRRGLCVTSGLTACRNHCR SCHRGDVGCVRCSNAQCTGFLGT TCTCINPCPRC	Inhibition of cell wall synthesis via binding to lipid II	[89]
Daptomycin	*Streptomyces roseosporus*	Extended, partially circular	C_9 fatty acid -WNDTGODADGS-WND-mGlu-Kyn	Cell membrane permeabilization, cell wall damage, cell division impairment	[90-92]
Θ-defensin (1 and 2)	*Macaca mulatta*	two antiparallel β-strands joined by two turns	G(F/V)CRCLCRRGVCRC(I/L)CTR	Inhibition of cell wall synthesis, cell wall disruption (by release of autolytic enzymes)	[93-95]
Pyrrhocoricin	*Pyrrhocoris apterus*	extended	VDKGSYLPRPTPPRPIYNRN	Inhibition of protein synthesis, binding to DnaK and GroEL	[96, 97]
Drosocin	*Drosophila melanogaster*	extended	GKPRPYSPRPTSHPRPIRV	Inhibition of protein synthesis, binding to DnaK and GroEL	[96]
Apidecin	*Apis mellifera*	extended	GNNRPVYIPQPRPPHPRI	Inhibition of protein synthesis, binding to DnaK, GroEL and ribosomal proteins	[96, 98] [99]
PR-39	*Sus scrofa*	extended	RRRPRPPYLPRPRPPPFFPPRLPPRI PPGFPPRFPPRFP	Inhibition of protein synthesis	[100]
Bac7(1-35)	*Bos taurus*	extended	RRIRPRPPRLPRPRPRPLPFPRPGP RPIPRPLPFP	Inhibition of protein synthesis, binding to DnaK, GroEL and ribosomal proteins	[65, 101]
NCR247	*Medicago truncatula*	extended, stabilized by two sulphide bonds	RNGCIVDPRCPYQQCRRPLY-CRRRRNGSIVDPRSPYQQSRR-PLYSRRR	Inhibition of protein synthesis, binding to ribosomal proteins, GroEL	[102, 103]
Pleurocidin	*Pseudopleuronectes americanus*	α-helix	GWGSFFKKAAHVGKHVGKAALT HYL	Inhibition of protein synthesis	[20]
Oncocin	*Oncopeltus fasciatus*	extended	VDKPPYLPRP(X/P)PPRRIYN(NR)	Inhibition of protein synthesis, binding to DnaK, ribosome	[56, 99, 104]

proteins of the bacterial endosymbiont *Synorhizobium meliloti* hosted in root nodules. *In vitro* coupled transcription/translation assay and *in vivo* radioisotopes uptake assays confirmed the inhibitory activity of NCR247 on protein synthesis, even though it is not clear if translation is specifically inhibited or if also other pathways are involved [102].

The tetrapeptide GE81112 and the cyclic decapeptide GE82832 (the latter strictly related to dityromycin), selected by a high-throughput screening of an actinomycetes metabolites library, were shown to specifically inhibit prokaryotic translation *in vitro*, and to inhibit isotopes incorporation *in vivo*. They both act by binding the 30S ribosomal subunit, but the first blocks the translation initiation, while the latter inhibits the EF-G-catalyzed translocation [106-109].

5.2. DNA Binding and Transcription/Replication Inhibition

Several cationic antimicrobial peptides have been shown to interact *in vitro* with nucleic acids (DNA and/or RNA) [69, 73, 79, 110]. This fact is not surprising given that the two polymers are oppositely charged. A well-known example is represented by buforin II. In the original manuscripts of Park et al, it was reported that this H2A-histone derivative from the asian toad *Bufo bufo gargarizans* was able to bind DNA after its translocation inside *E. coli* without membrane damage [58, 69, 70]. Several studies using buforin variants have confirmed the *in vitro* binding of buforin to duplex DNA [68], reporting a general correlation between DNA affinity and antimicrobial potency [111]. Lan *et al.* showed that this peptide adopts an extended conformation when bound to DNA, although it has a helical structure in membranes closely mimicking the composition of gram-negative bacteria, suggesting that an α-helical structure is not required for the DNA binding activity of buforin II [68]. A correlation between cell membrane translocation capacity, DNA binding capability and antimicrobial activity [48] has been also observed using buforin variants with a reallocated central proline residue crucial for the internalization [112]. These peptides, however, showed a significant membrane permeabilization activity. In addition, other designed histone-derived AMPs (DesHDAPs 1 and 3) and their variants exhibited different mechanisms of action, the former similar to the mode of action of buforin II and the latter showing a membrane-permeabilizing activity which correlates with an increased antimicrobial activity [45]. To understand the mechanism of action it is thus important to verify the capability of an AMP to enter living cells, and whether its antibiotic activity occurs in conditions under which it does not depolarize and/or permeabilize the cytoplasmic membrane. For example, a DNA binding ability has been reported for the 17 amino acid peptide tachyplesin I, isolated from the horseshoe crab [41, 73]. Gel-retardation assays and footprinting analysis showed that tachyplesin I interacts *in vitro* with the minor groove of duplex DNA [73]. By using confocal microscopy, TEM and SEM, Hong *et al.* clearly showed that cell membrane is the target of this peptide in *E. coli* cells and that it forms pores in the cell membrane with outflow of cytoplasmic content [53].

Recently, some synthetic polymers with a polyamide backbone (SAMPs) have been shown to possess a mode of action independent from membrane-permabilization and to have a selective bactericidal action against *Mycobacterium smegmatis*. *In vitro* gel retardation but also DNA synthesis inhibition assays suggest that DNA is the target of these peptides: in fact, when SAMPs bind to it, they impede PCR amplifications of DNA extracted from peptide-treated cells [60].

Another example of an AMP with DNA binding properties is the 13-mer cathelicidin peptide indolicidin of bovine origin. Its mechanism of action is however controversial; it appears to act by promoting significant membrane depolarization and lysis [77, 78] but also by inhibiting DNA synthesis [76, 79]. Very recently, the structural details of the interaction of indolicidin with DNA have been unraveled at an atomic resolution [75]. A study on the indolicidin derivative CP10A underlines the ability of this peptide to interact with membranes but also to affect intracellular synthesis of proteins, RNA, and DNA at 2- and 10-fold the MIC value. Electron microscopy showed minimal effects of the peptide on the cell wall, however, membrane depolarization by CP10A was very efficient. Results suggest multiple intracellular targets in the mode of action of this peptide [66]. Multiple intracellular modes of action for AMPs have also been evoked for a series of short arginine, lysine and tryptophan containing lipopeptides such as C10-RIKWWK and C10-RKWWK [113]. In addition to the small size, potent bactericidal activity, low membrane disruption ability, they apparently retarded the migration of DNA on agarose gel in the DNA-binding assay.

Gel retardation assay very commonly show that AMPs have an effect on mobility of DNA/RNA in agarose gel. However, in several cases the addition of the AMP seems to induce the precipitation of the DNA, which remains in the agarose wells, more than shift the mobility of the DNA bands. It has been reported that capacity to bind DNA may be unrelated with the mode of action. For example, quite different cationic cathelicidin peptides, including the non-lytic proline-rich peptide $Bac7_{1-35}$ and the lytic peptides human LL-37 and bovine BMAP-27 interact *in vitro* with plasmid DNA in a gel retardation assay in the same concentration range, despite only $Bac7_{1-35}$ inhibited *in vitro* transcription/translation, while the lytic peptides did not [65]. Thus, interaction between AMPs and DNA may reflect a general and unspecific electrostatic binding and it cannot be sufficient to explain the intracellular effects of these peptides. In another case, the tryptophan-rich antimicrobial PuroB peptides, derived from the puroindoline proteins, showed a good correlation between antibacterial activity and affinity for the plasmid DNA, suggesting that binding to DNA has a functional role [110].

5.3. Inhibition of Cytokinesis

Inhibition of cytokinesis has been indicated to be part of the mechanism of action of some AMPs. The α-helical peptide CRAMP, the mouse ortholog of the LL-37, was found to impair *Salmonella* cell division *in vitro* as well as in bacteria phagocytized by macrophages, resulting in long filamentous structures [80]. Interestingly, CRAMP shows some sequence similarity with a 40-amino acid peptide from *Bacillus subtilis* that inhibits the tubulin-like protein FtsZ, preventing inappropriate Z-ring formation during sporulation [81]. It has

therefore been speculated that CRAMP works in part by inhibiting cytokinesis, although it is known to also be an amphipathic helical lytic peptide [82]. An extremely elongated morphology indicating that peptide-treated cells are unable to undergo cell division has also been observed with the proline-rich PR-39 and the tryptophan-rich indolicidin, although the molecular mechanism of septum inhibition is unknown and may be connected to inhibition of DNA replication [10].

5.4. Cell Wall Inhibition

The cell wall is an essential bacterial structure. Different AMPs have been found to target components of peptidoglycan. Since this is not present in eukaryotic cells, compounds that inhibit its synthesis are interesting for therapeutic applications, and this is a mode of action of important antibiotic classes.

AMPs that are well known to interfere with cell wall formation are lantibiotics, a class of post-translationally modified bacteriocins, produced by gram-positive bacteria that contain unusual amino acids [83]. Despite most of these being membrane-acting agents, many bacteriocins show specific target-mediated modes of action. The membrane-bound cell-wall precursor lipid II has been identified as a principal target for these peptides [83, 84]. Some lantibiotics, such as nisin, are characterized by a dual mode of action: they initially form a complex with the ultimate cell wall precursor lipid II, thereby inhibiting cell wall biosynthesis, they then aggregate by incorporating further peptides and forming a pore in the bacterial membrane. Nisin variants that do not form pores but bind to lipid II have also been described [85, 114], suggesting that the binding and relocation of lipid II in the membrane blocks cell wall synthesis and causes cell death *per se*. For example a non-pore-forming mode of action has been demonstrated for the lantibiotic mutacin 1140. Hasper *et al.* showed by fluorescence microscopy that the addition of this lantibiotic to fluorescently labeled lipid II molecules in giant unilamellar vesicles (GUVs) resulted in hot spots of lipid II-associated fluorescence appearing in the membranes, while the impermeant soluble fluorescent marker Texas Red remained extracellular, thus indicating that no permeabilization has occurred. The lantibiotic-induced lipid II segregation seen in GUVs was also confirmed *in vivo*, both in *Bacillus* and *Lactococcus* cells, suggesting that this mechanism may be responsible for the inhibition of cell wall synthesis and ultimately cell death [86]. Type B lantibiotics, in particular mersacidin, have also been shown to function without forming pores and are believed to bind to lipid II, thus inhibiting the cell wall assembly [115]. On the other hand, lantibiotics such as plantaricin C, gallidermin and epidermin, have been shown to induce pore formation in a strain-specific manner, suggesting that the *in vivo* activity of lantibiotics cannot be assigned to a single factor, e.g. interaction with isolated cell wall precursors such as lipid II. Rather, cell wall synthesis inhibition and pore formation may contribute differently to the antimicrobial activity depending on the target strain [116, 117].

Another example of an AMP targeting cell wall components is plectasin, a defensin of fungal origin, that shows potent *in vitro* and *in vivo* activity against gram-positive bacteria. A wide range of genetic and biochemical approaches identified cell wall biosynthesis as the target pathway and *in vitro* inhibition assays for cell wall synthesis further identified Lipid II as the specific cellular target. Plectasin, similarly to glycopeptide antibiotics (e.g. vancomycin) and lantibiotics, may form a stoichiometric complex with the substrate rather than inhibiting the enzyme [23]. The human β-defensin hBD-3 also inhibits cell wall biosynthesis but to a lesser extent [118].

The AMP copsin, a novel defensin from the basidiomycete *Coprinopsis cinerea*, binds to lipid II but, differently from nisin, does not cause carboxyfluorescein efflux from lipid II loaded liposomes, nor potassium efflux from *B. subtilis* cells, thus indicating it does not lead to pore formation [89]. Unlike lantibiotics and other defensins, copsin binds to a different position of the lipid II pentapeptide.

Srinivas *et al.* have recently developed a series of epitope mimetics of protegrin I that specifically target bacteria via a mechanism of action that is distinct from the membrane-disrupting activity of the parent compound. Several rounds of optimization resulted in a lead compound that was specifically active in the nanomolar range against *Pseudomonas spp*., but was largely inactive against other bacteria [119]. Biochemical and genetic studies showed that some peptidomimetics (L27-11 and POL7001) target a homologue of the ß-barrel protein LptD (Imp/OstA), which functions in outer membrane biogenesis. LptD is an essential outer-membrane protein, widely distributed in gram-negative bacteria, which functions in the assembly of LPS in the outer leaflet of the outer membrane. No fluorescence increase was apparent when *P. aeruginosa* cells were exposed to SYTOX green in the presence of these peptides, suggesting no significant permeabilizing activity. L27-11 and POL7001 bind to LptD and impede its function resulting in outer membrane structure and biogenesis defects, internal accumulation of membrane-like materials and lipid A alterations. To our knowledge, this is the first report of an OM component as a principal target for an AMP.

5.5. Inhibition of Enzymatic Activity

Several studies characterized the interaction between PR-AMPs and the heat-shock protein DnaK, as well as other chaperones. By using a resin-immobilized form of the insect pyrrhocoricin, Otvos' group first reported that a PR-AMP specifically bound DnaK and less specifically the chaperonin GroEL from *E. coli* protein lysates. These interactions were confirmed using fluorescence polarization also for the insect PR-AMPs drosocin and apidaecin [96]. Further investigations showed that the interaction between DnaK and pyrrhocoricin was stereospecific and decreased the ATPase and the refolding activity of this protein [120]. Inhibition of DnaK activity was proposed to be at least co-responsible for the antimicrobial activity of pyrrhocoricin, since the capability of some fragments of this peptide to bind DnaK synthetic fragments correlated with their antimicrobial potency against different bacterial strains [121]. The protein-peptide interaction was characterized by using synthetic fragments, rational sequence modification of both pyrrhocoricin and DnaK, and biochemical and fluorescence polarization assays. It has been suggested that the peptide inhibits DnaK mainly by sterically

blocking the chaperone folding activity, and to a minor extent by competing with proteins for its active site [120, 121]. Others also suggested that the action of pyrrhocoricin consisted in a competition with proteins for the natural substrate-binding site [122], but a third model now proposes that pyrrhocoricin inhibits DnaK by a dual mode of action, based both on this competition and on the steric blocking of the chaperone folding capacity, showing that the two phenomena are linked [123]. It has been speculated that this mechanism of action against DnaK could be extended also to apidaecins [124]. The interaction of proline-rich peptides with DnaK, with particular attention to the substrate-binding domain, was further characterized by crystallographic analyses, with the aim to confirm an intracellular target for peptides, such as oncocins [104] apidaecins [55] and a wide panel of other PR-AMPs [125]. The involvement of chaperons in apidecin antimicrobial mechanism was further confirmed, by the fact that overexpression in *E. coli* of GroEL–GroES and DnaK–DnaJ–GrpE decreased bacterial sensitivity to this peptide [126]. Interestingly, mammalian PR-AMPs were also found to have DnaK as intracellular target. It has been shown by affinity chromatography, that the N-terminal fragment of the bovine PR-AMPs cathelicidin $Bac7_{1-35}$ bound strongly and stereospecifically DnaK from an *E. coli* lysate. It was also shown that it inhibited the protein refolding activity of the DnaK/DnaJ/GrpE/ATP complex [101]. The binding to DnaK was further confirmed and characterized by crystallographic studies [127]. On the other hand it has been shown for both insect and mammalian derived PR-AMPs that inhibition of DnaK is not sufficient on its own to explain their antimicrobial activity, since DnaK null-mutants remained sensitive to PR-AMPs [99, 101]. Some discrepancies were recently found in correlating DnaK-binding and antibacterial activity also for some apidaecin derivatives [31]. The DnaK binding capacity of PR-AMPs led to synthetic chimeric peptides, such as A3-APO, that combine features of different PR-AMPs in order to further optimize and maximize their antimicrobial potency [128, 129].

Plant peptides were also shown to bind bacterial chaperones. NCR247 is a symbiotic random coiled peptide of *Medicago truncatula,* stabilized by two disuplhide bridges. NCR247 presents *in vitro* antimicrobial properties and was shown to bind GroEL even though the significance of this interaction is not fully understood [102].

A recent study suggested a possible link between the α-helical magainin-2 and other amphibian AMPs and inhibition of bacterial ATP synthase. Although magainin is considered a typical membrane lytic peptide [27, 71], an inhibitory effect of magainin-2 on both purified F_1 ATPase and membrane bound F_1F_0 ATP synthase was observed at sub-MIC concentration [130]. Similar results were also obtained with magainin-1 and an its analogue on the F_1F_0 ATP synthase in plasma membrane vesicles of *Mycobacterium tuberculosis* [130, 131]. However, in these experimental conditions no data about integrity of the cell membrane were reported.

5.6. Other Membrane-Independent Mechanisms

In the last couple of years an increasing number of studies on novel/modified AMPs have reported antibacterial activity conducible to a non-membranolytic mode of action. While in most cases the intracellular targets of these peptides are either unknown or need to be confirmed, these data underline the complexity of interactions occurring at the molecular level between AMPs and bacterial structures.

For example, a model synthetic hexapeptide RWRWRW-NH_2 named MP196, representing a minimal pharmacophore of positively charged and hydrophobic amino acids, showed an unusual mechanism of inhibition [64]. MP196 did not act by forming pores nor releasing K ions from *B. subtilis* cells even though it concentrated at the membrane and inserted into phospholipid bilayers. Proteome and Western blot analyses suggested that membrane integration of the peptide caused the delocalization of peripheral membrane proteins essential for respiration and cell-wall biosynthesis, limiting cellular energy and undermining cell-wall integrity. This mechanism has been called the "sand in a gearbox" [132], and indeed Wenzel *et al.* suggest that delocalization of membrane proteins is a general mechanism extending to membrane-targeting peptides of other structural classes [64]. It may underlie the inhibiting effects of some AMPs on cell wall biosynthesis [118].

Confocal microscopy on *Bacillus subtilis* showed that another lipopeptide, daptomycin, alters bacterial envelope architecture before killing. This lipopeptide, which has an extended and partially circular conformation, apart from targeting the bacterial membrane and cell wall, also misregulates the recruitment of the cell division protein DivIVC. Despite the fact that daptomycin promotes leakage of ions and to some extent affects the permeability of bacteria to Sytox green, bacteria lysis occurs as a secondary event, so it may be the late consequence of a general cell dysfunction, and not the main mode of action of this lipopeptide [90].

It has recently been reported that θ-defensins, apart from interfering with cell wall biosynthesis, also cause the premature release of autolytic enzymes from their anchoring points on lipoteichoic acids, thus leading to cell wall disruption [93]. Wilmes *et al.* referred that the membrane activity of θ-defensins depends on the bacterial membrane potential, since peptide-induced potassium leakage from whole cells was blocked immediately after the addition of the ionophore CCCP. TEM analysis evidenced cell wall degradation in the septum area between two daughter cells, pointing to the autolysin Atl as the major protein responsible for the degradation of the cell wall. A mechanism based on bacterial lysis induced by the release of cell wall hydrolases (autolysins) was proposed similarly to what happens for the bacteriocin Pep5 [133, 134].

Synthetic PR-AMPs, presenting isobutyl-modified proline repeats showed good antimicrobial activity. β-galactosidase leakage assay suggested a non-membranolytic mechanism but the details are still unknown [135]. These peptides have a better antimicrobial activity when arranged as branched dimers, but in this form and at concentration above MIC, they showed a significant permeabilizing activity [136] resembling the dual mode of action observed for other PR-AMPs [21].

In addition to the membrane-permeabilizing activity of magainin-2, recently the morphological changes induced by

magainin-2 in *Escherichia coli* have been analysed with respect to typical apoptosis hallmarks, such as phosphatidylserine externalization from the inner to outer membrane surface, DNA fragmentation and chromatin condensation, suggesting the possibility of a bacterial apoptosis-like death [72].

CONCLUSION AND PERSPECTIVE

Among the huge number of known natural or artificially designed AMPs, only a few classes of peptides seems to act mainly through genuine, non-membranolytic mechanisms (Fig. **1**). While it is quite clear that the killing event due to the activity of these AMPs is not based on the disruption of membrane integrity, the understanding of their internalization, subcellular localization and detailed molecular action remains fragmentary or incomplete for most of them and is only speculative for others. Data on the real concentrations reached by the peptides inside the cells are very scarce, as well as those on their *in vivo* activity. Interestingly, some peptides seem to be directed towards a single target while others work in a relatively unspecific manner on multiple targets. Due to the incomplete information on the former type of peptides, it could be speculated that all the intracellular-acting peptides could interfere with multiple targets within the bacterial cells. This behavior may be considered an extension of the "sand in a gear box" mechanism [132] to the intracellular environment.

Understanding the mode of action of AMPs is the key to future developments of peptides as new drugs. In the last decade some AMPs have failed to achieve New Drug Application (NDA) approval, despite clinical efficacy. Now the urgency to develop novel antibiotics with different modes of action promotes a new phase of clinical experimentation and a renewed interest by the leading biopharma companies also based on a better understanding of the mode of action of the peptides and fuller recognition of the challenges in translating R&D leads into a new class of products [137].

Interestingly, the most promising AMPs currently in clinical trials or in preclinical development include non-membranolytic peptides whose mechanism of action has been defined. For example, the lipopeptide surotomycin (CB-315), related to the cyclic lipopeptide daptomycin, is in phase 3 clinical trials as a treatment for *C. difficile* infections [137]; the indolicidin-derived MBI-226 (omiganan) has completed two separate phase III clinical trials demonstrating significant efficacy in topical antiseptic prevention of catheter infection [138]; the epitope mimetics of protegrin I, POL7080, has been designated by U.S. FDA as a Qualified Infectious Disease Product for the treatment of Ventilator-Associated Bacterial Pneumonia caused by *Pseudomonas aeruginosa*, and the lantibiotic MU1140 shows promising activity against both actively growing and dormant *Mycobacterium tuberculosis* and is under preclinical evaluation [137].

In conclusion, non-membrane permeabilizing peptides are an important source of potential antibiotics with diversified, and likely novel, modes of action. To increase attractiveness as future drugs and the probability of success a better understanding of their modes of membrane translocation, cytoplasmic active concentration, and molecular interactions with internal targets is required.

CONFLICT OF INTEREST

Marco Scocchi, Mario Mardirossian, Giulia Runti and Monica Benincasa declare that this article content has no conflict of interest.

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