# 7 Antimicrobial and Cell-Penetrating Peptides: How to Understand Two Distinct Functions Despite Similar Physicochemical Properties

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**Abstract**

Antimicrobial and cell-penetrating peptides are both classes of membrane-active peptides sharing similar physicochemical properties. Both kinds of peptides have attracted much attention owing to their specific features. AMPs disrupt cell membranes of bacteria and display urgently needed antibiotic substances with alternative modes of action. Since the multidrug resistance of bacterial pathogens is a more and more raising concern, AMPs have gained much interest during the past years. On the other side, CPPs enter eukaryotic cells without substantially affecting the plasma membrane. They can be used as drug delivery platforms and have proven their usefulness in various applications. However, although both groups of peptides are quite similar, their intrinsic activity is often different, and responsible factors are still in discussion. The aim of this chapter is to summarize and shed light on recent findings and concepts dealing with differences and similarities of AMPs and CPPs and to understand these different functions.

**Keywords**

Antimicrobial peptides · Cell-penetrating peptides · Plasma membranes · Drug delivery · Lipid-peptide interaction

## Abbreviations

| | |
|---|---|
| AMP | Antimicrobial peptide |
| CD | Circular dichroism |
| CPP | Cell-penetrating peptide |
| CS | Chondroitin sulfate |
| DSC | Differential scanning calorimetry |
| EM | Electron microscopy |
| EPR | Electron paramagnetic resonance |
| FDA | Food and Drug Administration |
| FMM | Functional membrane microdomain |
| GAG | Glycosaminoglycan |
| GPMV | Giant plasma membrane vesicle |
| GUV | Giant unilamellar vesicle |
| HS | Heparan sulfate |

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| | |
|---|---|
| IR | Infrared |
| $L_d$ | Liquid disordered |
| $L_o$ | Liquid ordered |
| LTA | Lipoteichoic acid |
| LUV | Large unilamellar vesicle |
| MALDI | Matrix-assisted laser desorption/ionization |
| MS | Mass spectrometry |
| NMR | Nuclear magnetic resonance |
| OBOC | One bead one compound |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| PG | Phosphatidylglycerol |
| PI | Phosphatidylinositol |
| PS | Phosphatidylserine |
| QSAR | Quantitative structure-activity relationship |
| ROS | Reactive oxygen species |
| STED | Stimulated emission depletion |
| SUV | Small unilamellar vesicle |

## 7.1 Introduction

Nature has breeded a group of fantastic molecules, namely peptides, which are able to interact with membranes and operate in processes of fundamental importance such as viral fusion, antimicrobial defense mechanisms, membrane poration, delivery across membranes, and hormone-receptor interactions, to name only few. Two groups of peptides fall into focus of this chapter: antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs). While AMPs are key components of the innate immune system and act against many pathogens, CPPs have been emerged as valuable tools to translocate cargos across biological barriers. Despite their different functions, both groups share a lot of similarities in structure, sequence, and, particularly, membrane activity rising the question how these discrepancies in function can be explained by a common set of physicochemical characteristics. In fact, both groups of peptides are strongly membrane-active and induce membrane fluctuations arguing that the activity of these membrane-active families simply represents different facets of what is a shared energy landscape (Last et al. 2013). Therefore it is not contradictory to notice AMPs that cross plasma membranes and CPPs that offer antimicrobial activity. Moreover, it has been shown in many studies that by careful amino acid substitutions, some CPPs can be turned into AMPs and vice versa, leading to the often posed question: how different are they? The goal of this chapter is to underpin the recently emerged hypothesis that activity of AMPs and CPPs is mainly related to cooperative effects emerging during binding of peptides to the lipid bilayer. Thus, the membrane is not any more seen as a passive layer that is simply affected when peptides approach but actively contributes by its curvature and partitioning into microdomains to the entire peptide-lipid interaction process. In this way it might be possible to explain these different systems by common physicochemical processes. After a short introduction to both groups of peptides, their interplay with biological membranes and resultant biological effects, methods to measure those activities, as well as ways to predict membrane activity and to design novel AMP or CPP sequences will be summarized.

### 7.1.1 Cell-Penetrating Peptides

Cell-penetrating peptides constitute a family of natural or synthetically generated peptides that are able to translocate across cellular membranes. Usually these peptides are relatively short (smaller than 35 amino acids) and mediate the transport of cargos into cells, which can be either covalently or non-covalently attached to the CPP. A vast sequence variety exists making classification of CPPs difficult. One possibility is to group them depending on their origin in protein-derived, chimeric, or synthetic peptides. Otherwise, they can be ordered based on their physicochemical characteristics into three main classes such as cationic, hydrophobic, and amphipathic. Whereby, this latter classification is more helpful in view of the

current understanding of CPPs' cellular entry mechanism. CPPs have emerged as a powerful technique to deliver various types of molecules into cells such as proteins, peptides, siRNA, DNA, liposomes, nanoparticles, or small organic drugs (Kalafatovic and Giralt 2017). Additionally, some CPPs have made it already in clinical applications (Feni and Neundorf 2017).

CPPs enter cells using different modes of action such as energy-dependent (endocytotic) or energy-independent (direct) entry pathways. The latter might be accompanied by toxic membrane activity based on the ability of the CPP to disrupt membranes. Endocytosis on the other side is mainly observed when large cargos are attached to CPPs. Still it is not clear which factors provoke the one or other pathway. Notably, in both cases peptides adsorb at the membrane surface, where they interact with negatively charged surface components, probably glycoconjugates, anionic lipids, or membrane proteins. This adsorption is the result of a structural rearrangement, and sometimes it leads also to an interaction with the interfacial zone of the lipid bilayer. After a critical (threshold) concentration is reached, membrane deformation occurs as a result of elastic stress and mass imbalance (Alvares et al. 2017). Likely, these process parameters are unique for each CPP-cargo complex/conjugate. Of note is that most CPPs are unstructured in aqueous solution but rapidly adopt defined secondary structures when coming in contact with the membrane lipid phase. Frequently, the formation of amphipathic helices is observed in this case (Di Pisa et al. 2015). Although the intracellular uptake of most CPP is not fully understood, it is of wide acceptance that CPP enter cells by using probably both pathways, direct entry and endocytosis, simultaneously and/or depending on physicochemical properties, concentration, charge, and length of the CPP, as well as characteristics of the cargo. Additional influences on the uptake mechanism of CPPs derive from properties, lipid composition, and protein content of the cell membrane. Moreover, CPP concentration is suggested to have an important role, while at low concentrations endocytosis and at high concentrations, direct entry processes are usually observed.

One pitfall when working with CPPs is their mainly endocytotic uptake. Once taken up via endocytosis, the peptide-cargo complex resides in endosomes, from which it has to be released for reaching its target site. Thus, the endocytosis mechanism represents one of the major disadvantages for the further development of CPPs. Problems concerning the efficient escape of CPPs from the endosomes still persist and are in any case present when large molecules are delivered by CPPs. To circumvent these problems, CPPs may be equipped with fusogenic sequences or other endosomolytic molecules (Neundorf et al. 2009). Still many efforts are made to develop more selective and efficient CPP sequences.

### 7.1.2 Antimicrobial Peptides

Antimicrobial peptides are usually short peptides (<50 amino acids) and present in all forms of life, where they play a major role in the innate immune system and act as the "first line of defense" against invading pathogens. This class of peptides is structurally highly divers and of amphipathic or cationic nature. AMPs share widespread toxicity against bacteria, yeasts, and fungi but are relatively inactive toward host eukaryotic cells at bactericidal concentrations. Membrane permeabilization is their main mechanism of action, but additional mechanisms have been supposed, including membrane destabilization, intracellular translocation, and inhibition of protein or nucleic acid synthesis. Often they display a polycationic nature supporting electrostatic interaction with negatively charged bacterial surface structures such as lipoteichoic acids (LTA). Classical mechanisms of antibiotic action include their penetration of the plasma membrane or cell wall, thus resulting in lysis or disruption of ionic gradients. Specifically, they gain access to the cytoplasmic membrane and interact with lipid bilayers, forming transmembrane pores that disrupt the cell

membrane, finally leading to cell death. Several models have been proposed that explain how AMPs induce these membrane-disrupting processes, including the *barrel-stave*, *toroidal pore* and *carpet* model (Sierra et al. 2017).

Although they act preferentially on the membrane level, AMPs may affect multiple biochemical processes in the pathogen. An increasing body of evidence has demonstrated that AMPs have also intracellular targets (Le et al. 2017). For instance, blocking of RNA or protein synthesis and inhibiting enzymes necessary for linking cell wall structural proteins are further mechanisms of AMPs leading to cell death. Some AMPs have the ability to translocate in eukaryotic cells without damage of the plasma membrane. Notably, they target cancer cells and find intracellular targets like mitochondria, where they inhibit, e.g., cellular respiration and induce reactive oxygen species (ROS) formation (da Costa et al. 2015).

Since toxicity of AMPs is in most cases mediated by a non-specific process, bacteria have difficulties to develop resistance against AMPs. However, development of AMP-resistant strains is of course inevitable once they have been put into clinic. Indeed, processes by which microorganisms have produced resistance mechanisms against AMPs have been already reported. Moreover, to survive the bactericidal action of AMPs, bacteria must sense the presence and adapt accordingly by controlling the expression of genes involved in AMP resistance. Generally, bacteria try to change the composition of the outer or inner membrane, or to modify their cell wall composition, thus making principal AMP targets less susceptible. In fact, bacterial defense mechanisms often rely on cell wall modifications, which usually alter the ionic cell wall potential leading to a reduced AMP binding (Maria-Neto et al. 2015). Although clear efforts have been made, more techniques are needed to fully understand bacterial resistance strategies. Nevertheless, owing to their remarkable properties AMPs are one of the most promising drug candidates in a foreseeable future to overcome the alarming rise in microbial drug resistance (da Costa et al. 2015).

## 7.2 Constitution of Biological Lipid Membranes and Its Relevance to the Activity of AMPs or CPPs

Cellular membranes regulate the in- and outflow of nutrients, give the cell its shape, and are responsible for many other important cellular functions like cell-cell communication and signaling processes. Furthermore, membranes constitute an impermeable barrier for large, charged, or hydrophilic exogenous molecules, like therapeutic oligonucleotides, proteins, or peptides. Still, it is one of the major challenges in pharmaceutical industry to find powerful techniques to overcome this barrier for an efficient drug delivery. The membrane itself is built up of a lipid bilayer that is composed of various lipids, proteins, and sugars. Different phospholipid classes are the most abundant molecules, and besides their structural function, they play important roles in regulating and controlling processes occurring throughout the membrane (Jobin and Alves 2014). Glycosaminoglycans (GAGs) are characteristic for mammalian cells, and especially the presence of heparan sulfate (HS) is thought to be important for CPP-cell interaction. Consistently it has been noticed that AMPs and CPPs act on two main membrane classes: mammalian (eukaryotic) and prokaryotic ones. Moreover, distinct activities for both groups of peptides have been found for tumor cells. All these cells are characterized by certain heterogeneities with differences in lipid bilayer composition, expression of various specific markers, glycosylation profiles at the outer surface layer, or the presence of cell walls in the case of bacteria and will be discussed briefly.

### 7.2.1 Eukaryotic Cell Membranes

Eukaryotic cell membranes are mainly composed of phospholipids, glycosphingolipids, and cholesterol. The different lipid species are segregated into different domains within the lipid membrane. Moreover, whereas the outer phase of the lipid bilayer is usually characterized by the presence of

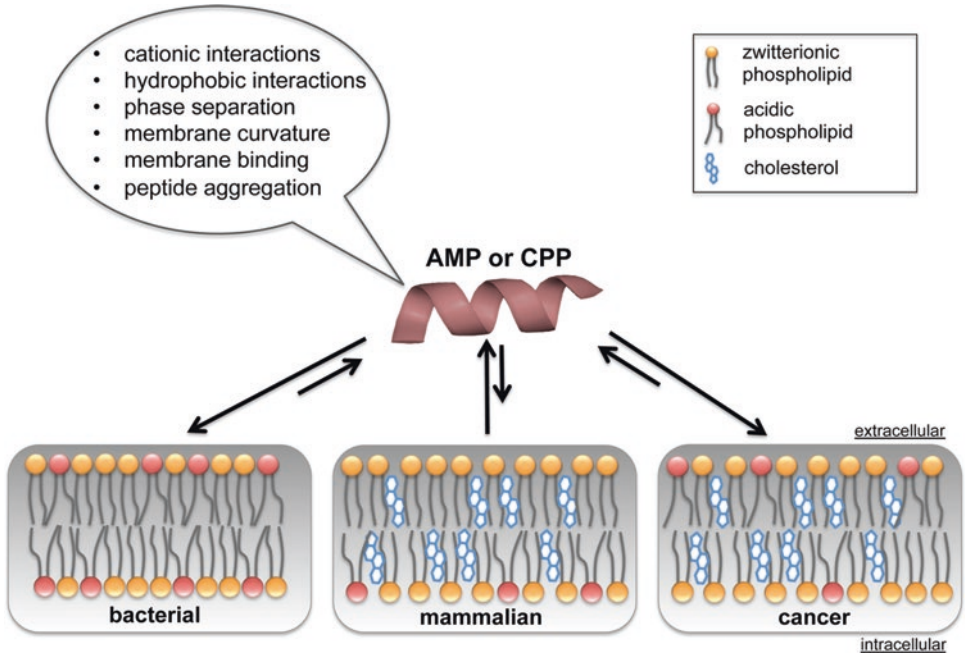


**Fig. 7.1** Sketch of different plasma membrane organizations in bacterial, mammalian, and cancer cells. Bacterial outer layers, as well as that of cancer cells, contain more negatively charged phospholipids and attract cationic AMPs or CPPs. Mammalian outer layers present zwitterionic phospholipids and attract cationic or amphipathic peptides by their phase separation and membrane curvature. However, for all membrane systems, the effects can be somehow interchangeable. Peptide orientation and aggregation are further important factors for following peptide association and partitioning within the membrane

zwitterionic phosphatidylcholine (PC), the inner layer contains essentially more negatively charged phosphatidylserine (PS) contributing to a characteristic asymmetry between outer and inner layer (Fig. 7.1). Additionally, cholesterol is a major constituent of eukaryotic plasma membranes and regulates, among other things, plasma membrane fluidity and endocytosis. Together with sphingomyelin, it occurs in so-called liquid-ordered domains, a liquid crystalline phase of a lipid bilayer. Nearly any eukaryotic cell contains a distinct membrane organization in membrane domains, which plays a role in many functional processes related to polarization, signal transduction, and membrane trafficking. Moreover, this temporal and spatial compartmentalization of membrane molecules in assemblies is crucial for membrane function (Simons and Vaz 2004). A number of observations corroborated the idea of the existence of so-called lipid rafts, relatively ordered subdomains, in which lipids and/or proteins are recruited and clustered. Cholesterol and sphingolipids are main components of such domains. With the presence of such lipid raft microdomains, it is hypothesized that lipids do not simply act as passive solvent but play a regulatory role in protein membrane assembly. However, still the raft hypothesis is highly discussed owing to the lack of methods for direct observation of such domains (Sezgin et al. 2017). For many CPPs an involvement of lipid rafts and the relevance of cholesterol for their uptake have been demonstrated (Pae et al. 2014; Watkins et al. 2009). Cholesterol depletion with methyl-β-cyclodextrin significantly affects translocation of many CPPs across the plasma membrane, either by affecting endocytosis pathways such as macropinocytosis or clathrin-/caveolin-mediated uptake or by influencing the overall membrane fluidity and, thus, direct translocation.

Other critical molecules that are exposed at the outer surface of the lipid bilayer and important for

CPP cell entry are glycosaminoglycans and proteoglycans. Particularly, heparan sulfate and other sulfated GAGs attract cationic CPPs by their negative charges, thus acting as primary binding site for CPPs. Generally, it is hypothesized that arginine-rich CPPs are able to associate at the membrane by bidentate-dependent binding at binding sites or partners, possibly represented by sulfate groups of HS. Furthermore, it has been recently shown that heparan sulfate proteoglycans or syndecans, another group of transmembrane proteoglycans, may act as CPP receptors (Chen et al. 2015; Letoha et al. 2010; Kawaguchi et al. 2016).

Interestingly, although it is relatively clear that electrostatic interaction is one of the key events for CPP membrane association, cell surface targets mediating this process remain surprisingly unknown. Therefore, it is of undisputable need to find suitable techniques and to further elucidate the role that distinct membrane constituents play in CPP uptake mechanisms.

### 7.2.2 Bacterial Cell Membranes

The bacterial cell wall is a complex polymeric structure with essential roles in defense, survival, and pathogenesis. The outer leaflet of the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria is surrounded by a mesh-like peptidoglycan sacculus (Caveney et al. 2018). The lipid bilayer of the plasma membrane includes proteins, associated RNA, and the common phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin, while PC and phosphatidylinositols (PIs) are less frequent. Cardiolipin and PG are negatively charged and contribute to the negative charge of the membrane. One additional major difference between eukaryotic and prokaryotic cell membranes is the existence of cholesterol in eukaryotic cell membranes and its complete absence in bacterial cell membranes (Fig. 7.1). Although it has been supposed that the presence of cholesterol suppresses the activity of AMPs, recently, this effect was put into perspective when it was shown that AMPs significantly disrupt heterogeneous lipid structures that contain cholesterol-enriched lipid rafts. Therefore, it is likely that cholesterol is not as important in determining the selectivity of AMPs toward bacterial membranes once supposed. However, which exact role cholesterol plays in the toxicity of AMPs or if unknown additional factors are necessary has to be elucidated in more detail (Brender et al. 2012; McHenry et al. 2012). Nonetheless, also prokaryotic cells contain functional membrane microdomains (FMMs) such as the lipid rafts in eukaryotic cells (Lopez 2015). For instance, it has been demonstrated that membrane-bound sensor kinases are organized in polyisoprenoid lipid containing lipid phases. As is with lipid rafts, these FMMs are able to resist detergent disaggregation when using a mixture of nonionic detergents (Brown 2002). However, the existence of such lipid subdomains suggests an important role of lipid organization in all domains in life.

External to the lipid membrane of Gram-positive bacteria exists a thick peptidoglycan layer, whose major constituent is lipoteichoic acid (LTA). The structure of LTA varies between the different species. Owing to its anionic nature caused by the presence of carboxyl and phosphate groups of the LTA as well as carboxyl groups of the muramyl peptides, a first contact with cationic AMPs is feasible. In fact, this electrostatic interaction is thought to be the primary mechanism for antimicrobial activity. Following, AMPs promote membrane damage and cell lysis either by membrane thinning, pore formation, or bilayer disruption (Pushpanathan et al. 2013). Gram-negative bacteria, on the other side, present another outer lipid membrane, which is separated by the periplasm. It consists of phospholipids, lipopolysaccharides, integral membrane proteins, and lipoproteins. In a multistep process, AMPs are first attracted by negatively charged groups of the lipopolysaccharides, following disruption of the outer membrane to gain access to the periplasmic space. As is the case with Gram-positive bacteria, electrostatic attraction by negatively charged groups lead then in a further step to AMP membrane binding. However, owing to the more complex structure, Gram-negative bacteria are usually more difficult to target, and only few exceptional AMPs exist that show activity against Gram-

negative bacteria. Additionally, also Gram-negative bacteria have developed various mechanisms to resist AMPs, like proteolytic degradation of AMPs, shielding of the bacterial surface, modification of the bacterial outer membrane, and pumping AMPs in or out of the cell (Gruenheid and Le Moual 2012).

### 7.2.3 Tumor Cells

Tumor cells are characterized by a phenotypic distinct membrane organization compared to healthy cells. In fact, compared to healthy cells, tumor cells display a higher overall net negative charge on their outer cell surface. This specific phenotype results from a number of key factors making cationic and amphipathic peptides susceptible to tumor cells. One is the presence of an increased amount of anionic phospholipids in the outer layer of the plasma membrane (e.g., PS) (Fig. 7.1) (Ran et al. 2002). Thus, the natural asymmetry between the inner and outer membrane leaflets is lost in tumor cells. Elevated reactive oxygen species (ROS) and hypoxia lead to dysregulation of phospholipid transporters, supporting this imbalance in the maintenance of the plasma asymmetry (Baxter et al. 2017). This involves activation of a putative scramblase and inactivation of a putative ATP-dependent phospholipid translocase. Moreover, several anionic cell surface glycoproteins are highly expressed in cancers and additionally contribute to an increased level of negative surface charge (Utsugi et al. 1991; Ran et al. 2002). Examples include mucins and heparan sulfate proteoglycans, which both promote electrostatic interaction of positively charged peptides at the outer surface of tumor cells. Furthermore, changes in membrane fluidity and pH, increased surface area and transmembrane potential, as well as a higher number of microvilli contribute to this specific characteristic of tumor cells.

#### 7.2.3.1 Anticancer Activity of Membrane-Active Peptides

Cancer is one of the major causes of death worldwide, and although many efforts have been made concerning the development of new anticancer therapeutics, the field is still in problems owing to upcoming resistances and low specificity of currently available drugs. One alternative approach that has come up during the last years is to use anticancer peptides. Many of these sequences derive originally from host-defense peptides (i.e., AMPs), but also for cell-penetrating peptides, an inhibition of cancer cells has been observed. Because of their alternative mode of action including their specificity to cell membranes as their primary target site, resistance and cytotoxicity are less likely to occur.

For AMPs it has become more and more evidenced that they are more than just alternative antibiotic weapons. Indeed, a lot of studies demonstrate a clear anticancer activity, and many anticancer peptides are often based on AMPs (Patel and Akhtar 2017; Deslouches and Di 2017; Roudi et al. 2017). Efforts are being made in order to understand the targeting mechanism of antimicrobial peptides, which would enable an improved design. Again, certainly structure plays a central role in their activity, while AMPs adopt a defined, often alpha-helical, structure when in presence of cancer cell membranes (Felicio et al. 2017). Moreover, the activity of anticancer peptides is in most cases driven by their cationic charge, while the presence of an amphipathic helix, i.e., spatial segregation of cationic and hydrophobic residues, seems also to be essential (Roudi et al. 2017). Notably, AMPs trigger distinct killing mechanisms based on membranelytic events or such without membrane-lytic events. In this way, necrosis occurs after cell membrane lysis and apoptosis in the case of mitochondrial membrane lysis. In both cases the presence of anionic lipids such as PS or cardiolipin is indispensable. Binding of AMPs to surface-exposed PS leads probably to membrane depolarization and cell death. Additionally, those anticancer peptides can present other intracellular targets, either targeting essential cell proteins, inhibiting angiogenesis, or recruiting immune cells to attack cancer cells (Wu et al. 2014).

On the other side, CPPs are usually not cell selective and, thus, controlled targeting strategies using internal or external stimuli to selectively

increase the activity of CPPs at the target site are necessary. With respect to tumor targeting, several such strategies have been successfully developed making use of activatable CPPs, attachment of ligands to CPPs that act as address labels, or localized hyperthermia, to name only few (Raucher and Ryu 2015; Bergmann et al. 2017; Splith et al. 2012). One other alternative targeting approach for tumor cells is based on the intrinsic properties of cationic CPPs. Their targeting mechanisms rely on the same parameters as those explained for AMP-derived anticancer peptides that act by their positive charge on the negatively charged surface of tumor cells. Several efforts have been made, in which such cationic anticancer peptides were successfully used to target and affect cancerous cells, also in vivo (Szczepanski et al. 2014; Gronewold et al. 2017). Moreover, such anticancer CPPs may also have intracellular targets. Thus, pore formation in the presence of high electrical potential at the mitochondrial membrane might be the basis for their activity (Rodriguez Plaza et al. 2014).

However, for such anticancer peptides, different activity levels might exist, and a careful selection, depending, e.g., on the tumor to be treated, has to be performed. For instance, for many anticancer peptides, it is shown that the interaction with glycosaminoglycans, HS, and chondroitin sulfate (CS), which are present on the outer surface, is one of the key steps during their action. However, it was recently demonstrated that HS at the outer surface of cancer cells sequesters anticancer peptides, in this case bovine lactoferricin, away from the phospholipid bilayer and thereby impede their ability to induce cell lysis (Fadnes et al. 2009). The results let further conclude that poorly differentiated tumors, with low expression of HS, are more susceptible to treatment with anticancer peptides, an interesting hypothesis that should be investigated in in vivo studies. Additionally, by generating modified versions of bovine lactoferricin, the cytotoxic activity against HS- and CS-expressing tumor cells was regained, demonstrating the need of such detailed structure-activity relationship studies (Fadnes et al. 2011).

Other obstacles that have to be faced with for a future application of anticancer peptides are adverse effects such as high toxicity to healthy cells and immune response. For this reason it is still necessary to dissociate the toxicity to mammalian cells from antimicrobial/anticancer activity. Moreover, as is the case with all possible peptide drug candidates, their high susceptibility to proteases is a major challenge that has to be tackled. One solution could be the design of modified peptides and peptide conjugates to increase selectivity and lower proteolytic degradation (Reinhardt and Neundorf 2016; Feni and Neundorf 2017). In this way, some cancer-targeting peptides are now in clinical trials, and the future will show if approvals will arise during the next years.

## 7.3 Experimental Methods to Classify Membrane-Active Peptides

Studying peptide-membrane interactions constitutes a challenging topic up to now, and besides all processes that have been already uncovered, their detailed understanding is still elusive. This is partially based on the complexity of the membrane composition, and additionally dependent on the favored arrangement of the peptide (also the cargo in the case of CPPs) when in the presence of the lipid phase, and its following membrane insertion at the same time. Regardless, it is of singular importance to reveal the biophysical and biochemical processes behind the function of membrane-active peptides helping to design more potent molecules with tailored functionalities that may be applied as active therapeutics. Several techniques have been developed and applied to biological samples or artificial membrane systems in combination with peptides. They differ in their sensitivity, resolution, and sample preparation and are often combined to gain complementary information. Roughly one can probably divide those methods into the three following categories: (i) quantification methods to unravel, e.g., peptide content in cells, (ii) methods to determine binding constants and affinities when peptides

interact with lipids, and (iii) visualization techniques and methods yielding structural information to track membrane-deforming events after peptide binding.

One focus has to be set on the choice of suitable membrane models to examine the specific lipid-peptide interaction. Various different such artificial membrane systems are presented, among those are unilamellar vesicles, mainly giant unilamellar vesicles (GUVs) and large unilamellar vesicles (LUVs), which represent the most relevant model systems to study membrane structures and dynamics. Herein, different lipid compositions are usually tested that mimic either bacterial membranes (containing mainly PG), healthy human cell membranes (containing a mixture out of PC:PE), or cancer cell membranes (containing a mixture out of PC:PE:PG). By adding sphingomyelin or cholesterol, the membrane fluidity can be further modulated, and by including appropriately labeled phospholipids, the vesicle membrane can be stained. As such it is possible to visualize membrane deformation processes or disruption after peptide addition by using fluorescence microscopy. Furthermore, encapsulating dyes within the vesicles allows for performing dye-release assays, which are easily conducted using flow cytometry or fluorescence spectroscopy. In addition to that, many researchers have made use of giant plasma membrane vesicles (GPMVs). GPMVs comprise a biologically more complex model and display a versatile tool to study membrane translocation of CPPs in conditions lacking endocytosis processes (Pae et al. 2014). Since GPMVs are released from cells after chemical induction, their lipid and protein content resembles that of the plasma membrane of living cells. Moreover, by applying low temperature, it is possible to segregate the membrane of GPMVs into different co-existing phases, namely, liquid-ordered ($L_o$) and liquid-disordered ($L_d$) membrane microdomains. By cholesterol depletion it is thus possible to determine the influence of these phases (ordered/disordered) to the lipid interaction of membrane-active peptide. For instance, Pae et al. demonstrated that amphiphilic CPP crosses more efficiently membranes that are partially depleted from cholesterol or are less ordered. On the other hand, arginine-rich CPPs translocated dependent on membrane proteinaceous components (Pae et al. 2014).

**Table 7.1** Methods to classify membrane-active peptides

| Method | Applied in context of… |
|---|---|
| *Determination of binding constants, affinities of peptides to lipids* | |
| Isothermal calorimetry (ITC) | Information about binding constants, affinity, thermodynamic parameters |
| Differential scanning calorimetry (DSC) | Phase transition measurements to yield thermodynamic parameters |
| Fluorescence spectroscopy | Peptide insertion into membranes, dye-release assays |
| Surface plasmon resonance (SPR) | Kinetic profiles of membrane lipid interaction |
| *Quantification methods* | |
| Flow cytometry | Association of peptides to membranes, uptake into cells |
| Mass spectrometry (MS) | Quantification of peptide cellular uptake or uptake into liposomes |
| *Structural studies and visualization methods* | |
| Infrared (IR) spectroscopy | Secondary structure of peptides in lipid phases |
| Circular dichroism (CD) spectroscopy | Secondary structure of peptides in lipid phase; possible in the presence of membrane vesicles or bacteria |
| Nuclear magnetic resonance (NMR) spectroscopy | Topology and three-dimensional structure of peptides in lipid phases; either solid or solution NMR in combination with artificial membranes or membrane lipids |
| Fluorescence microscopy | Cellular uptake; interaction with membrane vesicles |
| Electron microscopy (EM) | Membrane organization, peptide distribution at membranes |
| Atomic force microscopy (AFM) | In-depth membrane structures |

There are several biophysical techniques available that help to study membrane-active peptides and their adsorption, location, and orientation relative to a lipid bilayer (Table 7.1).[1]

[1]A few examples are listed in the text and in Table 7.1; however, this list is not exhaustive, and also alternative methods have been used.

Circular dichroism (CD) and infrared (IR) spectroscopy give information about secondary structures of peptides. Nuclear magnetic resonance (NMR) spectroscopy yields three-dimensional structure parameters. Particularly with solid-state NMR, it is possible to gain important insights in peptide depth and positioning within model membranes. Also solution-phase NMR is frequently applied to analyze peptides in the presence of lipid micelles or other membrane mimetics. Herein, small unilamellar vesicles (SUVs) are often used as versatile models, since owing to their small size, they possess a large surface curvature and, thus, differ in their membrane topology from those found in natural membranes. Complementary, electron paramagnetic resonance (EPR) spectroscopy is useful to obtain measures about peptide-lipid interaction on the molecular level (Galdiero et al. 2013; Alves et al. 2010).

Effects on phase transitions, membrane affinities, and binding of peptides can be determined using differential scanning calorimetry (DSC), isothermal calorimetry (ITC), and surface plasmon resonance (SPR). Indeed, SPR has been applied in many studies, in which membrane-like structures are immobilized on sensor chips to measure molecular interaction with peptides. In another method internal tryptophan residues of peptides or fluorophore-labeled peptides or lipids are investigated by fluorescence spectroscopy for determining binding constants and partition coefficients. Moreover, membrane leakage or fusion can be followed by this technique.

Microscopical techniques, such as electron microscopy, atomic force microscopy, or fluorescence microscopy, are useful to track morphological changes of cells or vesicles after binding of peptides. Whereas electron and atomic force microscopies live from their high spatial resolution, classical fluorescence spectroscopy might be limited by the sensitivity of the used dyes and low resolution. However, several recent improvements like stimulated emission depletion (STED) super-resolution microscopy have pushed applications in this latter field forward (Vicidomini et al. 2018).

For quantifying the intracellular peptide content, or the amount of peptides that has crossed the membranes of liposomes, mainly fluorescent methods, such as flow cytometry, or fluorescent microscopy or spectroscopy have been applied. A smart alternative method was offered recently and uses matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) (Walrant et al. 2013). Since MS is not a quantitative method, an internal standard labeled with a stable isotope is added to the sample. Therefore, deuterium-labeled groups are introduced into the peptide sequence.

Another method is to perform electrical measurements on bilayers providing a tool for evaluating the increase in conductance due to the formation of pore structures at low peptide concentrations. By determining the ionic current distribution, it is thus possible to analyze the pore-forming activity according to membrane phospholipid composition, for instance, with respect to cholesterol and sphingomyelin content. In this way, direct evidence for the binding and pore-forming activity of AMPs in either anionic or zwitterionic bilayers can be delivered (dos Santos Cabrera et al. 2012).

## 7.4 Prediction of AMP or CPP Activity by Bioinformatics and Library Screenings

Recently, several methods have been developed to predict antimicrobial as well as cell-penetrating peptides and to establish a link to their physicochemical properties (Brand et al. 2018; Lee et al. 2018). In silico approaches have further contributed to the design of highly effective engineered peptides with cell-penetrating, antimicrobial, or even anticancer activity (Gautam et al. 2013; Tyagi et al. 2013; Deslouches et al. 2013). For instance, based on the examination of structure-function relationship studies of synthetic peptides, a library of Arg and Val or Trp composed motifs that fold into helical amphipathic structures in the presence of lipid membranes were developed (Deslouches et al. 2005; Deslouches

et al. 2013). Additionally, within a recent study, physical properties of around 750 CPPs were analyzed, and it was concluded that they are median 14 residues in length and mainly cationic, the latter promoting their interaction with negatively charged outer plasma membrane constituents.

To facilitate the understanding of membrane interaction and the discovery of cell-penetrating and endosomolytic peptides, Carney et al. reported on a combinatorial library screen with liposomes (Carney et al. 2017). The authors employed an OBOC (one-bead one-compound) approach that was easily adapted to a variety of buffer and liposome compositions mimicking cellular biomembranes. By choosing the right lipid/sterol composition and by measuring the binding to zwitterionic liposomes, it was thus possible to discover several novel peptides and to successfully test them for their siRNA delivery ability. The presented method is easy to expand not only in regard of library size but also with respect to specifying the uptake pathways of the identified peptides.

Machine learning enabled antimicrobial peptide discovery, and design has been summarized recently by Lee et al. (2016). For realization, the authors trained computational models on large and high-quality data sets to perform high-throughput virtual screenings. In principle, such machine learning models fall all under the umbrella of quantitative structure-activity relationship (QSAR) models. Nicely, they help to design novel antimicrobial peptides and to discover membrane activity in diverse peptide families. Introduction of interpretable QSAR models permits also mechanistic understanding of underlying processes and provides predictable data about membrane activity.

On the other hand, several databases have sprouted during the last years that specifically compile sequence, structure, function, activity, etc. of CPPs and AMPs and give more comprehensive information (Table 7.2). Thus, the vast majority of those databases offer additional useful physicochemical parameters, like charge, isoelectric point, hydrophobicity, etc., which might be helpful for own rational peptide design strategies. Some have a particular focus on clinical or patent information or are provided as a tool to design novel AMP or CPP sequences. To date only one extensive database dedicated to CPPs has been developed (Gautam et al. 2013). *CPPsite* has more than 1700 entries, and peptides can be searched by their sequence, name, and source or defined by delivered cargo, modification, or used cell lines. Furthermore, also cyclized peptides are included in this collection. It has to be pointed out that increasing interest lies in the development of cell-permeable, small, often cyclized peptides, which, after cellular entry, interact selectively with proteins. Actually, inhibitors of protein-protein interactions in cells indeed count to a rapid developing field in pharmaceutical research (Kauffman et al. 2015). Thus, researchers have to find and determine the physical properties for a peptide that provides it with adequate membrane activity promoting binding at the lipid interface and favorable perturbance of the membrane structure. Such parameters may be found within this database and used for further peptide design. Notably, the same authors also offer another database including FDA-approved proteins and peptides (*THPdb*) (Usmani et al. 2017). Herein, some membrane-active peptides can be recovered, as, for instance, from the gramicidin family, and thus, this database might be a worthwhile extension. The number of available AMP databases is relatively large, and only some of them are listed in Table 7.2. They differ mainly in their collection of AMPs concerning source (APD offers mainly natural AMP sequences) or additional including information about, e.g., pharmacokinetic parameters and therapeutic index. Actually, it seems that these databases belonging to AMPs do not act as a network but rather in competition. It is a pity, since by merging and by updating all these data, differences and redundancies in entries would be diminished. As a consequence, researchers would greatly benefit by a more comprehensive picture about structure and function of given peptides or peptide families and their cellular mechanisms.

**Table 7.2** Useful databases for membrane-active peptides

| Database | Description | Link | Reference |
|---|---|---|---|
| *Cell-penetrating peptides* | | | |
| CPPsite | Contains around 1700 CPPs along with their structures, delivered cargos, and used cell lines | http://crdd.osdd.net/raghava/cppsite/ | Gautam et al. (2013) |
| CPC Scientific | Commercial supplier that provides tool to assist in custom CPP design. Adapted to THPdb and CPPsite | https://www.cpcscientific.com/resources/cell-penetrating-peptide-database/ | Gautam et al. (2013) and Usmani et al. (2017) |
| *Antimicrobial peptides* | | | |
| The Antimicrobial Peptide Database (APD) | Contains 2987 peptide entries from six kingdoms, mainly natural AMPs | http://aps.unmc.edu/AP/main.php<br>https://omictools.com/apd-tool | Wang et al. (2016) |
| Collection of Antimicrobial Peptides (CAMP) | Design of new AMPs, links databases for sequence alignment | http://www.camp.bicnirrh.res.in/ | Waghu et al. (2016) |
| Database of Antimicrobial Activity and Structure of Peptides (DBAASP) | Manually curated database providing information and analytical resources to develop antimicrobial compounds with high therapeutic index | https://dbaasp.org/ | Pirtskhalava et al. (2016) |
| Data repository of antimicrobial peptides (DRAMP) | Collection of AMPs with special focus on patent and clinical information containing 17,608 entries (thereof 4833 general AMPs, 12,704 patents) | http://dramp.cpu-bioinfor.org/ | Liu et al. (2017) |
| *Other useful databases* | | | |
| THPdb | Collection of FDA-approved therapeutic peptides and proteins providing information on sequence, indication, mechanism of action, pharmacodynamics, toxicity, metabolism, absorption, half-life, etc. | http://crdd.osdd.net/raghava/thpdb/index.html | Usmani et al. (2017) |

To mention is further that the accuracy of these in silico tools highly depends on the applied template structure, quality of sequence alignment, and prediction method. Hence, careful analysis of the data is necessary to allow a better understanding of physicochemical and functional properties. Nonetheless, these tools are highly valuable to expand the knowledge about AMPs and CPPs and to provide new leads for the translational design of new-generation antibiotics or drug transporters. Particularly in view of production costs, concerning future clinical applications, such in silico tools offer valuable alternatives to modulate and improve natural peptide sequences.

## 7.5 Functional and Mechanistic Redundancy of CPPs and AMPs

Although CPPs and AMPs share physicochemical properties, like their often alpha-helical structure, cationic charge, and amphipathicity, it is still not clear why these peptides display different activities. CPPs have only limited toxicity to eukaryotic cells and the ability to cross cellular plasma membranes in both energy-independent and endocytosis processes. Although many of these share also amphipathic characteristics, the overall interfacial hydrophobicity of most CPPs is not favorable for spontaneous partitioning into

membranes. Concluding that the observed activities are far more dependent on the chemical structure of a CPP, and that membrane lipid composition, applied concentration of CPPs but also lipid concentration and other bilayer physical properties play important factors for peptide-membrane interaction. Indeed, certain threshold values are indispensable for cellular uptake, even for direct translocation or for endocytosis. On the other hand, AMPs target and effectively kill bacterial cells. Arguing that the presence of bacterial cells might switch the activity of a CPP, several CPPs have indeed been tested and demonstrated to have antibacterial activity, too (Splith and Neundorf 2011). Interestingly, the same mechanistic models leading to pore formation, which have been proposed for AMPs, are postulated for CPPs when acting on bacterial cells. In many cases, helical structure or hydrophobic content is tuned by small sequence modifications leading to the one or other activity. Hereby, substitutions with positively charged arginine or aromatic residues play an important role (Piotrowska et al. 2017). However, considering their therapeutic potential, both groups share some disadvantages that often come along when using peptides such as toxicity, stability, and cost issues for their clinical and commercial development.

In spite of this observation, one might ask if the distinct membranes (prokaryotic versus eukaryotic) modulate their function. In fact, studies using artificial membrane vesicles illustrate the need of certain phospholipids present at the water-lipid interface directing the activity of the peptides in the one or other direction. Typically, CPPs and AMPs have only weak interaction with zwitterionic, synthetic membranes and strongly interact with anionic membranes. In addition, dependent on the CPP sequence, different effects after membrane binding can occur, such as vesicle aggregation and fusion, curvature formation, lipid flip-flop, membrane disruption, and many others. Indeed, some AMPs and CPPs share lipid-mixing abilities, concluding that they might be capable of triggering membrane fusion in vitro (Wadhwani et al. 2012). However, within this study of Wadhwani et al., fusion activity could not be correlated with the obtained secondary structure of membrane-bound peptides. Thus, it was hypothesized that not the particular type of secondary structure might be crucial but rather the extent of the conformational change upon membrane binding that correlates with the fusion activity. It was concluded that the driving force for fusion is the energy released when a formerly disordered, soluble peptide binds to a vesicle and acquires a secondary structure by H-bond formation (Wadhwani et al. 2012). Although pre-folded peptides are suggested not to promote much vesicle fusion, this well-defined fold might be nonetheless supportive for other functions of CPPs, like lipid-phase interaction and cargo delivery of cyclic peptides (Horn et al. 2016; Lattig-Tunnemann et al. 2011). Also charge distribution patterns were found to play a significant role for peptide-membrane interaction, particularly for membrane insertion of lytic and cell-penetrating peptides (Chen et al. 2017). In fact, charge distribution alongside the amphipathic helix might be the key factor affecting peptide insertion ability and thus bilayer disruption. Moreover, surface pressure is likely increased by both groups of peptides, CPPs and lytic peptides, whereas for the latter the pressure declines owing to molecule rearrangements after peptide insertion into the membrane.

On the other side, many AMPs have mainly deforming effects on the lipid phase, leading often to total membrane rupture. The response of AMPs to synthetic lipid compositions can be easily modulated by the presence of distinct molecules like cholesterol, sphingomyelin, or cardiolipin within the lipid phase. By the presence of cholesterol, eukaryotic membranes can be mimicked, and usually the activity of AMPs is decreased (Reinhardt et al. 2014). More recent findings have already demonstrated that for some AMPs their lack in cell selectivity might be based on aggregation behavior in solution and propensity to proteolytic degradation. For instance, LL-37 exists in equilibrium between monomers and oligomers in solution and is highly resistant

to proteolytic degradation when bound to both zwitterionic and negatively charged membranes. The oligomerized state might support a detergent-like effect also in the presence of zwitterionic membranes (Oren et al. 1999).

One more parameter that is strongly linked to membrane insertion activity is the spontaneous lipid curvature triggering response of membrane-active peptides. Recently, Koller and Lohner perfectly discussed how interfacially active peptides induce membrane curvature and which factors facilitate its formation (Koller and Lohner 2014). Membrane curvature is a result of a complex interplay between membrane proteins, lipids, and physical forces that are applied to the membrane surface. Nevertheless, membrane phospholipids itself have an intrinsic property to adopt planar or curved lipid molecular shapes leading to the formation of positive or negative curvature. Since the bilayer contains an asymmetrical lipid distribution, the different physical parameters of, e.g., PE at the inner monolayer and PC at the outer monolayer, induce curvature to a different extent. Membrane-active peptides induce curvature after incorporation in a bilayer; however, curvature induction by a peptide may differ for different lipid systems. Several parameters play a role, like H-bonding, electrostatic repulsion, monolayer surface area, and lateral pressure. Otherwise membrane curvature triggers peptide response, what becomes noticeable in peptide secondary structure formation. Thus, not only is membrane curvature spontaneously changed upon incorporation of a guest peptide, but lipid curvature can also influence the preference of a given peptide to insert into the host lipid matrix. For instance, the orientation of a peptide in the membrane is a key parameter determining the subsequent mechanism of action (*toroidal pore*, *barrel stave*, etc.). Hence, different modes of peptide-lipid interaction can be expected depending on the different cell types, which differ in terms of lipid composition and as a result in the amount of lamellar and non-lamellar phase-forming lipids present in the target membrane. PE exhibits a negative spontaneous curvature and is more prone to membrane disruption by interfacial active peptides. Given that PE is more abundant in the cytoplasmic membrane of Gram-negative bacteria and less present at the outer monolayer of eukaryotic cells, the selectivity of such peptides targeting membrane curvature can be nicely explained.

## 7.6 Conclusions

Membrane-active peptides are a class of peptides with undisputable relevant functions. Antimicrobial peptides are negotiated as highly promising new weapons against bacterial infections, while cell-penetrating peptides act as versatile delivery tools. Although both groups of peptides share physicochemical properties, their intrinsic function and mechanism of action can only hardly be predicted. It is relatively certain that in both cases lipid composition and constitution play major roles in peptide interaction and that the nature of the lipid phase somehow defines the orientation of the peptides. So, size of the phospholipid headgroups and bilayer elastic properties and dynamics are important determinants. On the other side, it is clearly demonstrated that charge is a further important key to attract cationic peptides to membrane surfaces. One way to achieve peptide selectivity would thus be to equip it with positive charge, making binding to anionic bacterial membranes (or to that of tumoral cells) more probable. Additionally, structural rearrangements during peptide-lipid interplay that help to bind and to position the peptide within the lipid bilayer are certainly essential. As already mentioned, folding into amphipathic helices might stabilize the membrane-bound state and increase the probability of the peptide to partition into the bilayer. Following, the interaction of AMPs or CPPs with lipid bilayers causes either local rupture (AMP) or transient permeation (CPP). How one can predict and exactly determine these two functions has to be one of the main foci within this research field in the future.

However, several tools that link those different functions are nowadays available, like databases, molecular dynamic studies, and other experimental classification systems. In this regard, it might be possible to use AMPs as a constructive template to design CPPs and, of course, vice versa. Therefore, it is hoped that in the near future, a more obvious picture can be drawn to unravel the

differences of AMPs and CPPs and that this knowledge will help in the engineering and discovery of more efficient and safer peptide sequences.

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