11 Selectivity of Antimicrobial Peptides: A Complex Interplay of Multiple Equilibria

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Abstract

Antimicrobial peptides (AMPs) attack bacterial membranes selectively, killing microbes at concentrations that cause no toxicity to the host cells. This selectivity is not due to interaction with specific receptors but is determined by the different lipid compositions of the membranes of the two cell types and by the peculiar physicochemical properties of AMPs, particularly their cationic and amphipathic character. However, the available data, including recent studies of peptide-cell association, indicate that this picture is excessively simplistic, because selectivity is modulated by a complex interplay of several interconnected phenomena. For instance, conformational transitions and self-assembly equilibria modulate the effective peptide hydrophobicity, the electrostatic and hydrophobic contributions to the membrane-binding driving force are nonadditive, and kinetic processes can play an important role in selective bacterial killing in the presence of host cells. All these phenomena and their bearing on the final activity and toxicity of AMPs must be considered in the definition of design principles to optimize peptide selectivity.

Keywords

Antimicrobial peptides · Host defense peptides · Selectivity · Toxicity · Peptide-membrane association · Aggregation · Hydrophobicity · Amphipathicity

11.1 Introduction

The scientific and medical interest for antimicrobial peptides (AMPs), short peptides produced by most organisms as part of their innate immune defenses, derives from their wide-spectrum bactericidal properties and their possible application to fight drug-resistant bacteria. However, in view of clinical applications, the absence of significant toxicity is almost as important as a good activity. In this respect, one of the appealing properties of many AMPs is their cell selectivity, i.e., the ability to kill bacterial cells at concentrations significantly lower than those causing damage to cells of the host organism, at least in in vitro tests. Still, potential toxicity is commonly listed as one of the challenges limiting the clinical application of AMPs as systemic drugs (Hancock and Sahl 2006; Eckert 2011; Yeung et al. 2011; Seo et al. 2012; Carneiro et al. 2015; Pachón-Ibáñez et al. 2017), and therefore, several research efforts are devoted to understand and further improve AMP selectivity.

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This chapter discusses AMP selectivity, the origin and the structural determinants of this property, the design strategies available to improve it, and the results of recent studies on the quantitative determination of peptide-cell association. Overall, the available data indicate that selectivity is the result of a complex interplay of several interconnected phenomena, including peptide association to target and host cells, peptide conformational equilibria, and AMP aggregation. Any modification to the peptide sequence and structure necessarily affects all of these processes, which must therefore be fully understood and considered in the rational design of new peptide or peptidomimetic molecules with improved selectivity properties. Our attempt is to give a critical overview of the available evidences, in order to provide a rationale for future efforts in this area. To this end, we have strived to derive, whenever possible, generalizations of the findings reported in the literature, but we have to stress from the beginning that the presence of exceptions to every rule is the norm, in such a diverse set as AMPs, also as a consequence of the complications mentioned above.

The different aspects of AMP selectivity have last been reviewed by Matsuzaki in 2009 (Matsuzaki 2009). Selectivity or toxicity has often been considered in general review articles on AMPs (Alba et al. 2012; Teixeira et al. 2012; Oddo and Hansen 2017; Hollmann et al. 2018). Some reviews have summarized our current knowledge on the structural determinants of AMP activity and selectivity (Takahashi et al. 2010; Huang et al. 2010; Strömstedt et al. 2010; Tossi 2011; Ruiz et al. 2014; Ebenhan et al. 2014a). Finally, for a recent discussion on how the interaction of AMPs with target and host cells determines their selectivity, see Savini et al. (2018).

As illustrated in other chapters of this book, AMPs have multiple functions, including anticancer, antifungal, and antiviral activities. For the sake of brevity and simplicity, in this chapter, we will essentially limit ourselves to discuss selectivity for bacterial versus host cells. Selectivity of anticancer peptides has been reviewed by Phoenix and coworkers (2012) and Harris et al. (2013).

11.2 AMPs Are Selective for Microbial Cells

AMPs have been isolated from natural sources based on their antimicrobial activity. The minimum inhibitory concentration (MIC, i.e., the lowest concentration of antimicrobial agent that inhibits the visible growth of a microorganism) (Wiegand et al. 2008) or the minimum bactericidal concentration (MBC, i.e., the minimal drug dosage killing at least 99.9% of the bacterial cells) (Lorian 2005) for AMPs is usually in the low μM range (Giacometti et al. 1998). AMPs are typically bactericidal, and therefore, the MIC and MBC values are usually similar (Giacometti et al. 1998).

The active concentration of a bioactive, therapeutically useful molecule must be much lower than the concentration causing toxic effects to the host cells. This property is quantified by the therapeutic index (TI), i.e., the ratio of the active concentration to the toxic concentration (see the legend to Table 11.1 for a detailed definition of these parameters). In the case of AMPs, whose main mechanism of bactericidal action is membranolytic (as discussed in Sect. 11.3), toxicity is most commonly assessed by measuring the lysis of erythrocytes (Fig. 11.1). Table 11.1 summarizes some TI values of natural and artificial AMPs, which are typically in the range 10–1000. However, it should be considered that unfortunately a strong variability is present in the literature regarding the definition of the toxic concentration, because different thresholds of lysed red blood cells (RBCs) are utilized to define the minimum hemolytic concentration (MHC), ranging from barely detectable to full hemolysis (see references cited in Table 11.1) (Bacalum and Radu 2015). In addition, MIC values depend on the specific strains tested in the assay.

When toxicity is assayed on other human cells, the results are generally not very different from those obtained using hemolysis (Table 11.2), but combining the two toxicity tests obviously

Table 11.1 Therapeutic index (TI) of natural and artificial AMPs

Name	Sequence	TI	Calculated as	References
Natural Peptides				
Temporin-L	FVQWFSKFLGRIL-NH_2	**1**	HC_{50}/MIC (Gram + &–)	Mangoni (2011)
Magainin-1	GIGKFLHSAGKFGKAFVGEIMKS	**2.5–4**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
PMAP-36	GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-NH_2	**3**	HC_5/MIC (Gram + &– and *C. albicans*)	Lyu et al. (2016)
Mastoparan X	INWKGIAAMAKKLL-NH_2	**3.4**	HC_5/MIC (*E. coli*)	Henriksen et al. (2014)
Arenicin-1	RWCVYAYVRVRGVLVRYRRCW	**5**	HC_{50}/MIC (Gram + &–)	Panteleev et al. (2015)
Polistes Mastoparan	VDWKKIGQHILSVL-NH_2	**6**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Magainin 2B	GIGKFLHAAKKFAKAFVAEIMNS	**9**	HC_{50}/IC_{50} (*B. anthracis*)	Dawson et al (2011)
Dermaseptin 1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	**0.83–7**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	**12**	HC_5/MIC (*E. coli, S. aureus*)	Luo et al. (2017)
Indolicidin	ILPWKWPWWPWRR-NH_2	**13** **4–11**	HC_{50}/MIC (Gram + &–) HC_5/MIC (*E. coli*)	Nan et al. (2009) Bacalum and Radu (2015)
Gramicidin-S	VOLFPVOLFP	**1–18** **2**	HC_{50}/MIC (Gram + &–) $HC1_{00}$/MIC (Gram + &–)	Swierstra et al. (2016) Kondejewski (1999)
XT-7	GLLGPLLKIAAKVGSNLL	**18**	HC_{50}/MIC (*E. coli)*	Kamech et al. (2012)
PGLa	GMASKAGAIAGKIAKVALKAL-NH_2	**24**	HC_{10}/MIC (Gram + &–)	Strandberg et al. (2007)
Tachyplesin I	KWCFRVCYRGICYRRCR-NH_2	**2–24**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	**26**	HC_{50}/IC_{50} (*B. anthracis*)	Dawson and Liu (2011)
Ascaphin-8	GFKDLLKGAAKALVKTVLF	**37**	HC_{50}/MIC (*E. coli*)	Kamech et al. (2012)
Papillosin	GFWKKVGSAAWGGVKAAAKGAAVGGLNALAKHIQ	**34**	HC_{50}/IC_{50} (*B. anthracis)*	Dawson and Liu (2011)
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	**23** **>57**	HC_5/MIC (Gram+ &–) $HC_{=0}$/MIC (Gram + &–)	Velduhizen (2017) Kang et al. (1999)
Ascaphin-1	GIRDVLKGAAKAFVKTVAGHIAN-NH_2	**>60**	HC_{50}/MIC (*E. coli*)	Juretic et al. (2009)
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	**3–82**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH_2	**20–130**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2	**177–579**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)

(continued)

Table 11.1 (continued)

Modified analogues of natural peptides				
Mastoparan-X Ala1	ANWKGIAAMAKKLL-NH_2	**7**	HC_5/MIC (*E. coli*)	Henriksen et al. (2014)
PMAP-36 T115	TRKRLKKIGKVLKWI-NH_2	**29**	HC_5/MIC (Gram + &– and *C. albicans*)	Lyu et al. (2016)
Gramicidin GS14K4	VKLkVyPLKVKLyP	**31**	HC_{100}/MIC (Gram + &–)	Kondejewski (1999)
D-Piscidin1 19K	ffhhifrgkvhvgktihrlvtg-NH_2	**33**	HC_{50}/MIC (*A. baumannii*)	Jiang (2014)
Arenicin-1 V8R	RWCVYAYRRVRGVLVRYRRCW	**80**	HC_{50}/MIC (Gram + &–)	Panteleev et al. (2015)
[K2, K16] XT-7	GKLGPLLKIAAKVGSKLL	**>130**	HC_{50}/MIC (*E. coli*)	Kamech et al. (2012)
Indolicidin-A7	ILKWKWKWWKWRR-NH_2	**190**	HC_{50}/MIC (Gram + &–)	Nan et al. (2009)
D-Dermaseptin S4 L7K,A14K	alwmtlkkkvlkakakalnavlvgana-NH_2	**219**	HC_{50}/MIC (*A. baumannii*)	Jiang (2014)
[I2, K19] ascaphin-8	GIKDLLKGAAKALVKTVLK	**>480**	HC_{50}/MIC (*E. coli*)	Kamech et al. (2012)
Gramicidin V3/A3	AKLkAyPLKAKLyP	**520**	HC_{100}/MIC (*C. xerosis)*	Kondejewski (2002)
	Designed peptides			
V13K	Ac-KWKSFLKTFKSAKKTVLHTALKAISS-NH_2	**163**	$HC_{>0}$/MIC (Gram + &–)	Chen et al. (2005)
P5	KWKKLLKKPLLKKLLKKL-NH_2	**>150**	$HC_{=0}$/MIC (Gram + &–)	Park et al. (2003)
Pep-1-K	KKTWWKTWWTKWSQPKKKRKV	**174**	$HC_{>0}$/MIC (Gram + &–)	Zhu et al. (2009)
PK-12-KKP	KKPWWKPWWPKWKK	**200**	$HC_{>0}$/MIC (Gram + &–)	Zhu et al. (2009)
D16	Ac-klkslltlskakkkklktllkalsk-NH_2	**890** **3355**	HC_{50}/MIC (*P. aeruginosa)* HC_{50}/MIC (*A. baumannii*)	Jiang (2011)

The TI is defined as the ratio of hemolytic to inhibitory peptide concentration. HC_x is defined as the peptide concentration causing the x% of hemolysis. $HC_{>0}$ is the minimal peptide concentration that produces detectable hemolysis; $HC_{=0}$ is the highest peptide concentration that causes no detectable release of hemoglobin. HC_{100} is the minimal concentration causing total lysis. MIC is defined as the minimum concentration that inhibits bacterial growth; when calculated on more than one strain, the geometric mean of the values is reported. Data presented as a range result from different MIC values reported in the literature for the same peptide. –NH_2 in the peptide sequence indicates amidation at the C-terminus, while Ac- indicates acylation at the N-terminus. *O* is pyrrolysine. Amino acids are colored in blue, red, orange, and green based on their cationic, anionic, polar, and hydrophobic character, respectively. Lowercase letters indicate D-amino acids

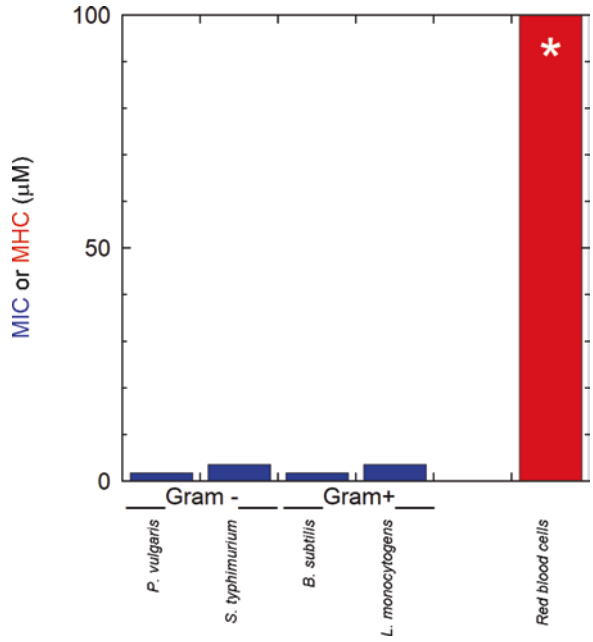

Fig. 11.1 Selective cytotoxicity in in vitro assays. Minimal inhibitory concentrations against different Gram- and Gram+ bacterial strains and minimal hemolytic concentration for the designed artificial AMP P5. The asterisk indicates that no hemolysis was observed for P5 in the peptide concentration range investigated (up to 100 μM). Adapted, with permission, from research originally published in Bobone et al. 2013, published by the European Peptide Society and John Wiley & Sons, Ltd.

provides a clearer picture of the selectivity of AMPs (Bacalum and Radu 2015).

AMP activity and toxicity are usually measured in separate assays performed under rather different conditions (for instance, regarding cell density, see Sect. 11.9) (Matsuzaki 2009). We have argued that experiments on bacterial and human cells in co-culture would provide a more stringent test of peptide selectivity (Savini et al. 2017, 2018). However, this approach has been employed only in a few cases. These studies, discussed in detail in Sect. 9.3, demonstrated that AMPs are selective even when acting on bacteria co-cultured with mammalian cells (Fig. 11.2a).

Several evidences indicate that AMPs are selective also in vivo. For instance, a large body of studies starting in 1999 (Welling) has shown that radiolabeled (Lupetti et al. 2003; Brouwer et al. 2008; Akhtar et al. 2012; Ebenhan 2014a) or fluorescent (Akram et al. 2015) AMPs can be used to image infections in vivo and can even discriminate between infection and inflammation, thanks to their specific binding to bacterial cells (Welling et al. 2000) (Fig. 11.2b, c). Among the peptides used for this purpose, there are defensins, cathelicidins, lactoferricins, histatins, artificial peptoids, and particularly sequences derived from ubiquicidin (Lupetti et al. 2003; Brouwer et al. 2008; Akhtar et al. 2012; Ebenhan 2014a, b; Dutta et al. 2017; Lei et al. 2018). Several imaging studies employed ubiquicidin 29–41 (Meléndez-Alafort et al. 2004; Akhtar et al. 2005; Vallejo et al. 2008; Gandomkar et al. 2009; de Murphy et al. 2010; Assadi et al. 2011; Ostovar et al. 2013; Saeed et al. 2013; Kahrom et al. 2014; Ebenhan et al. 2018; Bhatt et al. 2018), which has moderate activity and selectivity in the standard assays (MIC 40 μM, TI > 5) (Brouwer et al. 2006; Lupetti et al. 2008) but accumulates at the site of infection. For instance, one study reported overall values of sensitivity, specificity, and accuracy for infection detection of 100%, 80%, and 94% (Akhtar et al. 2005). Analogues of the ubiquicidin peptide have been used also for targeted

Table 11.2 Comparison of TI values determined with RBCs or with other eukaryotic cells

Name	Sequence	TI (cells)	Calculated as	TI (RBCs)	Calculated as	References
Natural peptides						
Magainin2-NH_2	GIGKFLHSAKKFGKAFVGEIMNS-NH_2	**6**	LC_{99} (3T3)/MIC (Gram+ &–)	**>130**	HC_{100} /MIC (Gram+ &–)	Javadpour et al. (1996)
Polistes Mastoparan	VDWKKIGQHILSVL-NH2	**7**	LC_{50}(PBMC)MIC (*E. coli*)	**6**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Dermaseptin 1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	**1–7**	LC_{50} (PBMC)/MIC (*E. coli*)	**1–10**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Lasioglossin III	VNWKKILGKIIKVVK-NH2	**>8**	LC_{50} (HUVEC)/MIC (Gram+ &–)	**>105**	HC_{50}/MIC (Gram+ &–)	Slaninová et al. (2012)
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	**7–12**	LC_{50} (PBMC)/MIC (*E. coli*)	**2.5–4**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Indolicidin	ILPWKWPWWPWRR-NH_2	**6–17**	LC_{50} (PBMC)/MIC (*E. coli*)	**4–11**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG	**17**	LC_{95} (HN)/MIC(Gram+ &–)	**55**	HC_{90}/MIC (Gram+ &–)	Skerlavaj et al. (1996)
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHLG	**18**	LC_{80} (HN)/MIC (Gram+ &–)	**59**	HC_{30}/MIC (Gram+ &–)	Skerlavaj et al. (1996)
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	**23**	LC_{30} (IPEC-J2)/MIC (Gram+ &–)	**23** **>57**	HC_5/MIC (Gram+ &–) $HC_{=0}$/MIC (Gram + &–)	Velduhizen (2017) and Kang et al. (1999)
Cecropin B-NH_2	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH_2	**29**	LC_{99} (3T3)/MIC (Gram+ &–)	**>86**	HC_{100} /MIC (Gram+ &–)	Javadpour et al. (1996)
Tachyplesin I	KWCFRVCYRGICYRRCR-NH_2	**3–30**	LC_{50} (PBMC)/MIC (*E. coli*)	**2–24**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Magainin2	GIGKFLHSAKKFGKAFVGEIMNS	**3–82**	LC_{50} (PBMC)/MIC (*E. coli*)	**3–82**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2	**27–180**	LC_{50} (PBMC)/MIC (*E. coli*)	**20–130**	HC_5/MIC (*E. coli*)	Bacalum and Radu (2015)
	Modified analogues of natural peptides					
A(A1R A8R I17K)	RIGSILGRLAKGLPTLKSWIKNR-NH_2	**1.5–3**	LC_{50} (L929)/MIC (Gram+ &–)	**10–16**	HC_{10}/MIC (Gram+ &–)	Zhang (2016)
Designed peptides						
D-LAK120 AP13	kklalalakkwlplakklalalakk-NH_2	**4**	LC_{50} (RAW 264.7)/MIC (Gram–)	**67**	HC_{50}/MIC (Gram–)	Vermeer (2012)
WK12	KWWKWWKKWWKK	**>10**	LC_{20} (PBMC)/MIC (Gram–)	**>10**	HC_{10}/MIC (Gram–)	Deslouches et al. (2016)
WR12	RWWRWWRRWWRR	**>20**	LC_{20} (PBMC)/MIC (Gram–)	**>20**	HC_{10}/MIC (Gram–)	Deslouches et al. (2016)
$(KLAKKLA)_2$	KLAKKLAKLAKKLA	**>45**	LC_{99} (3T3)/MIC (Gram+ &–)	**>125**	HC_{100} /MIC (Gram+ &–)	Javadpour et al. (1996)
$(KLAKLAK)_2$	KLAKLAKKLAKLAK	**>86**	LC_{99} (3T3)/MIC (Gram+ &–)	**>125**	HC_{100} /MIC (Gram+ &–)	Javadpour et al. (1996)

LC is the lethal peptide concentration. LC_x is defined as the peptide concentration killing *x%* of the cells. For HC and MIC definitions and for the color code used for the sequences, please see Table 11.1. When data are presented as a range, they result from different MIC values reported in the literature for the same peptide. *HBRC* human red blood cells, *PBMC* peripheral blood mononuclear cells ($3 \cdot 10^6$/mL), *3T3 cells* murine fibroblast cell line ($2 \cdot 10^5$/mL), *HUVEC cells* human umbilical vein endothelial cells ($2 \cdot 10^4$/mL), *HN* human neutrophils ($4 \cdot 10^6$/mL), *IPEC-J2* porcine intestinal epithelial cells ($1.5 \cdot 10^5$/mL), *L929* mouse fibroblast cells ($1.5 \cdot 10^4$/mL), *RAW 264.7* murine macrophage cells ($3 \cdot 10^5$/mL). The density of RBCs used in the hemolytic activity assays (when specified in the original reference) ranged from 10^6 to 10^9 cells/mL. Lowercase letters indicate D-amino acids

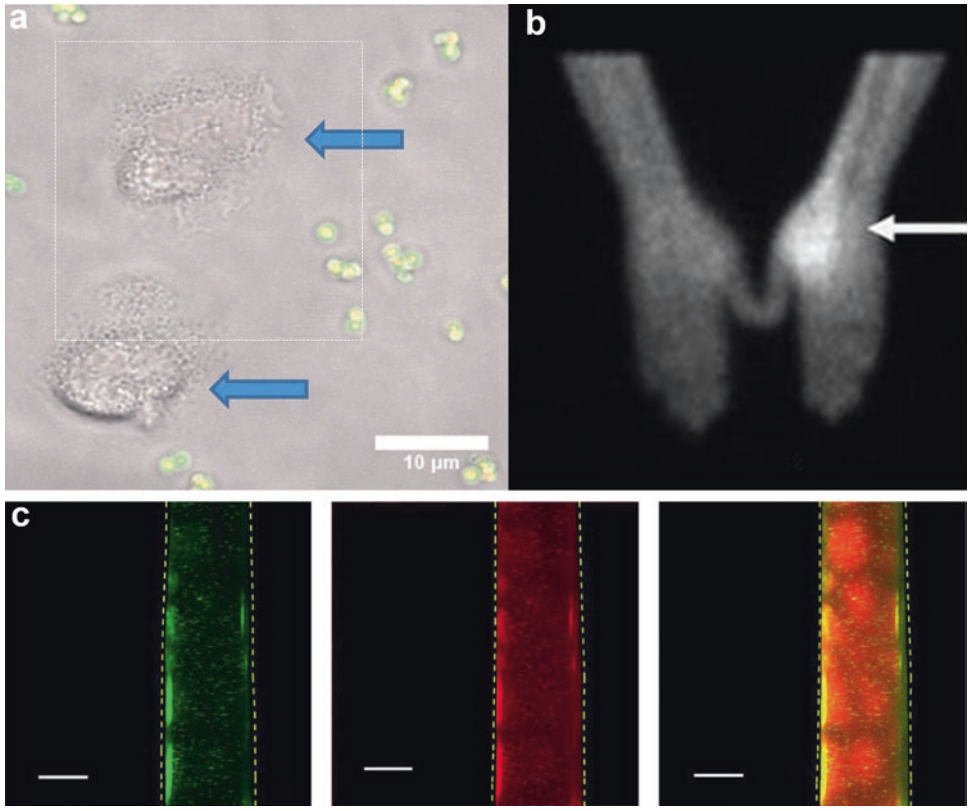

Fig. 11.2 Selective targeting of bacterial cells by AMPs in vitro and in vivo. (**a**) Optical and fluorescence microscopy image of a labeled ubiquicidin analogue (visible by the green fluorescence) selectively binding to *S. aureus* bacteria, in co-culture with isolated human neutrophils (blue arrows) Adapted, with permission, from research originally published in Akram et al. 2015, published by The Royal Society of Chemistry. (**b**) Positron emission tomography image of a patient with an infection in the left hand (indicated by the arrow), traced with a radiolabeled ubiquicidin analogue. No significant peptide uptake in the contralateral hand was noted. The image was obtained 30 min after tracer administration (Reproduced, with permission, from research originally published in Akhtar et al. 2012). (**c**) Visualization of in vivo targeting of human α-defensin 5 (HD5) toward *E. coli* cells. The mesenteric vein was imaged intravitally in mice by two-photon laser scanning microscopy, 30 min after injection of *E.coli* cells expressing a green fluorescent protein (visualized in the left panel), and treatment with HD5 labeled with a red fluorescent probe (imaged in the center panel). Colocalization is demonstrated by the overlapped images (right panel). Scale bars, 50 μm. Adapted, with permission, from Lei et al. 2018. Copyright (2018) American Chemical Society

delivery of traditional antibiotics to the infection site (Chen et al. 2015).

Although in vivo studies of the activity of AMPs abound, similar investigations characterizing their toxicity are more sparse (Mahlapuu et al. 2016). Some TIs derived from animal studies are summarized in Table 11.3, and the range of values is similar to that obtained in vitro.

The selectivity of AMPs for bacterial cells is demonstrated also by the fact that these peptides have been exploited in sensing elements that can detect infection (Mannoor et al. 2010; Shriver-Lake et al. 2012; Silva et al. 2014; Hoyos-Nogués et al. 2018), even in whole blood (Shi et al. 2017) or other complex biological samples (Qiao et al. 2017).

Overall, the results collected in the literature support an interesting selectivity of AMPs for target versus host cells. The origin of this property is necessarily related to the mechanism of action of AMPs.

Table 11.3 TI values obtained from in vivo studies

Name	Sequence	TI	Calculated as	Bacterial strain	References
Natural peptides					
P3	VNFKLLSHSLLVTLASHL	**3**	LD_{50}/ED_{60}	*E.coli*	Zhang (2015)
OH-CATH30	KFFKKLKNSVKKRAKKFFKKPRVIGVSIPF	**12**	LD_{50}/ED_{80}	*E.coli*	Li (2012)
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHLG	**47–55**	LD_{50}/ED_{100}	*P.aeruginosa*	Benincasa (2003)
		190–220	LD_{50}/ED_{100}	*E.coli*	
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG	**24–27**	LD_{50}/ED_{100}	*E.coli*	Benincasa (2003)
		47–55	LD_{50}/ED_{100}	*S.aureus*	
Modified analogues of natural peptides					
HD5-myr	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR-myr	**>3**	$LD_{=0}/ED_{90}$	*E. coli*	Lei (2018)
RN7-IN8	FLGGLIKWPWWPWRR-NH_2	**4**	LD_{100}/ED_{50}	*S. pneumoniae*	Jindal (2017)
JH3	RRFKLLSHSLLVTLASHL	**4.5**	LD_{50}/ED_{90}	*E.coli*	Zhang (2015)
TP3	FIHHIIGGLFSVGKHIHSLIHGH	**7**	MTD/ED_{60}	*A.baumannii*	Pan (2015)
		7	MTD/ED_{70}	*K pneumoniae*	Pan (2015)
		>24	MTD/ED_{100}	MRSA	Huang (2015a)
D-OH-CATH30	kffkklknsvkkrakkffkkprvigvsipf	**8**	LD_{50}/ED_{100}	*E.coli*	Li (2012)
OH-CM6	KFFKKLKKAVKKGFKKFAKV	**10**	LD_{50}/ED_{70}	*E.coli*	Li (2012)
TP4	FIHHIIGGLFSAGKAIHRLIRRRRR	**20**	MTD/ED_{90}	*A.baumannii*	Pan (2015)
		20	MTD/ED_{90}	*K pneumoniae*	Pan (2015)
		>24	MTD/ED_{100}	MRSA	Huang (2015b)
Designed peptides					
A3-APO	(Chex-RPEKPRPYLPRPRPPRPVR)$_2$-Dab-NH_2	**2.5**	LD_{50}/ED_{100}	*E.coli*	Szabo (2010)
Onc72	VDKPPYLPRPRPPROIYNO-NH_2	**>20**	LD_{50}/ED_{50}	*E.coli*	Knappe (2012)
$(LLKK)_2C$	LLKKLLKKC	**28**	LD_{50}/ED_{50}	*A.baumannii*	Huang (2012)
$C(LLKK)_2C$	CLLKKLLKKC	**34**	LD_{50}/ED_{50}	*A.baumannii*	Huang (2012)

TI was calculated as the ratio of the toxic or lethal dose to the effective dose of peptides in mice. LD_x is the minimum dose that was lethal for at least x% of animals; $LD_{=0}$ is the highest peptide concentration that caused no deaths; MTD is minimum dose that caused toxicity (narrowing of the eyes was taken as symptom), MLD is the minimum lethal dose, and ED_x is the effective dose, i.e., the minimum dose causing the survival of at least x% of the animals. Bacteria and peptides were injected intraperitoneally, except for Huang (2015a, b) and Pan (2015), where toxicity tests were performed by intramuscular injection. -myr indicates myristoylation at the C-terminus. *Chex* 1-amino-cyclohexane carboxylic acid, *Dab* 2,4-diamino-butyric acid, *O* pyrrolysine. MRSA is methicillin-resistant *S. aureus*. For the color code used for the sequences, please see Table 11.1. Lowercase letters indicate D-amino acids

11.3 Cellular Membranes Are the Main Target of AMPs

Selectivity is not surprising when a biomolecule associates to a specific receptor or protein (Le Joncour and Laakkonen 2018). However, this is not the case for most AMPs. In general, natural AMPs and their enantiomers comprising all D-amino acids have a comparable antimicrobial activity (Fig. 11.3), while any interaction of a peptide with a protein, due to the chirality of both systems, would be favored for one enantiomer over the other. As is often the case for AMPs, exceptions to this rule have been reported (Otvos et al. 2000; Bulet and Stocklin 2005; de la Fuente-Núñez et al. 2015), showing that the mechanism of action of a minority of AMPs could be receptor mediated. On the other hand, microbiological assays of membrane permeability and microscopic imaging of bacteria treated with AMPs clearly show that cell membranes are damaged and that, as a consequence, transmembrane gradients are dissipated (Tiozzo et al. 1998; Arcidiacono et al. 2009; Hartmann et al. 2010; Agrawal and Weisshaar 2018) (Fig. 11.4). Usually, membrane perturbation and bacterial killing are correlated, further supporting membrane disruption as the main bactericidal mechanism. However, also in this case, exceptions exist, indicating that a subclass of AMPs might act through different killing mechanisms (He et al. 2014; Friedrich et al. 2000). Finally, it is worth mentioning that AMPs usually are able to perturb the permeability of artificial membranes, comprising only phospholipids (Fig. 11.4c) (Orioni et al. 2009; Bocchinfuso et al. 2011; Braun et al. 2017; Savini et al. 2018). This observation demonstrates that the membrane-perturbing activity is purely the result of physicochemical interaction between the peptide and the lipid bilayer, and not the consequence of some biological process.

Overall, literature data clearly demonstrate that membrane perturbation is the main mecha-

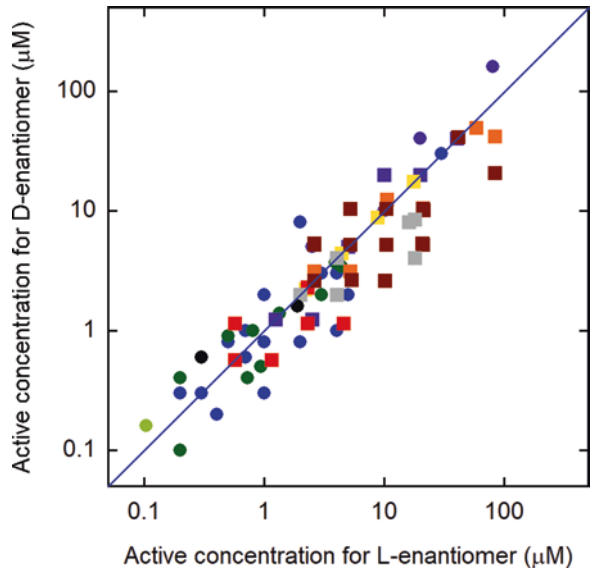

Fig. 11.3 Activity of natural AMPs and their enantiomeric analogues. Comparison of the antibacterial activities (circles for MBC, squares for MIC) of enantiomeric peptides. Data refer to magainin 2 (Bessalle et al. 1990) (violet), cecropin A (Wade et al. 1990) (blue), melittin (Juvvadi et al. 1996) (dark green), LL-37 (Dean et al. 2011) (light green), KSLK (Hong et al. 1999) (yellow), temporin A (Wade et al. 2000) (orange; data refer to IC_{50} values), camel 48 (Oh et al. 2000) (red), V681 and analogues (Chen et al. 2006) (dark red; data refer to LC_{50} values), lactoferricin B analogues (Wakabayashi et al. 1999) (silver), and cecropin B (Bland et al. 2001) (black). The blue line is the diagonal of the plot (corresponding to identical activity for D and L enantiomers) and not a fit. A version of this figure with a more limited set of data has been published previously (Savini et al. 2018)

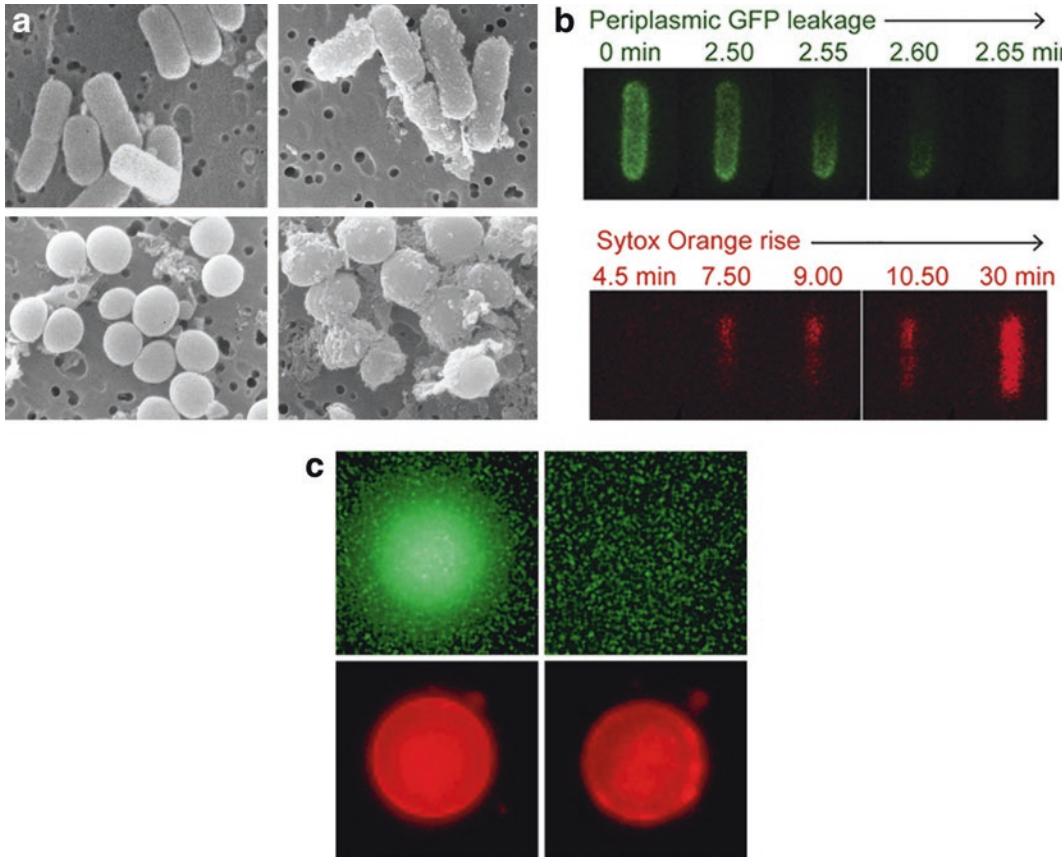

Fig. 11.4 Fluorescence and electron microscopy images of the effects of AMPs on bacterial and artificial membranes. (**a**) Scanning electron micrographs of *E. coli* (top) and *S. aureus* (bottom) before (images on the left) and after (images on the right) treatment with the synthetic AMP PGYa (30 min, 10 μM). The images show a considerable roughening of the bacterial membranes and formation of blebs on the cell surface, in contrast to the smooth surfaces of untreated bacteria, providing a strong indication that the membrane is being considerably altered by the peptide. Adapted, with permission, from research originally published in Tiozzo et al. 1998 © Elsevier. (**b**) Fluorescence microscopy images of an *E. coli* cell attacked by the AMP cecropin A (0.5 μM). The leakage of periplasmic green fluorescent protein (GFP), shown by the green fluorescence, indicates perturbation of the outer membrane, while uptake of the DNA stain Sytox Orange (red fluorescence) demonstrates pore formation in the plasma membrane (Adapted, with permission, from research originally published in Agrawal 2018 © Elsevier). (**c**) Fluorescence microscopy images of perturbation of a giant unilamellar vesicle by the AMP PMAP-23. The top panels report the green fluorescence emission from carboxyfluorescein molecules entrapped inside the GUV, which were completely released after peptide addition (right). By contrast, the vesicle was still present after peptide addition, as indicated by the red fluorescence of rhodamine-labeled phospholipids located in the GUV bilayer (bottom panels). Taken together, these images demonstrate pore formation by the AMP. The vesicle diameter is about 20 μm. Adapted, with permission, from research originally published in Orioni et al. 2009 © Elsevier

nism of direct bacterial killing for most AMPs. Even for those AMPs that act through a different antibacterial mechanism (Nicolas 2009; Otvos 2017), the cell envelope is the first cell component that the peptides encounter, and they have to cross the extracellular membrane (when present) and the cell wall to reach the plasma membrane and eventually the cell interior.

Incidentally, the fact that AMPs target microbial membranes determines their broad-spectrum activity, their bactericidal, rather than bacteriostatic, mechanism of action and also the higher difficulty for bacteria in developing resistance against them (compared to resistance against conventional antibiotics acting on a protein target) (Perron et al. 2006; Otvos 2017).

11.4 Bacterial and Host Cells Have Different Membrane Structure and Composition

If membranes are the target, then it is conceivable that selectivity arises from a difference in membrane composition of the various cell types. Indeed, bacterial and eukaryotic cells have very different cell envelopes (Wang 2017). Bacteria can be divided into Gram-positive and Gram-negative, depending on whether they are colored by the Gram stain or not. This assay reflects differences in the composition of the cell envelope. In both cases, the plasma membrane is surrounded by a cell wall. However, in Gram-positive bacteria, this is formed by a thick peptidoglycan and lipoteichoic acid layer (40–80 nm). By contrast, in Gram negatives, a thin peptidoglycan layer (8 nm thick) is contained in a second (outer) membrane, with asymmetric composition: phospholipids are the main components of the inner leaflet, while the outer layer is mainly formed by lipopolysaccharides (LPS). On the other hand, eukaryotic cells only have the plasma membrane, with asymmetric lipid composition in the two leaflets of the bilayer (Fig. 11.5).

In addition to the different structures of the cell envelope, important differences are present in the lipid composition of the cellular membranes. Tables 11.4 and 11.5 summarize the lipid content of bacterial and RBC membranes.

Bacterial membranes contain a significant fraction of negatively charged lipids: in Gram negatives, both membranes contain phosphatidylglycerol (PG, ~20% overall) and cardiolipin (CL, ~5% overall); in Gram-positive bacteria, the content of anionic lipids is much higher, again with PG and CL being the most important components (Malanovic and Lohner 2016). However, the membranes of these cells can also contain the positively charged L-lysyl-PG (LPG). In both cases, the main zwitterionic component is phosphatidylethanolamine (PE), and no sterols are present. Of course, the values for the composition reported in Table 11.4 are only approximate, since they change with the specific strain and growth conditions. In addition, lipid composition is not homogeneous over the cell surface (Renner and Weibel 2011; Oliver et al. 2014).

Human cells contain cholesterol and have no anionic phospholipids in the outer leaflet of their cell membrane. Some negatively charged glycolipids, such as gangliosides, are present on the cell surface (Miyazaki et al. 2012), but they are minor components in most cell membranes (with the exception of nerve cells) (Storch and Kleinfeld 1985). These properties are exemplified by RBCs, which are commonly used to test toxicity and selectivity (Table 11.5). For eukaryotes, the main zwitterionic components are phosphatidylcholine (PC), sphingomyelin (SM), and PE.

Overall, we can translate these differences in lipid composition in distinct physicochemical properties. Bacterial membranes contain more anionic lipids in the outer surface of their bilayers than eukaryotic cells. This difference combines

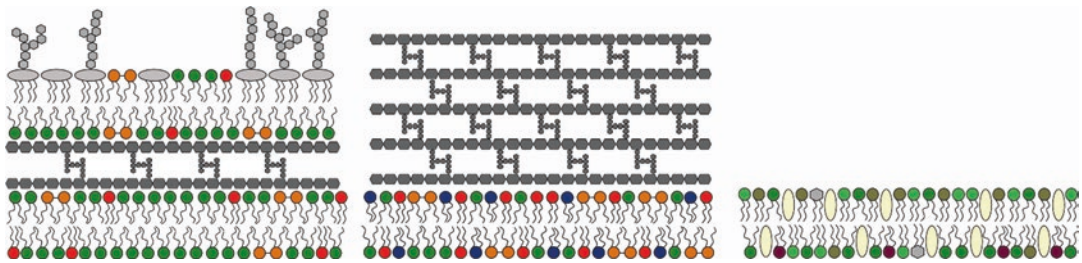

Fig. 11.5 Schematic depiction of the structure of the cellular envelope in different cell types. The three panels, from left to right, schematize the structure of the cellular envelope in Gram- and Gram+ bacteria and in human cells, respectively. Proteins, glycolipids, and lipoteichoic acids (in Gram+ bacteria) have been omitted, for the sake of clarity. LPS, light gray; peptidoglycan, dark gray; PC, light green; PE, dark green; SM, light brown; PI, light gray; PG, red; PS, dark red; CL, orange; L-lysyl PG, blue; cholesterol, beige

Table 11.4 Phospholipid composition of the membranes of Gram-negative (name highlighted in red) and Gram-positive (name highlighted in blue) bacteria

Phospholipid composition of bacterial membranes					
	PE	**PG**	**CL**	**PA**	**L-lysyl PG**
Total charge	0	−1	−2 (−1)	−1	+1
Intrinsic curvature	−	0	−	−	+
E. coli					
(both membranes)					
(Ames 1968)	69	19	6.5		
(Raetz 1986)	75–85	10–20	5–15		
(Morein et al. 1996)	79	17	4		
(Rowlett et al. 2017)	78	12	6	<3	
E. coli					
(cell membrane)					
(Morein et al. 1996)	75	19	6		
S. typhimurium					
(both membranes)					
(Osborn 1972)	86	13	1		
(Ames 1968)	78	18	3		
S. typhimurium					
(cell membrane)					
(Osborn 1972)	76	21	3		
B. subtilis					
(Op den Kamp et al. 1969)*	30	36	12		22
(Bishop et al. 1967)**	34	49	11		
S. aureus					
(Hayami 1979):					
(strain Newman)		49	13	0.6	34
(strain Tazaki)		47	10	1	38

Phospholipid composition data are expressed as molar percentages. In the case of Hayami et al. (1979), data were calculated converting the % of phosphorus to molar % by considering two P atoms per CL molecule and one for the other lipids. In the case of Osborn et al. (1972), data were calculated converting the % of [2-^{3}H] glycerol to molar %, by considering three ^{3}H atoms per CL molecule, two per PG, and one for the other lipids. Data on total charge at physiologic pH and intrinsic curvature were taken from Marsh (1990), McMahon and Boucrot (2015), Malanovic and Lohner (2016), and Boyd et al. (2017)

PE phosphatidylethanolamine, *PG* phosphatidylglycerol, *CL* cardiolipin, *PA* phosphatidic acid, *L-lysyl PG* L-lysyl phosphatidylglycerol

*Indicates a growth condition without glucose and sulfate

**Indicates that 6% of lipo-amino acids were also recovered.

with the additional negative charges conferred to bacterial cells by teichoic and teichuronic acids and LPS. Furthermore, the transmembrane potential of bacterial cells is more inside-negative than that of normal mammalian cells (Yeaman and Yount 2003). For all these reasons, bacteria have stronger electrostatic interactions with positively charged molecules than eukaryotic cells. Another difference is that bacterial membranes are more disordered and less well packed than those of eukaryotes, due to the lack of cholesterol. In addition, they contain larger amounts of "non-bilayer" lipids, with negative or positive values for the "intrinsic curvature," such as PE, CL, and PA, or LPG, respectively (McMahon and Boucrot 2015; Malanovic and Lohner 2016). This property depends on the relative sizes of the phospholipid head-groups and acyl chains. Lipids where the cross-sectional area occupied by head-groups and tails is similar (e.g., PC, PG, PS) are said to have a cylindrical shape and pack well in locally flat bilayer structures (zero intrinsic curvature).

Table 11.5 Phospholipid and cholesterol content of human erythrocyte membrane

Phospholipid composition of human RBC membranes						
	PC	**PE**	**SM**	**PI**	**PS**	**PA**
Total charge	0	0	0	−1	−1	−1 (−2)
Intrinsic curvature	0	−	0	+	0	−
Both leaflets						
(Dodge and Phillips 1967)	29.2±1.5	27.5±1.5	25.4±1.4	0.6±0.5	14.8±1.7	1.1±0.5
(Broekhuyse 1969)	28.3±2.1	26.7±1.0	25.8±1.7	1.9±0.6	12.7±1.3	
(White 1973)	34.7	28.0	20.1		14.3	
(Verkeleij 1973)	28	26	24		13	
(Van Meer 1981)	29.5	25.9	25.3		12.2	
Outer leaflet						
(Verkeleij 1973)	42	10	40		0	
(Virtanen et al. 1998)	44.8	11.1	42.1			
Inner leaflet						
(Verkeleij 1973)	14	42	8		26	
(Virtanen et al. 1998)	14.0	43.9	9.1	1.2	29.6	2.2
Asymmetry of distribution (% in the outer leaflet)						
(Verkleij 1973)	76	20	82		0	
(Zwaal et al. 1973)	62		83			
(Gordesky and Marinetti 1973)		15			0	
(Zwaal et al. 1975)	75					
(Gordesky et al. 1975)		33			0	
(Van Meer 1981)	78	20	80			
(Bütikofer et al. 1990)				24		
(Gascard et al. 1991)				20		

Cholesterol content as cholesterol/phospholipid molar ratio	
(Cooper 1975)	0.95
(Ballas and Krasnow 1980)	0.75
(Chabanel 1983)	0.80

Phospholipid composition data are expressed as molar percentages. Data from Verkeleij (1973) were derived from a figure in the cited reference. Data on total charge at physiologic pH and intrinsic curvature were taken from Marsh (1990) and McMahon and Boucrot (2015)

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *SM* sphingomyelin, *PI* phosphatidylinositol, *PS* phosphatidylserine, *PA* phosphatidic acid

By contrast, lipids where the head-group is smaller than the tails (e.g., PE or PA) favor concave shapes of the monolayer (negative curvature). The opposite is true for lipids with comparatively larger polar heads (e.g., LPG) which have a positive curvature (Koller and Lohner 2014).

The differences in lipid composition and in physical properties between bacterial and human cell membranes are considered to be the origin of AMP selectivity. Similar considerations on membrane composition (particularly regarding the content of anionic lipids and sterols) have been proposed to explain the selectivity for cancer cells (Hoskin and Ramamoorthy 2008; Schweizer 2009; Phoenix et al. 2012; Gaspar et al. 2013), fungi (van der Weerden et al. 2013; Rautenbach et al. 2016), protozoa (Rivas et al. 2009), and enveloped viruses (Aloia et al. 1993; Findlay et al. 2013), since in all cases the lipid distribution is different from that of a normal eukaryotic cell.

11.5 Lipid Composition Determines the Affinity of AMPs for Lipid Bilayers

The hypothesis of a selectivity based on differences in lipid composition has been tested by studying the interaction of AMPs with model membranes mimicking the composition of the natural bilayers. With liposomes, it is possible to vary the lipid composition at will and to measure both peptide-membrane association and peptide-induced membrane permeability (Bocchinfuso et al. 2011; Savini et al. 2018). The role of various membrane properties in AMP selectivity is summarized in the following sections.

11.5.1 Membrane Charge

In model membranes, the presence of anionic lipids increases peptide association to the bilayer and, as a consequence, peptide-induced leakage (Matsuzaki et al. 1989, 1995; Gazit et al. 1995; Abraham et al. 2005; Sood et al. 2008; Russell et al. 2010; Bobone et al. 2013; Golbek et al. 2017; Maturana et al. 2017). On the other hand, the positively charged lipid lysyl-PG, present in Gram+ bacteria, inhibits AMP activity (Nishi et al. 2004; Andra et al. 2011). These findings are a straightforward consequence of electrostatic interaction of the membranes with the positively charged AMPs (see Sect. 11.6). Regarding the anionic gangliosides present in the outer leaflet of eukaryotic membranes, Matsuzaki and coworkers (2012) demonstrated that, although their acidic moieties favor the association of AMPs to model membranes, this interaction does not lead to strong membrane perturbation, since the peptides remain trapped in the sugar region.

11.5.2 Cholesterol Content

Several studies also reported an AMP inhibitory effect of cholesterol. For instance, the presence of cholesterol inhibits the membrane-perturbing activity of magainin, pardaxin, LL-37, temporin L, human defensin HNP1, and other AMPs (Matsuzaki et al. 1995; Tytler et al. 1995; Hallock et al. 2002; Sood et al. 2008; Sood and Kinnunen 2008; Gonçalves et al. 2012; Verly et al. 2008; Wu et al. 2010; McHenry et al. 2012). The membrane-ordering effects of cholesterol in fluid bilayers are well established: insertion of the rigid ring structure of the sterol limits the possibility for trans-gauche isomerization for adjacent phospholipid tails, leading to an increase in bilayer order, packing, thickness, and rigidity (Henriksen et al. 2006; Mouritsen and Zuckermann 2004). All these effects could contribute to reduce peptide binding and membrane perturbation (McIntosh et al. 2002). However, the relevance of cholesterol for AMP selectivity has been recently questioned. While all the investigations listed above were performed on simple lipid mixtures, a comprehensive study by Ramamoorthy and coworkers on more realistic lipid compositions showed that cholesterol's protective effect against AMPs does not occur in lipid systems containing raft domains and presenting phase separation (McHenry et al. 2012; Brender et al. 2012). There are examples where the activity of AMPs is not affected by the presence of cholesterol even in simple lipid mixtures (Bobone et al. 2013). On the other hand, Matsuzaki et al. (1995) demonstrated an AMP-inhibiting effect of cholesterol in real cells, by artificially varying the cholesterol content of RBCs.

It is worth mentioning that the inhibitory effect is specific of cholesterol, while ergosterol, present in fungal membranes, does not appear to inhibit peptide binding and activity to the same extent, in agreement with the specific activity of antifungal peptides (Sood and Kinnunen 2008; Gonçalves et al. 2012) and with the comparatively smaller effects of ergosterol on membrane order (Henriksen et al. 2006).

11.5.3 Intrinsic Curvature

The situation is less clear regarding the effect of the presence of negative curvature lipids (PE) in bacterial membranes. PE has been shown to inhibit pore formation by magainin, melittin,

alamethicin, PMAP-23, and mastoparan X (Matsuzaki et al. 1998; Allende et al. 2005; Lee et al. 2005; Bobone et al. 2012). On the other hand, the activity of some AMPs is favored by the presence of PE (Schröder-Borm et al. 2003; Epand et al. 2006; Leite et al. 2015). Inhibition of pore formation by PE can be understood by considering that the peptides act by inserting in the head-group region of the membrane, thus imposing a positive curvature strain, which is released after a threshold of membrane-bound peptide concentration is reached, through the formation of membrane defects or pores. The presence of lipids with negative intrinsic curvature would counteract this mechanism (Matsuzaki et al. 1998, Lee et al. 2005). Similar considerations, on the other hand, suggest that PE can favor membrane binding of AMPs, by reducing the intrinsic curvature strain needed for peptide insertion in the polar region of the bilayer; an increased binding to PE-containing membranes has been reported for some AMPs (Schröder-Borm et al. 2003; Phoenix et al. 2015). However, reasoning only in terms of intrinsic curvature might be misleading. For instance, the peculiar lipid-lipid interactions made possible by the structure of PE could inhibit peptide insertion: this phospholipid contains a primary amine (lacking in PC), which allows it to form strong hydrogen bonds with phosphate or CO groups in other lipids (Lewis and McElhaney 2005). This H-bond network is responsible for the melting temperatures of PE lipids being higher than those of their corresponding PC analogues (Lewis and McElhaney 2005). This difference might lead to an increase in the energy needed to insert a peptide in the bilayer, or to open a pore, in the presence of PE. Finally, an additional mechanism invoked to explain different activities on vesicles lacking or containing PE involves peptide-induced formation of lipid domains (Epand et al. 2006). Overall, these considerations can explain why different final effects are observed on the membrane-perturbing activity of AMPs, depending on which of the various phenomena predominates in each specific case. In any case, it is difficult to ascribe a well-defined role in AMP selectivity to the PE content.

11.6 Thermodynamics of Peptide-Membrane Association

In principle, selectivity for different membrane compositions could result from two effects. AMPs could have a higher affinity for bacterial membranes than for human bilayers, or they could be more effective in perturbing the former, once inserted (Wimley and Hristova 2011). The data on model membranes presented above clearly indicate that differential binding is an important aspect of AMP selectivity.

AMPs are usually short (about 10–50 residues in length), and their sequences and structures have no common features, except for the cationic charge (most AMPs fall in the range of +2 to +4 e), and amphipathic character, with an overall content of about 50% hydrophobic residues (Wang 2017). The role of these properties is easily rationalized: charge imparts selectivity toward bacterial versus eukaryotic membranes, and apolar residues provide a hydrophobic driving force for binding and insertion into membranes, leading to perturbation of bilayer integrity. Indeed, peptides interacting only electrostatically usually do not cause significant membrane leakage, because their depth of insertion is too shallow (Wimley 2010a).

11.6.1 Hydrophobic and Electrostatic Driving Forces Are Nonadditive

Different treatments are used in the literature to describe peptide-membrane interactions, and comprehensive reviews are available on this topic (White and Wimley 1999; Wieprecht and Seelig 2002; Simon and McIntosh 2002; Santos et al. 2003; Seelig 2004; Wimley 2010a). Here we will just briefly mention that, since peptide-membrane association does not have a specific stoichiometry, it is not correctly described by a binding equilibrium, and it is better treated as a partition equilibrium between the water and the membrane phase (White and Wimley 1999; Wieprecht and Seelig 2002; Santos et al. 2003; Wimley 2010a). In this view, the main effect of Coulombic inter-

actions can be described as an increase in local peptide concentration in the vicinity of the bilayer, according to Gouy-Chapman theory (Beschiaschvili and Seelig 1990; Wieprecht and Seelig 2002; Seelig 2004). However, the thermodynamic contributions of electrostatic and hydrophobic effects to the driving force of peptide water-membrane partition are not simply additive (Ladokhin and White 2001). This finding is due mainly to the different depths of polar and aliphatic moieties of phospholipids in the bilayer: charged groups are located on the surface of the membrane, well separated from the hydrocarbon core, and the physicochemical properties of the bilayer vary steeply in the head-group region (Wimley 2010a). As a consequence, the depth of insertion of a peptide in the membrane is determined by the interplay between hydrophobic effect and Coulombic forces (Wimley 2010a). Highly charged, hydrophilic molecules sit on the membrane surface, and strongly hydrophobic peptides insert into the hydrocarbon core, while cationic, amphipathic peptides are located at an intermediate position, which depends on their specific properties (Bocchinfuso et al. 2009; Farrotti et al. 2015). In turn, the depth of insertion in the bilayer modulates the intensity of electrostatic and hydrophobic contributions: strongly simplifying, one could say that the position of a peptide in the membrane determines the average distance between the peptide and the charged lipid moieties and the degree of insertion of the peptide in the water-free hydrocarbon core. An increase in peptide hydrophobicity ultimately reduces the effect of electrostatic interactions; on the other hand, augmenting the Coulombic forces diminishes the hydrophobic contribution to the binding free energy (Ladokhin and White 2001).

11.6.2 Multiple Interconnected Equilibria Modulate Peptide Activity and Selectivity

Several artificial peptides were designed having the required characteristics of cationic charge and amphipathic character. However, in many cases, such peptides turned out to be highly toxic (Dathe et al. 1996; Cornut et al. 1994; Bobone et al. 2013). These findings demonstrated that a cationic charge is not sufficient for specificity and provided a first indication of the complexities of peptide-membrane interaction discussed above. Peptides in solution can assume different conformations and aggregation states, and once membrane-bound, they can change conformation, orientation, insertion depth, and aggregation state (Fig. 11.6). All these phenomena are regulated by interconnected equilibria, and therefore they contribute in determining the final membrane-perturbing activity (Stella et al. 2004; Mazzuca et al. 2005; Gatto et al. 2006; Bobone et al. 2013). Every modification in peptide properties can affect all these processes (Gatto et al. 2006). In our opinion, this is the reason why the rational design of peptides with improved selectivity has met with limited success, and it has progressed through a trial-and-error process. Even so, several useful principles for the optimization of AMP selectivity have been defined.

11.7 Selectivity of AMPs Is Determined by Their Physicochemical Properties

Helical peptides are the most abundant and best-characterized class of AMPs. Investigations on the structural determinants of AMP selectivity have mostly focused on this type of peptides. They are usually disordered in solution but attain a helical conformation when membrane-bound, with a spatially amphipathic distribution of the side chains, where most of the hydrophobic residues face toward the membrane center and the polar and charged residues are oriented toward the water phase. From a physicochemical point of view, they can be characterized by several parameters, such as charge, hydrophobicity, or amphipathicity. In addition to the considerations discussed in the previous section, on the multiple processes involved in peptide-membrane interactions, investigations on the role of each of these parameters are complicated by the fact that varying the sequence by a single

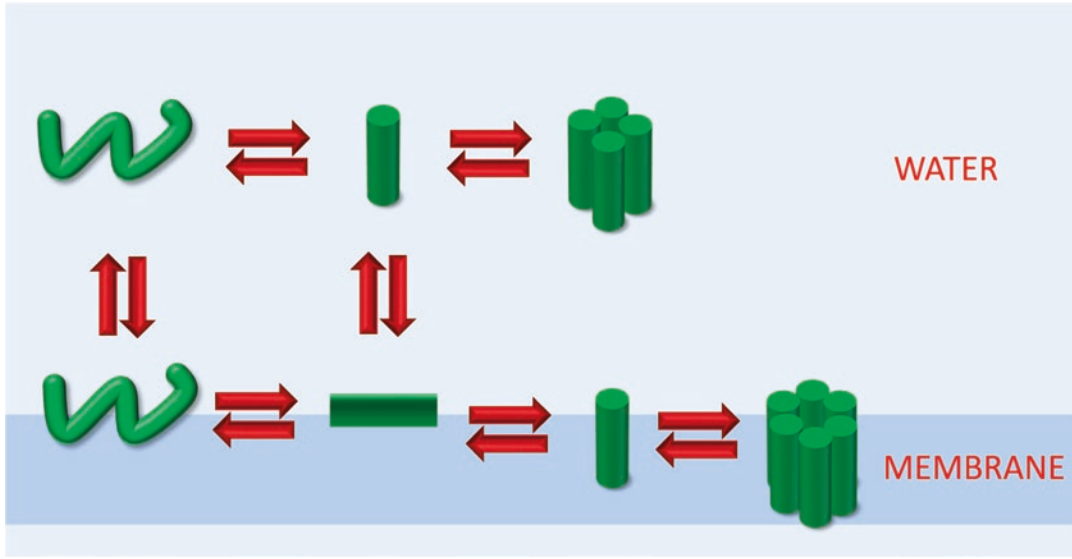

Fig. 11.6 Schematic depiction of the phenomena involved in peptide-membrane interaction. In addition to water-membrane partition, conformational, orientational, and aggregation equilibria are at play in determining the final peptide activity and selectivity

amino acid substitution usually causes a variation in multiple physicochemical properties of the peptides (Wieprecht et al. 1997a; Dathe et al. 2001). For instance, inserting an additional cationic residue does not vary only the peptide charge but also its hydrophobicity and amphipathicity. However, some systematic studies have been performed where the authors tried to compare peptide sequences where multiple substitutions were inserted to cause the significant variation of one parameter only, while the others were kept as constant as possible (Dathe et al. 1997, 2001, 2002; Wieprecht et al. 1997a, b, c; Dathe and Wieprecht 1999; Giangaspero et al. 2001; Zelezetsky et al. 2005; Zelezetsky and Tossi 2006).

11.7.1 Cationic Charges Favor Selectivity

Based on the results on the importance of anionic lipids for the membrane activity of AMPs, it is not surprising that a positive correlation between peptide positive charge and antimicrobial activity and selectivity has often been described (Bessalle et al. 1992; Matsuzaki et al. 1997; Dathe et al. 2001; Giangaspero et al. 2001; Zelezetsky and Tossi 2006; Bobone et al. 2011). However, several studies reported that increasing cationicity above a certain level (+5, +8, or +9 depending on the specific case) is not beneficial and might even cause a decrease in activity or selectivity (Dathe et al. 2001; Giangaspero et al. 2001; Zelezetsky and Tossi 2006; Jiang et al. 2008). This last finding might be due to an overly shallow insertion of the peptide in the bilayer (Wimley 2010a) and to the nonadditivity of electrostatic and hydrophobic effects, discussed in Sect. 11.6.

One of the ways to increase the total positive charge of the peptide is C-terminal amidation, which is frequent in natural sequences and has the additional advantage of reducing susceptibility to proteolytic degradation (Huang et al. 2010; Mura et al. 2016). However, this approach to increase peptide selectivity is not generally valid, possibly because it also affects the stability of helical conformations in solution (Dennison et al. 2009) (see Sect. 11.8).

11.7.2 Hydrophobicity Is Necessary for Activity but Correlates with Toxicity: The Two Thresholds

The other main parameter influencing peptide affinity for membranes is hydrophobicity. Several studies concur to support the view that two hydrophobicity thresholds exist (Dathe et al. 1997; Kondejewski et al. 1999, 2002; Stark et al. 2002; Chen et al. 2007; Glukhov et al. 2008; Mojsoska et al. 2015; Uggerhøj et al. 2015). A first threshold hydrophobicity value must be reached to obtain peptides with significant membrane binding and insertion and thus endowed with antimicrobial activity. However, if hydrophobicity surpasses a second, higher threshold, toxicity is observed, because binding to neutral membranes becomes significant. The difference between these two thresholds is due to the electrostatic contributions to peptide binding to bacterial membranes. Therefore, an optimal range of hydrophobicity values exists, in which peptides exhibit antimicrobial activity, but no significant toxicity. Above a third, even higher threshold, activity decreases, because of peptide aggregation and lack of solubility (Gatto et al. 2006; Chen et al. 2007; Chu-Kung et al. 2010; Wimley 2010a). It is difficult to provide quantitative values for these thresholds, since different hydrophobicity scales are used in the literature. Just as an example, Deber and coworkers identified values of 0.4 and approximately 2 in the Liu-Deber scale for the activity and toxicity thresholds, respectively, for the hydrophobicity of the core segment of a series of model peptides (Glukhov et al. 2008).

It is interesting to note that hydrophobicity affects binding to neutral membranes more than to charged bilayers and hemolysis more than bactericidal activity (Wieprecht et al. 1997a; Dathe et al. 2002). The rationale underlying this finding is not immediately obvious, since the hydrophobic driving force is present for both membrane types, and therefore any variation in hydrophobicity should affect both antimicrobial activity and toxicity to the same extent. The experimental observations can be explained based on the nonadditivity of electrostatic and hydrophobic effects (Sect. 11.6).

In the case of highly hydrophilic peptides, modifications that increase hydrophobicity can enhance the antimicrobial activity (first threshold), without inducing strong toxicity (second threshold). Malmsten and coworkers have reported addition of hydrophobic oligopeptide stretches to the N- or C-terminus of the sequence as a way to improve peptide activity and selectivity (Pasupuleti et al. 2009; Schmidtchen et al. 2009, 2011, 2014). Comparison of different hydrophobic modifications indicated that tagging by oligo-Trp sequences at the C-terminus is the most effective one, leading to a substantial increase in activity, without significant enhancement of toxicity. Trp residues have peculiar properties, since they are known to have an affinity for membrane interfaces, thanks to their ability to interact both with hydrophobic moieties and with charged groups (through cation-aromatic interactions) (Yau et al. 1998). It has been speculated that the specificity-enhancing effect of Trp might be linked to the difficulty of inserting such a bulky residue in the tightly packed, cholesterol-containing membranes of eukaryotes (Pasupuleti et al. 2009; Schmidtchen et al. 2009, 2011, 2014). However, preferential interaction of Trp with cholesterol has also been hypothesized (de Kruijff 1990), although it is disputed (Holt et al. 2008), and in some cases, introduction of Trp residues has been linked to enhanced peptide toxicity (Oddo and Hansen 2017; Matsuzaki et al. 1997). Another common approach to increase the hydrophobicity of highly hydrophilic peptides is lipidation (Gatto et al. 2006). Shai's group demonstrated that highly polar peptides, originally devoid of antimicrobial activity, can become antimicrobial, but not toxic, after this modification (Avrahami and Shai 2004; Malina and Shai 2005; Makovitzki et al. 2006, 2008). However, in other cases, lipidation led to strong toxicity (Chu-Kung et al. 2004; Laverty et al. 2010), or even to loss of activity, when it compromised peptide solubility (Toniolo et al. 1996; Gatto et al. 2006; Chu-Kung et al. 2010). These findings highlight the fine-tuning of AMP hydro-

phobicity needed for optimal activity and selectivity properties.

11.7.3 Excessive Amphipathicity Causes Toxicity

The total quantities of charged and hydrophobic residues provide only a very rough measure of peptide properties, since also their position in the sequence and structure are obviously important. Amphipathicity measures the degree of asymmetry in the distribution of polar and hydrophobic residues. This property can be quantified by the hydrophobic moment. This quantity is usually defined assuming an ideal helical structure and summing the vectors indicating the position of each residue with respect to the helix axis, multiplied by their respective hydrophobicity values (in analogy with the definition of an electric dipole). To compare sequences of different lengths, the mean hydrophobic moment can be obtained by normalizing for the number of amino acids (Eisenberg et al. 1982; Phoenix and Harris 2002). Peptide amphipathicity is a very important parameter for determining the free energy of membrane binding (Fernández-Vidal et al. 2007). As early as 1981, De Grado demonstrated that amphipathicity is sufficient to induce lytic activity in a helical peptide (De Grado et al. 1981). The specific value of the hydrophobic moment becomes particularly important for selectivity in an intermediate range of hydrophobicity values, when the hydrophilic or hydrophobic components of the peptide do not predominate in determining its behavior (Dathe and Wieprecht 1999; Dathe et al. 2002). Similar to what has been reported for hydrophobicity, an increased hydrophobic moment affects the activity on neutral membranes more than that on charged bilayers (Wieprecht et al. 1997b; Dathe et al. 2002). Increasing amphipathicity above a critical threshold results in strong interaction with neutral membranes, leading to toxicity (Wieprecht et al. 1997b; Dathe and Wieprecht 1999; Fernández-Vidal et al. 2007; Kindrachuk and Napper 2010). As discussed above for hydrophobicity, also in this case, it is difficult to provide a quantitative, generally valid value for this threshold.

Another measure of the distribution of polar and hydrophobic residues is the angle subtended by the polar face of the amphipathic helix, again assuming an ideal conformation and looking along the helix axis (Uematsu and Matsuzaki 2000). The available data on the role of this property in selectivity are limited, but a comprehensive study by Dathe and coworkers (2002) provided some indications. As discussed above, hydrophobicity and hydrophobic moment mostly affect the affinity for neutral membranes and thus the toxic activity. By contrast, in model membranes, the polar angle affects AMP ability to perturb the bilayer, after membrane binding: in charged bilayers, peptide-induced membrane leakage decreases with increasing polar angle, while it is essentially unaffected in neutral membranes (Dathe et al. 2002). However, the effects of the polar angle in cellular assays of activity and toxicity are more limited (Dathe et al. 2002).

11.8 Conformational and Aggregation Equilibria Play an Important Role in Membrane Selectivity: The Concept of Effective Hydrophobicity

All the considerations reported in the previous section are based on hydrophobicity values determined from the peptide amino acid composition and on amphipathicity calculated assuming an ideal helical conformation. In addition, a monomeric peptide state is always considered. However, as discussed in Sect. 11.6, peptides in solution and in the membrane attain specific ensembles of conformations, which can deviate significantly from an ideal alpha-helix. In addition, amphipathic peptides have a strong tendency to aggregate (Fig. 11.6). Conformational equilibria and self-assembly affect the degree to which the hydrophobic moieties of AMPs are exposed to the water phase and therefore modu-

late the hydrophobic driving force for membrane binding (Bobone et al. 2013). Similarly, water-membrane partition is affected by the peptide conformation, orientation, and depth of insertion in the bilayer. Based on these considerations, in our opinion, AMP selectivity is not determined by the "ideal" peptide hydrophobicity or amphipathicity but by what we call "effective" hydrophobicity and amphipathicity, i.e., the value these parameters assume in the actual conformation and aggregation state attained by the peptide in solution and in the bilayer (Bobone et al. 2013; Uggerhøj et al. 2015).

If peptide conformation and aggregation influence peptide hydrophobicity, the opposite is also true: high hydrophobicity and amphipathicity values favor a stable secondary structure by allowing the formation of intramolecular interactions between apolar residues (Fernández-Vidal et al. 2007). Peptide structure is influenced by self-assembly processes, too (Sal-Man et al. 2002). Therefore, in order to fully understand the determinants of peptide selectivity, conformational and self-assembly equilibria should be considered.

11.8.1 Helicity Correlates with Toxicity

A correlation between peptide helicity and toxicity has been reported in many studies (Tossi et al. 2000; Giangaspero et al. 2001; Zelezetsky et al. 2005; Chen et al. 2005; Khandelia and Kaznessis 2006; Zhang et al. 2011; Mangoni et al. 2011; Chapuis et al. 2012; Bobone et al. 2013; Cherry et al. 2014). In addition, helix-destabilizing Gly or Pro residues are often present close to the center of the sequence of natural, selective AMPs that attain a helical conformation in membranes (Tossi et al. 2000, Bobone et al. 2013). These amino acids are important for peptide selectivity, since their deletion, substitution, or insertion significantly affects toxicity, through the perturbation of the secondary structure (Thennarasu and Nagaraj 1996; Zhang et al. 1999; Shin et al. 2001; Yang et al. 2002, 2006a, b; Lee et al. 2004, 2007; Song et al. 2004; Carotenuto et al. 2008; Bobone et al. 2013; Wang et al. 2015). An increase in selectivity with a reduction in helical structure has been reported for other helix-breaking strategies, such as the insertion of D-amino acids (Shai and Oren 1996, 2001; Oren and Shai 1997; Papo et al. 2002; Chen et al. 2005; Zhu et al. 2007c; Kaminski and Feix 2011; Nan et al. 2012; Huang et al. 2014) or peptoid residues (N-substituted glycines, which lack a H-bonding proton on the N backbone atom and comprise a flexible main-chain methylene group) (Song et al. 2005; Zhu et al. 2007a, b; Kim et al. 2010). Incidentally, these non-proteinogenic residues have the added advantage of reducing peptide susceptibility to proteolysis (Papo et al. 2002; Kim et al. 2010).

The correlation between helicity and toxicity was tentatively explained by proposing that a stable helical conformation enhances the peptide propensity to aggregate (Kindrachuk and Napper 2010; Vermeer et al. 2012): in helical amphipathic peptides, the hydrophobic face of the helix is totally exposed to the aqueous phase, and therefore aggregation is hydrophobically favored. Aggregation, in turn, would inhibit crossing of the LPS layer and cell wall and thus access to the plasma membrane of bacteria (see below). However, helicity normally affects toxicity, rather than antibacterial activity. In addition, in the peptides we investigated, aggregation was significant only at concentrations higher than the membrane-perturbing values, and therefore it was not relevant for activity (Bobone et al. 2013). Probably a higher tendency to aggregate and an enhanced toxicity are just two independent consequences of the hydrophobicity induced by a stable helical structure, but the lack of selectivity in helical peptides is not caused by peptide aggregation (see also below). Based on a systematic study in which a central proline residue was moved along the sequence or deleted, we obtained data supporting an alternative explanation (Bobone et al. 2013). In a perfectly helical, amphipathic structure, the apolar residues are completely exposed on the hydrophobic face of the helix. Even though short peptides are often unstructured in water, helical conformations can be at least partially populated, also thanks to the sta-

bilization due to the interaction between hydrophobic side chains aligned along the helix (a motif often called "leucine zipper" (Asthana et al. 2004)). White and coworkers reported a strong correlation between the amphiphilicity of a peptide sequence in an ideal helical conformation and both the degree of helicity in solution and the affinity for neutral membranes (Fernández-Vidal et al. 2007). Destabilization of the helical conformation allows the peptide to fold onto itself, hiding the apolar side chains from the water phase, and reducing the effective hydrophobicity of the peptide, and thus the driving force for binding neutral membranes (Bobone et al. 2013; Büttner et al. 1992). Destabilization of the helix also increases the entropic cost of membrane binding, since association to the bilayer is normally followed by peptide structuring (Zelezetsky et al. 2005). Interestingly, once membrane-bound, the helix-destabilizing modifications do not preclude the attainment of an amphipathic helical conformation (Bobone et al. 2013; Orioni et al. 2009; Oren and Shai 2000). Therefore, variations in the free energy of membrane binding are determined essentially by changes in the solution conformation. Unstructured conformations would inhibit binding to neutral membranes but would affect only marginally the affinity for charged bilayers, leading to enhanced selectivity. Related to this interpretation is the concept of position-dependent hydrophobicity: hydrophobic residues in unstructured regions of the peptide contribute to the effective hydrophobicity and to toxicity less than those in helical segments (Tachi et al. 2002).

The effective, conformation-dependent hydrophobicity can be calculated from peptide structures (Gaillard et al. 1994), but it can also be determined experimentally. Reversed-phase chromatography retention times have resulted to be an accurate measure of the effective hydrophobicity of peptides (Krause et al. 1995; Zhou et al. 1990; Kim et al. 2005). Interestingly, strong correlation between RP-HPLC retention times and the hemolytic activity of AMPs has been reported (Blondelle and Houghten 1991; Kondejewski et al. 1999; Tachi et al. 2002).

11.8.2 Imperfect Amphipathicity Optimizes Selectivity

Another idea related to effective hydrophobicity is imperfect amphipathicity. Insertion of a polar/charged residue in the hydrophobic face of an amphipathic helix has proven to be a reliable method to increase AMP selectivity (Asthana et al. 2004; Chen et al. 2005; Ahmad et al. 2006, 2009a, b; Hawrani et al. 2008; Pandey et al. 2010, 2011; Jiang et al. 2011, 2014, 2018; Son et al. 2013; Dalzini et al. 2016; Zhang et al. 2016). Hodges and coworkers even termed these misplaced polar residues "specificity determinants" (Jiang et al. 2011, 2014, 2018), even though exceptions to the selectivity-improving effect of this approach have been reported (Wang et al. 2018). Interestingly, an imperfectly amphipathic structure is a common property of many natural, selective AMPs (Wimley 2010b; Orioni et al. 2009). Again, this approach reduces the hydrophobic driving force for binding neutral membranes. On the other hand, antimicrobial activity is usually not affected significantly by these changes: in charged bilayers, binding takes place all the same, thanks to the electrostatic attraction; once membrane-bound, the peptide is able to attain a (possibly distorted) helical conformation, as demonstrated by spectroscopic and simulative studies (Hawrani et al. 2008; Orioni et al. 2009). In the bilayer, imperfect amphipathicity will contribute to membrane disruption, by driving some polar head-groups in the hydrophobic core of the membrane, as we observed for PMAP-23 (Orioni et al. 2009) (Fig. 11.7). This type of membrane activity has been termed "interfacial activity" by Wimley (2010b). Finally, it is worth mentioning that we recently observed an effect of imperfect amphipathicity on toxicity also in the case of peptidomimetic antimicrobial molecules (Konai et al. 2018). Two small amphipathic, cationic molecules were characterized by the same compositional hydrophobicity but had very different selectivity. By combining molecular dynamics simulations and RP-HPLC retention times, we demonstrated that this was due to imperfect amphipathicity and lower effective hydrophobicity of the selective analogue compared to the toxic compound.

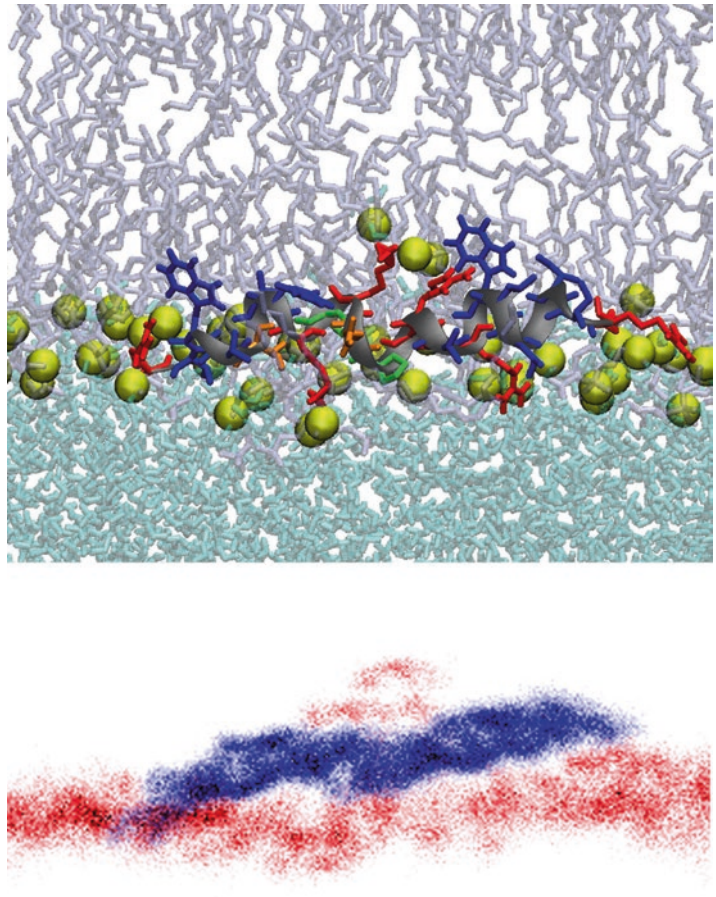

Fig. 11.7 Interfacial activity of an imperfectly amphipathic AMP. Effects of the imperfectly amphipathic AMP PMAP-23 on the structure of a lipid bilayer, as observed in MD simulations. Two charged residues located on the hydrophobic side of the helix drive three phospholipid head-groups and some water molecules into the hydrophobic core of the membrane. Water is represented in cyan, phospholipids in gray, and phospholipids' phosphorus atoms as yellow spheres. The peptide backbone is shown in gray, charged side chains in red, polar amino acids in orange, apolar residues in blue, and prolines in green. The lipid composition was POPG/POPC (1:3 mol/mol).The bottom panel reports the density map of the lipid phosphorus atoms (red) and of the peptide backbone atoms (blue). Adapted, with permission, from research originally published in Orioni et al. 2009 © Elsevier

11.8.3 Effects of Peptide Aggregation in the Aqueous Phase on Activity and Toxicity Are System Dependent

AMPs, due to their amphipathic nature, are susceptible to aggregation in water (Tian et al. 2015). Some peptides oligomerize through the interaction of the apolar sides of their amphipathic helices (Oren et al. 1999; Asthana et al. 2004; Raimondo et al. 2005; Ahmad et al. 2006), while others form micellar structures (Liu et al. 2009; Wang et al. 2010; Joshi et al. 2015; Lin and Grossfield 2015; Haney et al. 2017; Lei et al. 2018), fibrils (Tu et al. 2007; Chen et al. 2010; Chen and Liang 2013; Shankar et al. 2013; Chairatana and Nolan 2014; Ravi et al. 2015), or even hydrogels (Veiga et al. 2012; McCloskey et al. 2014; Haney et al. 2017). Often the aggregates disassemble into monomers once membrane-bound (Ghosh et al. 1997). The critical concentration for self-assembly can vary sig-

nificantly from one specific case to the other, also depending on the experimental conditions and particularly on salt concentration.

The results of experimental and theoretical studies on the effects of aggregation on peptide activity and selectivity are extremely contradictory. Both negative (Feder et al. 2000; Kustanovich et al. 2002; Chen et al. 2006, 2007; Daschbach et al. 2012; Lin and Grossfield 2015; Farrotti et al. 2017; Haney et al. 2017; Bagheri et al. 2018; Zou et al. 2018) and positive (Sal-Man et al. 2002; Avrahami and Shai 2002; Liu et al. 2009; Chen et al. 2010; Joshi et al. 2015; Ravi et al. 2015; Lei et al. 2018) correlations between aggregation and activity have been reported, as well as lack of activity changes following aggregation (Chen and Liang 2013). Similarly, some studies found that toxicity was not significantly affected by aggregation (Lei et al. 2018), while others reported an increase (Chen and Liang 2013; Lin and Grossfield 2015) or a decrease (Kustanovich et al. 2002; Chen et al. 2006; Chen et al. 2007) in selectivity upon self-assembly. Shankar et al. (2013) suggested that toxicity of self-assembled lipopeptides depends on the specific structure of the fibrillar aggregates.

The reported discrepancies are most likely due to the fact that peptide aggregation is usually controlled by varying the peptide properties or by modulating electrostatic interactions by changing the ionic strength of the solution. It is therefore difficult to discriminate between the direct effects of these changes (e.g., an increase in hydrophobicity) and the consequence of the variations they induce in aggregation. One approach to solve this problem is covalent linking of the monomers (Sal-Man et al. 2002; Dempsey et al. 2003), but it does not exactly mimic self-assembly driven by hydrophobic interactions.

Thermodynamic considerations on the interconnected equilibria involved in AMP activity indicate a possible positive role of aggregation in enhancing peptide selectivity. Aggregation, which is hydrophobically driven, reduces the effective peptide hydrophobicity by hiding the apolar moieties in the molecule from the aqueous phase. As a consequence, the hydrophobic driving force for membrane binding is reduced in the aggregates (Stella et al. 2004; Mazzuca et al. 2005; Gatto et al. 2006; Chen et al. 2007; Chu-Kung et al. 2010; Farrotti et al. 2017). Considering the various hydrophobicity thresholds discussed above, aggregation could therefore lead to a reduced toxicity. At the same time, preassembly of AMPs causes a local release of a high concentration at a single site in the membrane, and this could cause higher activity (Ravi et al. 2015). In addition, computational studies suggested that self-assembly could lead to membrane selectivity also by affecting the kinetics of membrane binding (Lin and Grossfield 2015): binding to host mammalian membranes will be slow and inefficient as long as the lipopeptides are micellized in solution, while binding to the bacterial surface will still be efficient, thanks to electrostatic interactions and to the higher fluidity of the membrane. On the other hand, in cellular assays, the large size of the aggregates, compared to monomers, could impair selectivity: preassembled AMPs might be unable to cross the LPS layer or the cell wall and thus to reach the plasma membrane of bacteria. At the same time, they would still be able to interact with the "naked" membrane of host cells (Oren and Shai 2000; Kustanovich et al. 2002; Sal-Man et al. 2002; Mangoni and Shai 2009).

Discussing aggregation, it is important to note that this phenomenon reduces susceptibility to proteolytic degradation and affects the pharmacokinetics and pharmacodynamics in vivo (Raimondo et al. 2005; Tu et al. 2007; Chen and Liang 2013; Lei et al. 2018). It is also worth mentioning that human α-defensin 6 (HD6) has negligible direct killing activity but prevents infections by self-assembling into a network of fibrils that capture pathogens and thus contrast microbial invasion (Chairatana and Nolan 2014).

11.9 AMP Binding to Cells

As discussed in the previous sections, AMP selectivity is usually interpreted essentially on the basis of the different affinities observed in liposome studies for bilayers mimicking the membranes of bacteria or eukaryotes. However, quite surprisingly, peptide affinities toward the

two types of cells are largely uncharacterized. In addition, if peptide activity is modulated by a cell-binding equilibrium, it should depend on the density of cells, but antimicrobial activity and toxicity assays are usually carried out using standardized, fixed cell densities, which are not necessarily representative of the cell concentrations present in a typical infection site (Savini et al. 2018). Finally, bactericidal and hemolytic activities are routinely determined in separate assays, but when the two cell populations are present at the same time, they compete for peptide association. All these aspects have received limited attention, until quite recently. Biophysical studies on model membranes allow the determination of both membrane-binding and bilayer-perturbing activities, while microbiological studies usually report activities only in terms of total peptide concentration. We recently reviewed the few studies that are trying to apply to cellular experiments the same quantitative approaches normally used with model systems (Savini et al. 2018). Here, only the aspects relevant to AMP selectivity are summarized.

11.9.1 AMPs Have a Higher Affinity for Bacterial Than for Eukaryotic Cells

Only a handful of studies reported data on AMP binding to bacterial and eukaryotic cells. As soon as 1988, Bruce Merrifield and his group (Steiner et al. 1988) measured binding of cecropin A and some of its analogues to *Escherichia coli*, *B. megaterium*, *B. thuringiensis*, and *P. aeruginosa* cells and to erythrocytes. While binding to the bacteria was significant (between 70% and 80% for the natural peptide, under the conditions studied), no detectable association was observed for RBCs, at a cell density corresponding to a membrane area similar to that present in the experiments with bacteria. Welling et al. (2000) measured the binding of defensins 1–3, ubiquicidin, and human lactoferrin to bacteria and activated murine peritoneal leucocytes. In the presence of the same cell density ($2 \cdot 10^7$ cell/mL), the peptides bound 5–500 times more efficiently to bacteria than to mammalian cells, even though the latter are much bigger. Similarly, Ferro-Flores et al. (2003) reported that, in the presence of $2 \cdot 10^7$ cell/mL, an ubiquicidin analogue was 35% bound in the case of bacteria (*S. aureus*) while less than 4% in the case of human tumor cell lines LS174T and ACHN (which, again, are significantly bigger than bacteria). Comparable results have been reported for two ubiquicidin analogues (approximately 45–100% binding to *S. aureus* while only 10% to leukocytes, in the presence of $2 \cdot 10^5$ cell/mL), although in this case, selectivity was surprisingly observed also for an anionic peptide used as negative control (Ebenhan et al. 2014b). Wimley and coworkers (Starr et al. 2016) measured the binding of the artificial AMP ARVA to *E. coli*, *S. aureus*, and RBCs. In all cases, association to bacteria was more favorable than to RBCs. After accounting for the differences in cell size, the authors estimated that the affinity for bacterial membranes was more than two orders of magnitude higher than for erythrocytes. Finally, an analogue of LL37 was reported to bind *E. coli*, *S. aureus*, and *M. smegmatis*, but not to hepatic cells, under conditions of comparable cell numbers (Dutta et al. 2017). Overall, these data indicate that the differential affinity routinely observed with model bilayers is present also for the membranes of real cells.

11.9.2 Activity and Toxicity Are Cell Density Dependent

Another aspect that has remained essentially uncharacterized until very recently is whether the activities of AMPs depend on the density of cells present in the assays. Based on a partition equilibrium, the fraction of membrane-bound peptide obviously depends on the concentration of cells in the sample. Therefore, it is to be expected that MIC/MBC/MHC values depend on the concentration of cells used in the assays. In broth dilution assays of antimicrobial activity, the recommended value for the initial cell density (inoculum) is $5 \cdot 10^5$ cell/mL (Patel et al. 2012), which was selected for minimizing false-positive

and false-negative results in the clinical practice (Wiegand et al. 2008). However, bacterial cell densities in clinically relevant infections range from 1 to 10^9 cell/mL. Similarly, hemolytic activity assays are normally performed with $5 \cdot 10^8$ cell/mL, which is 1/10 of the cell density in whole blood (Savini et al. 2018 and references therein). Matsuzaki (2009) pointed out that the cell densities in the two assays are very different, also considering that the membrane area of an erythrocyte is approximately ten times bigger than that of a typical bacterium. Therefore, he wondered if TI values such as those reported in Table 11.1 are an experimental artifact due simply to the fact that more peptide is probably needed to kill a higher number of bigger cells.

In the case of traditional antibiotics, it is well known that the MIC often depends on the size of the bacterial inoculum ("inoculum effect"). By contrast, in the case of AMPs, this possible dependence has been investigated only in very few studies (Savini et al. 2018). In the 1990s, Levison et al. (1993) reported that the bactericidal activity of magainins against *P. aeruginosa* was inoculum dependent above $3 \cdot 10^5$ cell/mL, but it did not vary if the inoculum was reduced below this value. Similarly, Jones et al. (1994) observed an inoculum effect for lactoferricin B against *E. coli*, with a plateau at inoculum densities below 10^6 cell/mL. Ulrich and coworkers measured MIC values for gramicidin S and PGLa at two cell density values and observed a cell density dependence (Hartmann et al. 2010). More recently, we measured the MBC values for a fluorescent analogue of PMAP-23 in the presence of different *E. coli* cell densities (Savini et al. 2017). Also in our case, the MBC increased with inoculum size but reached a plateau at densities of $5 \cdot 10^6$ cell/mL and below. A similar trend was reported by Poon and coworkers for pexiganan (Jepson et al. 2016). Finally, a recent study reported an inoculum effect also for LL-37 (Snoussi et al. 2018).

Overall, these studies show that AMP activity is strongly dependent on the density of cells in the assay, with a linear (Savini et al. 2017) or sublinear (Jepson et al. 2016) trend. If a threshold concentration must be reached in the membrane to form pores (Melo et al. 2009), it is obvious that more membrane-bound peptide molecules are needed to kill a higher number of cells (Savini et al. 2017, 2018). Therefore, under conditions of relatively high cell densities, where the peptide is completely bound to the cells, a strong cell density dependence of the activity is expected. Data by Jepson et al. (2016) and Snoussi et al. (2018) indicate that this effect might be due also to peptide sequestration by strong binding to killed bacterial cells. We tentatively explained the plateau in the low cell density regime as due to the cell-binding equilibrium. At low cell densities, some of the peptide remains free in solution, and this fraction increases with decreasing concentrations of cells. As a consequence, the two effects (when there are less cells to kill, also a lower fraction of peptide is cell-bound) cancel each other, leading to a plateau in the total peptide concentration needed in the sample to kill the bacteria (Savini et al. 2017, 2018).

Interestingly, we observed a cell density dependence also for the hemolytic activity, with a plateau at densities below 10^7 cell/mL (Savini et al. 2017).

11.9.3 Competition for Cell Binding in Co-culture Experiments Might Be Regulated by Kinetic Phenomena

Since both antimicrobial activity and toxicity seem to depend on the density of cells, the effective selectivity also depends on the value of this parameter used in the two assays (MIC and MHC). The quantity of peptide that binds to a type of cell or to the other is determined by the respective affinities but also by the concentration of cells of each type. In the end, the peptide is active and/or toxic if the respective threshold of bound peptide needed for membrane perturbation is reached. In principle, under conditions where the host cells are in large excess (as in the case of systemic treatment of an infection), lack of toxicity is expected even if the affinities for the two cell types are similar (Matsuzaki 2009; Savini et al. 2017).

A limited number of studies have tested peptide activity and toxicity in assays where both bacteria and mammalian cells were present. Mor and coworkers showed that AMPs bound to RBCs are able to transfer to microbial cells, exerting their activity (a phenomenon they termed "affinity-driven molecular transfer") (Feder et al. 2001). Derivatives of lentivirus lytic peptides killed *P. aeruginosa* bacteria interacting with cultured human airway epithelial cells, at peptide concentrations that only moderately affected the cell monolayer (Phadke et al. 2003). Fluorescence microscopy images showed that in a co-culture of *S. aureus* and human cells (endothelial cells or neutrophils), AMPs concentrate on the bacterial cells (Matsuzaki 2009; Akram et al. 2015) (Fig. 11.2a). Chen and Liang (2013) showed that the artificial AMP CL-1 is able to kill selectively *S. aureus* in co-culture with human cells, and Malmsten and coworkers reported activity without hemoliticity in bacteria-supplemented blood for an engineered AMP (Schmidtchen et al. 2011). A striking evidence of selectivity is provided by the fact that some AMPs (e.g., LL-37) are able to kill *S. aureus* bacteria internalized into mammalian cells (Noore et al. 2012). Selectivity in co-culture has been reported also for AMP-inspired systems, such as peptidomimetics, cationic peptidopolysaccharides, and peptide hydrogels (Salick et al. 2007; Li et al. 2012; Konai et al. 2018). Some co-culture data have been reported also for antifungal, antiprotozoan, and anticancer activities. For instance, an analogue of the antifungal peptide PAF26 concentrated on fungal cells in co-culture with human lung epithelial cells (Mendive-Tapia et al. 2016); an artificial anticancer peptide concentrated in cancer cells, co-cultured with primary cells (Chen et al. 2014). Some AMPs (e.g., dermaseptins or NK-lysin) are able to kill protozoan parasites such as *P. falciparum* or *T. cruzi* inside human cells, disrupting the plasma membrane of the intracellular parasites without harming that of the host (Ghosh et al. 1997; Krugliak et al. 2000; Jacobs et al. 2003; Gelhaus et al. 2008).

Based on association equilibria, when the two cell types are present at the same time, competition for peptide binding should take place: the antimicrobial activity and/or the toxicity of the peptides should be inhibited by sequestration of a fraction of the peptide molecules due to binding to the other cell population. Very surprisingly, we observed that this is not the case (Savini et al. 2017). We measured both bacterial killing and hemolysis for analogues of PMAP-23 and esculentin, in a mixed population of *E. coli* and erythrocytes, and compared these results with the traditional assays performed on the two cell types separately (Fig. 11.8). The activities on bacteria and on RBCs were essentially unaffected by the presence of the other cell population, in contradiction with the predictions based on binding equilibria. Data from Wimley's lab provide further support to the conclusion that out of equilibrium, kinetic phenomena are at play when two cell populations are present at the same time. They showed that the results of such experiments depend on the order of addition of the different components: like in our case, no change in the MIC was observed when AMPs were added to a mixture of bacteria and RBCs or to bacteria alone. However, the antimicrobial activity was significantly inhibited when the peptide was incubated with RBCs first, and then both were added to the bacterial culture (Starr et al. 2016) (Fig. 11.8). Actually, while equilibrium processes can be invoked before the cell membranes are perturbed, it is easy to realize that this approach is too simplistic in the case of bilayer disruption, which allows access to multiple additional binding targets (e.g., inside the cell) (Snoussi et al. 2018).

Overall, quantitative measurements of peptide interactions with cells confirmed that AMPs have a higher affinity for bacterial than for host cells. Experiments performed with varying cell densities indicated that both activity and toxicity depend on this parameter (even though a plateau is observed at low cell densities) and that therefore the measured selectivity depends on the specific conditions of the experiments. Finally, experiments with mixed bacterial and eukaryotic cells showed that, contrary to expectations based on equilibrium considerations, competition for peptide binding does not lead to a loss in activity.

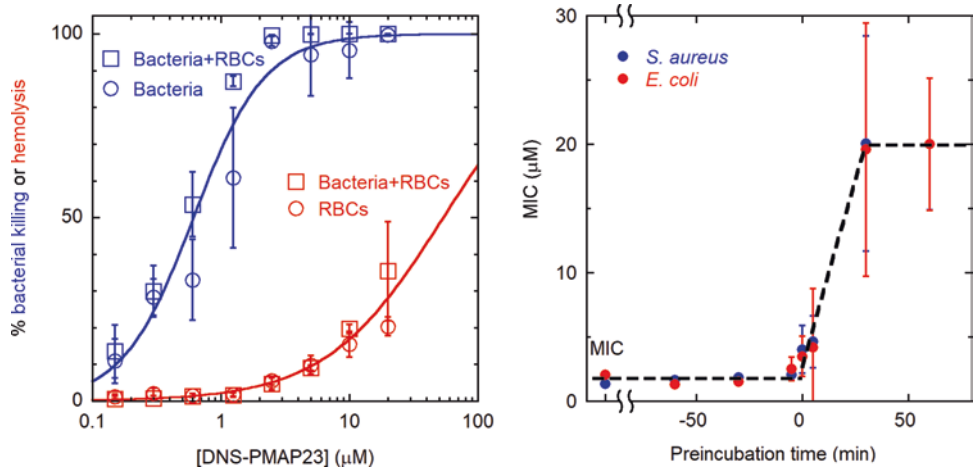

Fig. 11.8 Antimicrobial and hemolytic activity in assays with both bacterial and erythrocytes. Left panel, bactericidal (blue) and hemolytic (red) activities of the AMP DNS-PMAP23 in the presence of both bacteria and erythrocytes (squares) or of one cell type only (circles). Both activities are only slightly affected by the presence of the other cell population. 4.5×10^7 *E. coli* cells/mL, 4.5×10^8 RBCs/mL. (Reproduced with permission from Savini et al. 2017 https://pubs.acs.org/doi/abs/10.1021/acschembio.6b00910 (Copyright 2017, American Chemical Society)). Further permissions related to the material excerpted should be directed to the ACS. Right panel: effect of incubation time on MIC values in assays with both bacteria and erythrocytes. The artificial AMP ARVA-D was tested against *E. coli* (red) and *S. aureus* (blue) under various conditions. "MIC" represents measurements done in the absence of RBCs. All other experiments include 10^9 human RBC/mL. Time zero represents the experiments in which RBC and bacteria were first mixed, followed by peptide addition, i.e., no preincubation with either cell type. Negative times represent peptide preincubation with bacteria before the addition of RBCs. Positive times represent peptide preincubation with RBCs, followed by addition of bacteria. Points plotted at 20 μM had MIC values ≥20 μM. Significant inhibition of peptide antimicrobial activity due to the presence of RBCs was observed only in the case of preincubation with erythrocytes. Proteolytic degradation effects can be ruled out, since ARVA-D is a peptide comprising all D-amino acids. (Adapted with permission from Starr 2016 (Copyright 2016, American Chemical Society))

This finding definitely warrants further co-culture studies.

11.10 Concluding Remarks

A large body of studies has been devoted to characterize, understand, and improve the selectivity of AMPs. These data support the view that selectivity arises due to the different lipid composition of bacterial and host cell membranes. AMPs are able to discriminate between the two types of bilayers, thanks to their physicochemical properties. We can summarize here the main guidelines for optimization of peptide selectivity:

- Increasing the cationic charge, by C-terminal amidation, substitution of anionic residues, or insertion of cationic amino acids in the polar side of the peptide structure, leads to a better selectivity. However, an excessive increase in positive charge, above a threshold that depends on each specific case, might be ineffective or even detrimental.
- Reducing hydrophobicity, amphipathicity, and helicity is an effective strategy. These properties are necessary for activity, since they are responsible for the hydrophobic driving force for membrane binding and for insertion in the bilayer. However, several studies have demonstrated that they affect toxicity more than activity. This finding is probably a consequence of the nonadditivity of electrostatic and hydrophobic effects. Therefore, an intermediate range of hydrophobicity and amphipathicity values optimizes selectivity.
- The real determinants of selectivity are the effective, conformation-dependent, hydropho-

bicity and amphipathicity values (rather than the parameters determined based on an idealized conformation). They can be optimized by introducing:
  - Polar residues in the hydrophobic side of the peptide helix (imperfect amphipathicity)
  - Helix-breaking residues, such as Pro, Gly, D-amino acids, or peptoids
- Aggregation in the aqueous phase modulates selectivity, too.

In this chapter, we tried to generalize and simplify as much as possible the results collected over many years of studies. However, the readers that had the patience to follow us until here must have realized that the literature on AMP selectivity is full of exceptions and contradictions. Peptide association to bacterial and host cells is modulated (in a nonadditive way) by electrostatic and hydrophobic interactions. The effective peptide hydrophobicity is determined by peptide conformation and aggregation state. Therefore, AMP selectivity is finely regulated by interconnected binding, aggregation and conformational equilibria. Any variation in peptide property will affect all of these phenomena. Therefore, in order to predict, or at least to understand, the effect of peptide modifications on the final selectivity, all the possible processes involved in peptide behavior in the aqueous and membrane phases must be considered.

We still do not fully understand what happens when AMPs act on bacteria and human cells together. Recent data indicate that in this case even the complex scenario outlined above is overly simplified, since kinetic phenomena probably have to be taken into account. Further studies in this area are definitely warranted and hold the promise to provide a better understanding of AMP selectivity.

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