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Review

Control of cell selectivity of antimicrobial peptides

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ABSTRACT

Antimicrobial peptides (AMPs) are promising novel antibiotics, because they exhibit broad antimicrobial spectra and do not easily induce resistance. For clinical applications, it is important to develop potent AMPs with less toxicity against host cells. This review article summarizes the molecular basis for the cell selectivity (bacteria versus host cells) of AMPs and various attempts to control it, including the optimization of physicochemical parameters of peptides, the introduction of D-, fluorinated, and unusual amino acids into peptides, the constraining of peptide conformations, and the modification of peptides by polymers. Pros and cons of these approaches are discussed.

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1. Introduction

Several hundred antimicrobial peptides (AMPs) composed of <50 amino acid residues have been discovered in plants, insects, and vertebrates including humans, constituting host defense systems against invading pathogenic microorganisms [1–3]. Accumulating evidence suggests that these peptides not only directly kill pathogens but also modulate innate immunity and even bridge the innate and adoptive immune responses [3–6]. Many attempts have been made to

utilize AMPs as novel antibiotics, because they exhibit a broad spectrum of antimicrobial activity (against Gram-positive and negative bacteria, fungi, parasites, enveloped viruses, and even multidrug-resistant microorganisms) and do not easily induce resistance compared to conventional antibiotics [1,3], although they do eventually evoke resistance [7]. Indeed, more than a dozen companies have been commercially developing antibiotic peptides and peptidomimetics, several of which are in clinical trials. However, the trials are limited to topical applications, because peptides that have apparently negligible lethality for mammalian cells in vitro are usually toxic when injected into the bloodstream, although this issue has not been well documented [3,8]. Systemic application would certainly expand the usefulness of AMPs and therefore the market. Toward this goal, toxicities associated with systemic administration should be urgently and extensively investigated.

Abbreviations: AMP, antimicrobial peptide; MG-H1, GIKKFLHIIWKFIKAFVGEIMNS; MG-H2, IIKKFLHSIWKFGKAFVGEIMNI; MIC, minimal inhibitory concentration; PEG, polyethyleneglycol

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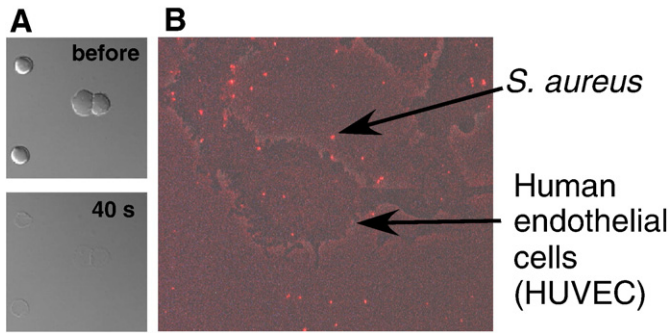


Fig. 1. Do AMPs really exhibit cell selectivity? (A) Magainin-induced hemolysis [17]. F5W-magainin 2 (10 μM) induces complete lysis of human erythrocytes at a cell density of 6×10^5 cells/mL. Differential interference contrast images of erythrocytes before (upper) and 40 s after (lower) addition of magainin. (B) Preferential interaction of magainin with bacteria in the coexistence of mammalian cells (M. Zasloff, unpublished work, with permission). A dye-labeled magainin selectively bound to *Staphylococcus aureus* but not to surrounding epithelial cells.

In this review article, I summarize the molecular basis for the cell selectivity (bacteria versus host cells) of AMPs and various attempts to control it. Only important original articles and recent papers are cited, because there are a number of excellent review articles [1–3,9–13].

2. Cell selectivity of AMPs

AMPs have been 'believed' to exhibit cell selectivity. That is, they selectively kill microorganisms without being significantly toxic to host cells. This concept, which coincides with roles of AMPs in innate immunity, comes from a plethora of observations that AMPs are nonhemolytic at concentrations well above their minimal inhibitory concentrations (MICs) against various microorganisms. For example, magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS) discovered in the skin of the African clawed frog *Xenopus laevis* inhibits bacterial growth in the

concentration range 2–50 μM , whereas the concentration at which it causes 50% hemolysis in human erythrocytes is as high as 1000 μM [14,15]. However, this apparent cell selectivity seems to be an experimental illusion. The conventionally used cell concentration is completely different between antimicrobial and hemolysis assays. Antimicrobial assays are usually carried out at a bacterial cell concentration of 5×10^5 colony-forming units/mL. In contrast, hemolysis assays are performed at hematocrit values of 1–10%, typically 5%. Given a volume of 86 fL per erythrocyte [16], a 5% hematocrit value corresponds to a cell concentration of 6×10^8 cells/mL, which is three orders of magnitude larger than that for antimicrobial assays. This difference is much larger on a cell surface area basis, taking the fact that erythrocytes ($\sim 7 \mu\text{m}$) are bigger than bacteria ($\sim 1 \mu\text{m}$) into account. It is clear that more peptides are needed to kill more cells. Indeed, when the erythrocyte concentration is reduced to 6×10^5 cells/mL, 10 μM of magainin is enough to completely lyse cells (Fig. 1A) [17]. Furthermore, 100 μM of magainins is enough to exert significant cytotoxicity in mammalian cells at a lower cell density of 0.5×10^4 – 1×10^5 cells/mL [18,19]. Therefore, strictly speaking, a 100-fold difference between MICs and hemolytic (cytotoxic) concentrations would not imply that AMPs exhibit cell selectivity. However, one may say that AMPs are 'practically cell-specific' because the hematocrit value in the blood (40–50%) is larger than the value at which in vitro hemolysis assays are performed.

Several studies clearly showed that AMPs do exhibit cell selectivity in actual situations. An in vitro example is shown in Fig. 1B (M. Zasloff, unpublished work, with permission). A dye-labeled magainin selectively bound to *Staphylococcus aureus* but not to surrounding epithelial cells. Similarly, a human antimicrobial ubiquicidin fragment peptide labeled with Technetium-99m accumulated more at sites of infection than at sites of inflammation in experimental animals, although the difference was only two-fold [20]. However, it should be stressed that AMPs are potentially toxic to mammalian cells in the absence of microorganisms, as discussed above. They may be rendered less harmful to the host simply by minimizing their interaction with

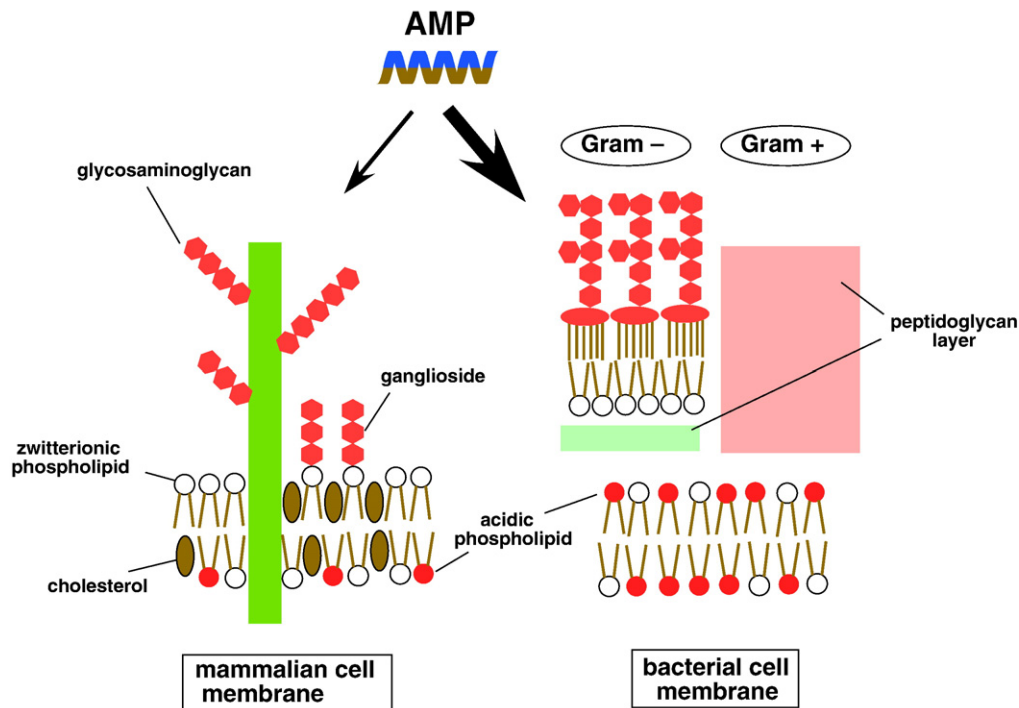


Fig. 2. Molecular basis of cell selectivity of AMPs. AMPs form amphipathic structures with a positively charged face (blue) and a hydrophobic face (brown). Electrostatic interaction between the positive charges of AMPs and negatively charged components (red) at the mammalian cell surface (left) and bacterial surface (right) is the major driving force for cellular association. Negatively charged sugar chains of glycoproteins (not shown) may also serve as binding sites for AMPs. In addition, hydrophobic interaction of the hydrophobic face with the lipidic moieties of membranes (brown) also drives peptide–cell binding.

host tissues [2]. For example, epithelial cells onto which many AMPs are secreted are relatively inert. Peptides in granules of phagocytic leukocytes kill pathogens within phagolysosomes, thereby obviating interaction with the extracellular milieu.

3. Molecular basis for cell selectivity

Despite their structural diversity, AMPs possess common physicochemical features; they are cationic and amphipathic [1,3]. The latter property allows AMPs to favorably interact with membranes, which are composed of amphipathic lipids (Fig. 2). Many AMPs are considered to exert toxicity by permeabilizing the lipid matrix of cell membranes [1,3,10], although intracellular targets have also been suggested for certain peptides [21–23]. Even in the latter case, peptide–membrane interaction is important for the cellular entry of the peptide.

The cationic property of AMPs mainly contributes to cell selectivity [10], because the surface of bacterial membranes is more negatively charged than that of mammalian cells (Fig. 2). The cell membranes of bacteria are rich in acidic phospholipids, such as phosphatidylglycerol and cardiolipin [24]. As an extreme case, phosphatidylglycerol composes ~90% of the phospholipids in the inner membrane of *Staphylococcus epidermidis* [25]. Bacteria with higher levels of negatively charged lipids are more susceptible to magainin [15]. The cell walls also contain anionic molecules, such as lipopolysaccharides in the outer membrane of Gram-negative bacteria and teichoic acids and lipoteichoic acids in the peptidoglycan of Gram-positive bacteria.

In contrast, acidic phospholipids are usually sequestered in the inner leaflets of plasma membranes in the case of mammalian cells (Fig. 2) [26]. The outer leaflets are mainly composed of zwitterionic phosphatidylcholine and sphingomyelin, although negatively charged gangliosides are present as minor species. Recently, Lee et al. showed that gangliosides play a pivotal role in the cellular entry of the buforin IIb peptide (RAGLQFPVG[RLLR]₃) [27].

Hydrophobic interaction between the hydrophobic face of an amphipathic peptide and zwitterionic phospholipids on the cell surface play a major role in the interaction of AMPs with mammalian cell membranes. We showed using several peptides a correlation between hemolytic activity and lytic activity against phosphatidylcholine liposomes [28]. This concept has been supported by a number of biophysical studies. Hemolytic peptides exhibit strong interaction with phosphatidylcholine, whereas nonhemolytic peptides do not (for example, [29–36]).

Recently, anionic sulfated glycosaminoglycans, such as heparan sulfate, have been suggested to serve as a molecular portal for the cellular entry of Arg-rich, cell-penetrating peptides [37]. AMPs with multiple Arg residues may be internalized via the glycosaminoglycan pathway. Arg-lacking magainin was reported not to interact with sulfated glycosaminoglycans [38].

Factors other than cell surface charge also contribute to cell selectivity [28]. The presence of membrane-stabilizing cholesterol in mammalian cells protects the cells from attack by AMPs. An inside-negative transmembrane potential facilitates membrane permeabilization probably by facilitating the insertion of positively charged peptides into membranes. The transmembrane potential of bacterial cells is more negative than that of normal mammalian cells [2].

As discussed in Section 2, AMPs may not exhibit cell selectivity in a strict sense. There are several plausible explanations why. First, in the case of bacteria, nontrivial amounts of cationic peptides are trapped by negatively charged molecules outside the cell membrane, the ultimate target (Fig. 2). Second, the mode of membrane permeabilization differs between bacteria and mammalian cells. We recently found that magainin forms a ~3 nm pore in the Gram-positive bacterium *Bacillus megaterium*, whereas it induces a huge (>23 nm) membrane defect in Chinese hamster ovary cells [17]. Third, in contrast to

bacteria, the permeabilization of plasma membranes may not be enough for cytotoxicity against mammalian cells. The cell selectivity issue should be seriously reinvestigated.

4. Control of cell selectivity

Various attempts have been made to improve the cell selectivity of AMPs. These include the optimization of physicochemical parameters of peptides, the introduction of D-amino acids, fluorinated amino acids, and unusual amino acids into peptides, the constraining of peptide conformations, and the modification of peptides by polymers.

4.1. Optimization of physicochemical parameters

A large family of AMPs assume cationic amphipathic helices that are characterized by several physicochemical parameters: net charge, helicity, hydrophobicity per residue (H), hydrophobic moment (μ) and the angle subtended by the positively charged polar helix face (Φ). Generally, an increase in positive charge of up to ~10 enhances antimicrobial activity without significantly affecting hemolytic activity [13]. Although many researchers have reported the structure–activity relationships for AMPs [9,12,13], it is difficult to estimate the contributions of other parameters to antimicrobial activity and cell selectivity, because these parameters are not necessarily independent. The group of Dathe clarified the importance of each parameter using carefully designed model peptides and magainin analogues [11,39,40]. The activity against Gram-negative bacteria is mainly determined by charge except for inactive peptides with low hydrophobicity. In contrast, the increase in H , μ , and Φ substantially enhances the activity against Gram-positive bacteria and hemolytic activity. Systematic studies by the group of Hodges [41,42] also revealed that hemolytic activity can be significantly decreased by simply reducing the hydrophobicity of the helix hydrophobic face. However, peptides with very low hydrophobicity have less antimicrobial activity. A correlation between hydrophobicity and hemolytic activity was also observed for cyclic peptides containing D-amino acids [43,44]. Recently, the disruption of the hydrophobic face by the introduction of a charged residue was reported to significantly reduce hemolytic activity without deteriorating antimicrobial activity [36].

The hydrophobicity not only affects the cell selectivity but also modulates the mode of peptide–membrane interaction. F5W-magainin 2 (GIGKWLHSAKKGKAFVGEIMNS) forms a toroidal pore with a diameter of 2–3 nm in lipid bilayers composed of phosphatidylglycerol/phosphatidylcholine [45,46], whereas its analogue MG-H1 with an increased hydrophobicity (GIKKFLHIIWKFVGEIMNS, Fig. 3) induces a larger membrane defect and the aggregation and/or fusion of lipid vesicles [30].

In most studies, the hydrophobicity of a peptide is calculated based on its amino acid composition. However, the hydrophobicity is dependent on the positions of hydrophobic residues. We designed the MG-H2 peptide (IIKKFLHSIWKFGKAFVGEIMNI, Fig. 3) with an amino acid composition identical to that of MG-H1 [30]. Between the two analogues, there are only two differences in the position of substitutions, i.e. S8I and G13I versus G1I and S23I. The hydrophobic residues are clustered in the central region of the sequence of MG-H1, whereas they are more evenly distributed along the sequence of MG-H2.

The other physicochemical parameters, such as the charge, H , μ , and Φ , are almost the same in these molecules. However, the two magainin peptides exhibited significantly different properties. The observed hydrophobicity (retention on a C₁₈ column and affinity for phosphatidylcholine bilayers) of MG-H1 was much larger than that of MG-H2 because of a tendency toward helix fraying near the termini. MG-H1 was more hemolytic, whereas MG-H2 was more potent against *E. coli* and *Leishmania donovani* promastigotes [47]. Thus, MG-H2 has a larger therapeutic index.

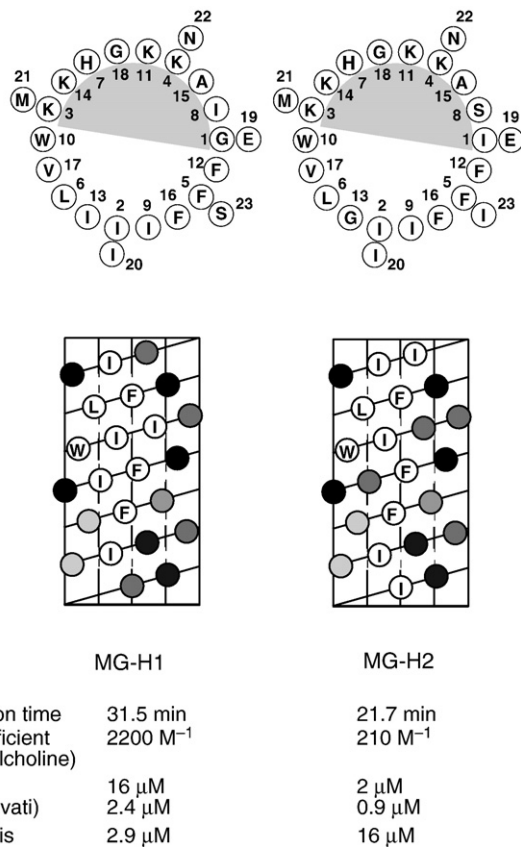


Fig. 3. Position-dependent hydrophobicity of magainin peptides [30,47]. Helical wheel (above) and helical net (middle) representations of the magainin analogs MG-H1 (left) and MG-H2 (right). The shaded areas indicate the polar surface of the amphipathic helix. The hydrophobicity of each amino acid is represented by a gray scale with the most polar residues in black. Physicochemical properties and biological activities of the peptides are also summarized (bottom).

4.2. Introduction of *D*-amino acids and fluorinated amino acids

Introduction of amino acids with the opposite chirality in the hydrophobic face of amphipathic secondary structures breaks the secondary structures, reducing hydrophobic interaction necessary for interaction with mammalian cells (Table 1). The substitution of several amino acid residues in hemolytic peptides with corresponding *D*-amino acids was found to improve selective toxicity. Shai and Oren replaced P⁷, L¹⁸, and L¹⁹ in a cytolytic paradaxin peptide with their *D*-counter parts. The hemolytic activity was reduced depending on the number of *D*-amino acids introduced, with the triple substituted analog practically nonhemolytic, whereas the attenuation of antimicrobial activity was within two-fold [31]. A similar strategy was used to increase the therapeutic index of bee venom melittin [32,33], synthetic amphipathic peptides mainly composed of K and L [34,48], and cyclic gramicidin S analogs [44]. Notably, this approach also significantly improves the *in vivo* efficacy of the intravenously administered amphipathic peptide LKLLKLLKLLKLLK-NH₂ [49].

Attempts to use enantiomeric peptides solely composed of *D*-amino acids have also been made to enhance the therapeutic index. The enantiomer of the 13-mer peptide pEM-2 (KKWRWWLKALAKK) reduced the endotoxin-induced lethality in mice, whereas the all L peptide exhibited no effect [50]. This effect can be explained by the fact that peptides containing *D*-amino acids are resistant to proteolysis [51,52].

Meng and Kumar recently found that the susceptibility of AMPs to proteolysis can be reduced by incorporating fluorinated amino acids

[53]. The substitution of 5, 5, 5, 5', 5', 5'-2S-hexafluorooleucine residues for two Leu residues in buforin peptides reduced the rate of peptide degradation by trypsin by ca. 50%. Furthermore, this modification enhanced antimicrobial activity without augmenting hemolytic activity. However, this strategy did not work for magainin. The hemolytic activity was increased without improving antimicrobial activity, and the susceptibility to trypsin was not changed.

4.3. Introduction of peptoid residues

As mentioned above, breaking secondary structures by introducing *D*-amino acids in the hydrophobic faces of amphiphilic peptides is a promising strategy to improve cell selectivity. The introduction of peptoid residues (*N*-substituted glycines) has also been reported to increase the therapeutic index. The secondary structures are disrupted because of the lack of a hydrogen atom attached to a nitrogen atom capable of forming a hydrogen bond, similarly to Pro. The original work by the group of Shin discovered that the substitution of Ala-peptoid residues for L⁹ and L¹³ of the α -helical KLW peptide (KWKKLLKLLKLLKLLK-amide) significantly reduced hemolytic and cytotoxic activities without weakening antimicrobial activity (Table 1) [54]. Interestingly, this substitution also changed the mode of action from membrane disruption to DNA binding. Similar approaches were used to enhance the selective toxicity of cathelicidin-derived Trp/Pro-rich peptides [55,56] and melittin [57]. In addition to peptides containing a few peptoid residues, peptide mimetics composed of peptoid residues [58–60] and α -peptide/ β -peptoid chimeras [61] have been examined as alternatives to AMPs.

4.4. Cyclization

The cyclization of linear peptides was found to increase selective toxicity. Oren and Shai cyclized the linear AMP CKLLKWLKLLKLC with an intramolecular disulfide bond (Table 1) [35]. The parent peptide was strongly active against Gram-positive bacteria and highly hemolytic. The cyclic peptide exhibited stronger antimicrobial activity even toward Gram-negative bacteria and significantly weaker hemolytic activity than the linear peptide, although the cyclization did not change the helical structure of the peptide in negatively charged membranes as evaluated by infrared spectroscopy. A combination of cyclization with *D*-amino acid substitution further enhanced the

Table 1

Examples of improvement of selective toxicity by various chemical modifications.

Peptide ^a	MIC ^b	Hemolytic activity	Reference
GFFALIPKIISSPLFKITLLSAVGSALSSSGGQE-(NH ₂) ₂	0.8–3 μM	100% at 10 μM	[31]
GFFALIPKIISSPLFKITIISSAVGSALSSSGGQE-(NH ₂) ₂	0.9–6 μM	~5% at 50 μM	
GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	0.3–20 μM	100% at 4 μM	[32]
GIGAVLkvlTTGLPALISWIKRKRQQ-NH ₂	0.8–12 μM	<5% at 50 μM	
GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	0.5–2 μM	10% at 0.78 μM	[33]
GIGAVIKVLTTGIPALISWIKRKRQQ-NH ₂	0.5–1 μM	10% at 6.25 μM	
LKLLKLLKLLKLLK-NH ₂	11–45 μg/mL	100% at 180 μg/mL	[49]
LKILKkLkLkLkLkL-NH ₂	5.6 μg/mL	0% at 180 μg/mL	
KWKkLkLkLkLkLkLkL-NH ₂	4–8 μM	55% at 25 μM	[54]
KWKkLkLkLkLkLkLkLkL-NH ₂	1–4 μM	0% at 100 μM	
VRRFPWWWWPFLRR-NH ₂	1–8 μM	70% at 200 μM	[56]
VRRFPkWWWWkFLRR-NH ₂	1–4 μM	0% at 200 μM	
CH ₃ -S-CKLLKWLKLLKLC-S-CH ₃	2–150 μM	100% at 10 μM	[35]
C*KLLKWLKLLKLC*	1–30 μM	50% at 50 μM	
C*KILKWLKILKLC*	1–20 μM	<10% at 50 μM	

^a Underlined letters indicate peptoid residues. Lower case letters indicate *D*-amino acids. Cysteine residues with asterisks are connected by a disulfide bond.

^b Range of minimum inhibitory concentrations against several microorganisms tested.

therapeutic index by almost nullifying the hemolytic activity. The cyclization method was successfully applied also to a melittin analogue [62]. Cyclization not only enhances selective toxicity but also increases protease stability. The half-life of a cyclic analog of the U-shaped indolicidin peptide analogue CP-11 in the presence of trypsin was increased by 4.5 fold [63].

However, this approach is not universal. Although cyclization eventually increased the therapeutic index, cyclization of the hexapeptide RRWWRF increased both antimicrobial and hemolytic activity with the former more significantly increased [64]. In contrast, the cyclization of a magainin analogue reduced both antimicrobial and hemolytic activity [62].

4.5. PEGylation

Attaching a polyethyleneglycol (PEG) moiety to peptide and protein drugs (PEGylation) is frequently used to improve the in vivo efficacy of these drugs [65]. The pioneering work by Guiotto et al. reported that PEGylation reduced the antimicrobial activity of nisin by more than two fold, although solubility was improved [66]. We investigated the effects of PEGylation of α -helical magainin 2 [19] and tachyplesin I having a cyclic β -sheet structure [67]. The attachment of a large PEG moiety (5 kDa) at the N-terminus reduced the membrane binding for both peptides. A reduction in membrane-permeabilizing activity occurred only for magainin, because the PEGylation destabilized the secondary structure. The structure of tachyplesin I was not affected by the PEGylation because it is stabilized by two disulfide bonds. Interestingly, the PEGylation did not change the essential mode of action against lipid bilayers. The PEGylation significantly reduced cytotoxicity and antimicrobial activity at the same time. The extent of the decrease depended on the peptide. The antimicrobial activity of magainin 2 was lowered only by 4-fold, whereas that of tachyplesin I was reduced much more. Optimization of the size of the PEG moiety may improve the therapeutic index of AMPs.

5. Conclusion

I would stress again that the dogma that AMPs exhibit cell selectivity should be reconsidered in a strict sense, because it originates from an experimental illusion. However, AMPs are practically cell-selective, and a great number of studies focused on the improvement of cell selectivity of AMPs were in the right direction. The results suggest that strong antimicrobial activity and less cytotoxicity can be achieved by increasing the net positive charge of the peptide with minimal hydrophobicity above a threshold. This is consistent with the hypothesis that the lipid composition of cell surfaces primarily determines cell selectivity. The hydrophobicity effectively responsible for cytotoxicity is that on the hydrophobic face of the amphipathic secondary structure formed upon binding to the membrane. Residues close to the ends of a helix do not fully contribute to the effective hydrophobicity. A reduction in hydrophobicity can be achieved by introducing charged residues, D-amino acids, or peptoid residues into the hydrophobic face or by cyclization of the peptide. Each method has pros and cons. The use of enantiomeric or unusual amino acids contributes to resistance to proteolytic degradation, however, it has a problem in biotechnological production. Furthermore, plausible in vivo toxicity is of deep concern. Conversely, AMPs exclusively with natural amino acids are suitable for mass production, but proteolysis is inevitable.

Despite extensive research, knowledge on the interaction of AMPs with mammalian cells is still very limited. Most researchers employ hemolytic assays as a measure of cytotoxicity. However, erythrocytes are specialized cells without extracellular matrices or intracellular organelles. Studies using mammalian cell lines have examined only cell viability. More subtle toxicities and the mode of AMP–cell interaction should be urgently investigated.

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